SCIENTIFIC BULLETIN
SERIES F. BIOTECHNOLOGIES
VOLUME XIX, 2015

University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Biotechnologies

SCIENTIFIC BULLETIN

SERIES F. BIOTECHNOLOGIES

Volume XIX

2015
BUCHAREST

PUBLISHERS:

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania – Faculty of Biotechnologies

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CERES Publishing House

Address: 1 Piaţa Presei Libere, District l, Zip code 013701, Bucharest, Romania
Phone: + 40 21 317 90 23, E-mail: edituraceres@yahoo.com, Webpage: www.editura-ceres.ro

To be cited: Scientific Bulletin Series F "Biotechnologies", Volume XIX, 2015

ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

International Database Indexing: COPERNICUS, CABI, GOOGLE SCHOLAR, DOAJ, Scipio, PBN (Polish Scholarly Bibliography), OCLC, Research Bible, Cite Factor (Academic Scientific Journals), Universal Impact Factor

SUMMARY

AGRICULTURAL BIOTECHNOLOGY

BIOTECHNOLOGY IN VETERINARY MEDICINE

FOOD BIOTECHNOLOGY

INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

FOOD SAFETY

MISCELLANEOUS

AGRICULTURAL BIOTECHNOLOGY

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

TREATMENT OF ECHINOCHLOA CRUS-GALLI (L.) BEAUV WEEDS BY TRICHODERMA HARZIANUM FUNGUS AND IMPROVEMENT OF GROWTH AND PRODUCTIVITY OF RICE (*ORYZA SATIVA* L.) ANBER -33

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Abstract

This study was conducted to evaluate the effect of biocontrol fungi - Trichoderma harzianum.austaralian(T.h.a). and Trichoderma harzianum.Rafai (T.h.n) and Chaetomium elatum (C.e) isolates in improvement of the growth of rice seedlings - class anber -33 and combating Echinochloa weeds which accompaniment to rice cultivation.To attain these aims , three laboratorial experements were carryied out. The first experement included isolation, purification and estimation of fungi frequency in rice field (AL-Najaf AL-Ashraf Governorate), while the second experiment included the use of fungi and their filtrates by planting rice and Echinochloa seeds in petri-dish containing biocontrol fungi. The third experiment acheived by using a spots that containing a soils (200 gm of field samples) treated with studied fungi (held on powerded wheat straw) and comparing the germination and growth (percentage of germination , lengths (cm), fresh and dry weights (gm) of plumile and radicle of rice and Echinochloa seedlings).These experiments was designed by completely randomized design(C.R.D) Results of this study can be summarized as follows.

1- The genous Aspergillus showed the frequency of 23.53% at the start of growing, while Trichoderma reached at the highest frequency of 39.00% at the end of growing.

2- T.h.t. and Rhizoctonia solani (non pathogenic isolate) and their filtrates showed an important results in the field of controlling of Echinochloa weeds grown in petri dishes and spots , Echinochloa germination percentage reached 8.22, 16.55% in petri dishes and 20.56, 12.65% in spots respectively as well as fungi filterates achieved 12.50, 17.50% in petri dishes respectively, in addition T.h.t. and R. solani attained a asignificant differences on rice seedlings growth parameters germination percentage reached 95.32, 80.16% in petri dishes and 93.50,77.50% in spots respectively, as well as fungi filterates acheived 93.51, 81.50% in petri dishes respectively. Also T.h.t. and R. solani and its filtrates attained asignificant results in combating with Echinochloa germination percentage and in reducing of plumile and radicle length ,fresh and dry weight in compare with control treatment.

Key words: *Trichoderma harzianum, Oryza sativa L., Echinochloa crus-galli (L.) Beauv, Germination percentage, Biological control.*

INTRODUCTION

The rice (*Oryza sativa* L.) is conceived as one of major and important cereal crops in the world, and its food importance comes of contains a high amounts of carbohydrates which was easy to digestion that needed by the man in the diet to supply energy. As well as rice proteins have a balanced content of essential amino acids, especially lysine acid in compare with other grains (Tai, 2000), as it contains 6.7-8% protein, 75-80% starch and 0.4% fat and 13.3% water and 0.9% of metals such as iron , calcium ,chlorine, phosphorus (salts and metals) and vitamin A, B (Al-Younis and Al- Shammaa, 1987).

The treatment of plants residues (wheat) with fungi were conceived one of the vital means of raising the nutritional values of the remnants (Van Wyk, Mohulatsi, 2003).

T.harzianum fungus was used in the treatment of a number of plant residues, including sawdust, barley, wheat straw, rice and wheat millet bran, (Khafaji, 1985; Dewan, 1989; Alwan, 2005). Evans, (2002) has pointed that rice cultivation with wheat residues increase the effectiveness of microorganisms and thus obtain a significant increase in plant height. Zeilinger, (2003) also noted that the use of organic fertilizers and the addition of growth promoting fungus(*T. harazianum*) to the soil, increases the rates of flowering of plants because of the mechanisms and enzymes

secretions that provides protection for seeds and provide ideal atmosphere for germination and increase the density of the root of plants. Wheat remnants (wheat straw) consists of the following chemical components: dissolved carbohydrates 5.60, semicellulose 25.20, cellulose 33.30, lignin 10.10, crude protein 13.30, and ash 10.50.

The strains of growth promoting fungi (non pathogenic) were used in cracking cellulosic links or increasing microbial mass, which leads to increase the protein in the waste that treated by fungi (Zeilinger, 2003), so these objects will be able to produce products free from toxins.

The distinctive role of the fungus *Trichoderma* spp. in the fighting against many pathogens as well as improvement plant growth and productivity, as the fungus worked to reduce the incidence and severity of radicals disease caused by fungus *Fusarium* spp in tomato, eggplant, potato , pean , wheat and rice plants (AL-Rawi, 1997 and Zobaie, 2000 and Harman, 2000 and Sarhan Jasim, 2000, Abdul Aziz, 2001), the mechanisms that used by *T.harzianum* fungus in the fight against diseases were paratisim, enzymes secretion (Chitinase, Cellulase, Protease, β 1,3gluconase), antagonisim, production of antibiotics (Trichodermol, Trichodermin, Gliotoxine, Emodin Chrysophancol), competition and plant growth inducing (Limon et al, 1999 and Harman et al,2004).

The presence of wheat remnants in the rice growing areas as aresult of succession cultivation of rice after wheat for several years which create a new bioavailable society, so must know the presence of biomass as the "quantity and quality" in these soils before and after cultivation and using the most efficiency fungi in the analysis of plant wastes as a result of their enzymatic abilities and increase the availabilities of some nutrients and improvement the soil structure and its retention of moisture.

Recently, a lot of agricultural research institutions interested with finding alternative means for chemical combating which is harmless to plants and have a highly efficient in influencing the aetiology of the plant and to avoid collateral damage left by the chemical pesticide in the environment and human health , as well as the emergence of strains of pathogens resistant to pesticides (Montealegre et al, 2003). AL-Shibli (1998) has been abled to biologically control a types of *Fusarium*spp fungus and *Rhizopus* spp which causing the death of rice seedlings and seeds rot diseases by using the *Trichoderma harzianum*, *Chaetomium elatum* and *Penicillium* spp).

The rice is very sensitive to the weeds, especially in the early stages of growth, the research results indicated that this crop losses of up to 70% of yeilds when do not control ,in addition to its poor quality (Slaton, Norman,2006). So the interesting is taken to combating these weeds by using chemical pesticides and achieved good results in this regard (Hill et al, 2008), but the use of pesticides frequently led to environmental, healthy problems, and appearance of some weed classes resisted to these pesticides (Baltazal and Smith, 1994), so the researchers Oriented to use of alternative methods to combat the weeds in order to reduce the side effects of chemical pesticides, and reduce costs to the lowest possible level, these alternatives ways include the use of biological control fungi.

Because of the scarcity of such studies especially the role of *Trichoderma harzianum* in combating *Echinochloa* weeds which accompanied rice cultivation To achieve this goal, has been to follow the following themes:

1. Isolation and diagnosis the fungi in rice fields.
2. The effect of studied fungi, fungi filterates in the germination and growth of rice and *Echinochloa* seedlings in petri dishes and spots.
3. Test the ability of some fungi in inhibition *Echinochloa* weeds growth.

MATERIALS AND METHODS

1- The laboratorial study carried out in Faculty of Agriculture laboratory / Kufa University, soil samples were given from.

*- Rice fields in the College of Agriculture.

*- Rice fields in AL-Hera district, the two fields are planting within the succession of rice - wheat crops.

2- Laboratery experements.

*- Measurement of physical and chemical properties of the soils .
*- Isolation, purification and estimation of fungi frequency in rice field.
*- Study the effect of studied fungi , fungi filterates in the germination and growth of rice and *Echinocholoa* seedlings in petri dishes and spots that containing a soils (200 gm of field samples).

ISOLATION, DIAGNOSIS OF FUNGI IN THE SOILS CULTIVATED WITH RICE

Soil samples were collected randomly from the study region before planting for adepths of 0-30 cm to conduct chemical , physical and biological analyzes, wheras the fungi were grown by using dilutions plate method on the media PDA To calculate the number of fungal genera and species in one gram of soil as dry weight, dishes were incubated at a temperature of 25 ± 2°C.
The colonies were purified on PDA, WA media For the purpose of diagnosis, according tothe approved taxonomic keys (Barmett, 1965; Moustafa, 1982; Moubasher and Aisha, 1987; Burgess et al 1988; Domsch et al, 2003).

THE EFFECT OF STUDIED FUNGI, FUNGI FILTERATES IN THE GERMINATION AND GROWTH OF RICE AND ECHINOCHLOA SEEDLINGS IN PETRI DISHES AND SPOTS.

Carling and Leiner (1986) method was adopted, PDA media was poured in Petri dishes 9 cm diameter that planted by *R. solani*, *F. pseudograme niarum*, *F.oxysporum* , *T. harzianum* (*T.h.a*) (*T.h.t*) and *C.elatum* fungi into three replicates of eachfungi then incubated at a temperature of 25 ± 2º, after 48 hours petri dishes and spots (200 gm of field samples treated with studied fungi (held on powerded wheat straw) were planted with rice and *Echinochloa* seeds, sterilized with sodiumhypochloride of concentration 4% for 4-5 minutes (25 seeds per each dish) just 1 cm from the edge of the dish. After 10 days,the seedling lengths, moist and dry weight for each of plumile and radicle were calculated .

The laborataries experiment was designed as completely randomized design (C.R.D), treatment means has been compared according to less significant difference (L.S.D) at 0.05.

RESULTS AND DISCUSSIONS

PHYSICAL AND CHEMICAL PROPERTIES

The results appeared that the surface soil texture in AL-Herra fields was Silty Clay, while it was Silty-Clay-Loam in Faculty of Agriculture fields (Table 1). The bulk density values for rice soils in the surface horizon (AP) has been up to 1.38, 1.35 gm. cm^{-3} in AL-Herra and Faculty of Agriculture soils, respectively, while the values of pH for all samples were between 7.83 and 7.60 in AL-Herra and Faculty of Agriculture soils, respectively, and this is consistent with what (Hayatsu, 1993) referred to, while salinity values were ranged between 2.1 and 2.6 ds.m-1 ECe in AL-Herra and the Faculty of Agriculture soils, respectively, so these good values for salinity because of the appropriate washing processes.

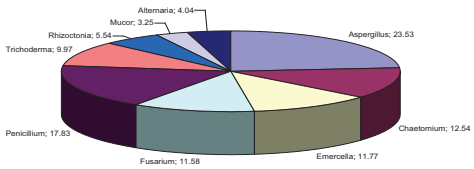
Table 1. Some physical and chemical properties of studied soils in the AL-Herra and the Faculty of Agriculture, for agriculture seasons 2010

Property	AL-Herra	Faculty of Agriculture
Soil Texture	Silty-clay	Silty-clay-Loam
Bulk density	1.38	1.35
pH	7.83	7.60
ECe $ds.m^{-1}$	2.1	2.6

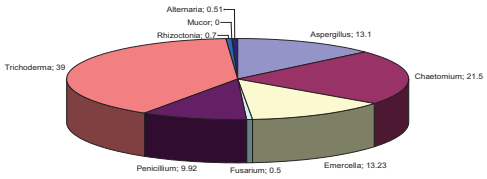
ISOLATION, DIAGNOSIS OF FUNGI IN THE SOILS CULTIVATED WITH RICE FREQUENCY OF THE FUNGI GENUS IN THE STUDIED SOILS

The results of isolation from the soil surrounding the rhizospher of rice in two regions appeared that there are 14 species of fungi within 9 genus which are *Alternaria*, *Aspergillus*, *Chaetomium*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizoctonia* and *Trichoderma* as shown in Figure (1), these results has coincided with previous studies which has

been isolated of a number of fungi from the soil cultivated with economic plants (Dewan, 1994 and AL-Helo, 1995 and Moussawi 2003). *Aspergillus* genus was the most frequen species of fungi before planting, as this percentage was reached 23.53%, while *Trichoderma* genus frequency was the most frequent species of fungi in the end of season, that ratios reached 39.00%.



Before planting



After planting

Figure 1. the percentage (%) for isolated fungi genus frequency in 1 gm dry soil of rice soils in AL-Herra and the Faculty of Agriculture fields before and after cultivation

THE EFFECT OF STUDIED FUNGI IN THE GERMINATION AND GROWTH OF RICE AND ECHINOCHLOA SEEDLINGS IN PETRI DISHES

The results found that fungi *T.h.a, T.h.t.* and *C.e* led to a significant increase in the percentage of germination of rice seedlings that attained 96.34, 95.32 and 92.30%, respectively, while it did not get any death of seedlings with these fungi as shown in the table (2), this is due to that the fungus (*T.h*) may secrete substances act on the decomposition of the outer shell of the grains, which facilitates the process of germination, such as enzyme Cellulase, or secretion motivational material to germinate and grow like Indole Acetic Acid (Dewan and others, 1994; Harman, 2000), these results agreed with Dewan, (1989) and al-Moussawi, (2005), who pointed to the existence of the motivational impact of growth promoting fungi in germination and growth of wheat and sunflower seedling, respectively.

It is noticeable, the fungus *(R. s*) achieved a germination rate of 80.16% and gave an increase in lengths of plumile and radicle of rice seedlings that reached 6.97, 2.21 cm, respectively, as well as in fresh weight and dry for them, and this result different if compared with previous studies that refers, the (*R.s*) fungus has ahigh pathogenesis - capacity (Diwan al-Bahadli, 1985). This may be due to genetic differences among them (Barreto et al, 2003).

The results showed , as shown in the table (2) that the fungus (*F.pg., T.h.t. , F. o* and *R. s*.) were the most influential fungi in reducing *Echinochloa* germination rate , which reached (8.10, 8.22, 12.33 and 16.55%) , respectively, in compared with control of 84.10%. Also notes the high proportion of dead seedlings when treated with *F. o., F. p., R. s*. And *T.h.t.* fungus, that reached 5.17, 4.57, 4.49, 2.05%, respectively, in compared control of 1.02% ,as well as (*F.pg., T.h.t., F.o* and *R.s*.) achieved significant reducing in the lengths (cm) , fresh and dry weights (gm) of plumile and radicle of *Echinchloa* plants grown in a petri dish table (2) , this result may be due to fungal parasitism of *F. pg*. And *R. s*. And analyst enzymes produced that lead to seeds rot, or the excretion of some toxic substances such as Fusaric acid Zearalenone and Trichothecin, which led to the killing of seeds embryos directly (Lauren and Greenhalph, 1987 and Dowd and others, 1989), which was due to high pathogenesis capacity of these fungi (Diwan, al Bahadli, 1985).

As can be seen from the mentioned table the effect of some studied fungi in length of plumile and radicle of *Echinochloa*, wheras *T.h.t*, *F. o*., *F. pg*, and. *R. s*. reduce plumile and radicle lengths, t the plumile lengths rates when treated with these fungi reached 1.89 , 1.65, 1.54 and 1.43 cm, respectively,in compared with control, which amounted to 4.75 cm, while radicle lengths amounted to 0.72, 0.66, 0.63 and 0.59 cm, respectively, in compared with control that was 2.15 cm, as for the fungi *T.h.a* And *C. e* led to a significant increase in plumile and radicle lengths, which amounted 6.95 , 6.83 cm for

plumile, and 3.38, 3.20 cm for radicle respectively in compared with control, 4.75, 2.15 cm for plumile and radicle respectively. With regard to combating *Echinochloa* weeds, it is noted in the table (3) that the fungus isolate *T.h.t* and non-pathogenic fungus *R.s*. were contributed significantly in reduction of the percentage of seeds germination of this isolation in its effective role in the combating the weeds biologicaly, this may be attributable due to genetic changes between isolates (Barreto et al, 2003).

Table 2. Effect of studied fungi in the germination rate, seedlings death , lengths (cm), fresh and dry weights (gm) of plumile and radicle of rice and *Echinochloa* plants grown in a petri dish

Fungi species		germination % rate	lengths (cm)		plumile weights (gm)		weights radicle (gm)	
			Plumile	radicle	fresh	dry	fresh	dry
A.niger	R	79.35	6.15	1.73	0.191	0.045	0.073	0.028
	E	74.30	2.12	0.86	0.130	0.038	0.043	0.018
A. terreus	R	63.25	5.92	1.43	0.181	0.041	0.064	0.025
	E	65.18	2.33	0.97	0.142	0.035	0.041	0.017
A. oryzae	R	60.64	5.30	1.36	0.110	0.037	0.042	0.011
	E	61.73	2.19	0.87	0.101	0.031	0.036	0.010
C globosumKunze	R	87.15	9.28	4.04	0.341	0.082	0.133	0.048
	E	87.21	6.65	3.01	0.251	0.062	0.071	0.035
C.elatum	R	92.30	9.92	4.33	0.362	0.092	0.140	0.050
	E	91.44	6.83	3.20	0.277	0.076	0.080	0.040
F.oxysporum	R	16.45	2.61	0.061	0.134	0.023	0.048	0.013
	E	12.33	1.65	0.66	0.071	0.019	0.024	0.008
F.pseudogramiarum	R	12.98	2.26	0.52	0.124	0.022	0.037	0.012
	E	08.10	1.54	0.63	0.065	0.017	0.021	0.006
P.griseofulvum	R	63.68	4.85	1.58	0.183	0.063	0.073	0.021
	E	66.22	3.75	1.25	0.135	0.037	0.040	0.020
P.oxalicum	R	92.65	7.91	3.04	0.321	0.068	0.113	0.035
	E	90.38	4.87	2.20	0.201	0.042	0.050	0.025
E.nidulans	R	89.53	7.92	3.15	0.325	0.071	0.119	0.036
	E	89.50	5.08	2.25	0.216	0.048	0.056	0.026
T.harzianum .t.	R	95.32	10.06	4.41	0.365	0.095	0.152	0.054
	E	08.22	1.89	0.72	0.082	0.020	0.026	0.011
T.harzianum .a.	R	96.34	10.14	4.62	0.372	0.106	0.161	0.062
	E	92.24	6.95	3.38	0.281	0.085	0.086	0.049
R. solani	R	80.16	6.97	2.21	0.173	0.081	0.087	0.029
	E	16.55	1.43	0.59	0.061	0.016	0.020	0.006
Control	R	87.40	7.43	2.91	0.311	0.066	0.110	0.032
	E	84.10	4.75	2.15	0.214	0.041	0.056	0.027
L.S.D. 0.01	R	5.715	2.475	1.479	0.0477	0.0149	0.0217	0.0124
	E	5.322	2.097	1.003	0.0619	0.033	0.011	0.010

Wheras R= Rice, E=Echinochloa Seedlings. radicle

THE EFFECT OF FILTRATES OF STUDIED FUNGI IN THE GERMINATION AND GROWTH OF RICE AND *ECHINOCHLOA* SEEDLINGS IN PETRI DISHES

Table (3) clarify that the filtrates of *T.h.a ,T.h.t.* And *C.e* fungi has led to a significant increase in the percentage of germination of seeds of rice, amounted to 94.87 , 93.51 and 93.12%, respectively, in compared with the

control, which was 85.32%. This explains the abilities of this fungi to secret some of the catalysts materials like growth regulators or increasing nutrients availability (Dewan, 1989; Ghisalberti et al, 1990; Musawi, 2005). The results showed also that the effect of *T.h.a ,T.h.t.*, and *C.e* fungi filtrates in increase the lengths of plumile and radicle in compared with the control, where gave significant differences amounted to 3.13, for plumile and 2.01,1.98,1.96 cm for radical. Also the result showed that the fungi *F.o* and *F.pg*. led to a reduction in the germination rates of rice seeds significantly that attained 24.43 , 20.23%, respectively, in compared with the control , which amounted to 85.32%. also the percentage of dead seedlings were amounted to 4.62 and 5.24%, respectively, in compared with control, which was 0% , these fungi have ability to secrete toxins that affect the growth indicators of the rice plant.

As the table shows , the affect of fungi *F.o* and *F.pg*. on plumile and radicle lengths ,that reached 0.43, 0.38 cm for plumile respectively, while the length of the radicle has reached 0.25, 0.23 cm, respectively, in compared with the control that was 1.86 cm for plumile and 1.15 cm for radicle, due to the ability of these fungi to secrete some toxic substances and inhibiting the roots when used in a high concentrations (Decal, 2000).The results are shown in the table (3) that the fungus *R.s*. attained a germination rate of 81.50% and an increase in plumile and radicle lengths stood at 1.76 and 0.94 cm, respectively, and in the fresh and dry weight of rice seeds, this result is inconsistent with many of the studies that indicate that the fungus *R.s.* due to its ability to secrete enzymes and toxins for plant (Dixon, 1993), this may be attributed to get agenetic changes in this isolates, or may be due to genetic modification between isolates (Barretoetal,2003).

The results also shown that fungi *F.pg., T.h.t., F.o* and *R. s*. were the most influential studied fungi in reducing germination ratio of Echinochloa seeds, which attained 9.20, 12.50, 14.34 and 17.50%, respectively, in compared to with control treatment that was 85.53%. It also notes raising of dead seedlings proportion of Echinochloa when treated with fungi *F.pg., F.o., R.s*. and *T.h.t.*, whereas reached 4.22, 3.95, 2.80 and 2.06%, respectively, in compared with control which reached 1.02%. As can be seen from the table(3) the effect of studied fungi filtrates on reduction of plumile and radicle lengths of *Echinochloa* significantly when treated with *T.h.t, R.s., F.o.,* and *F.pg*., that reached 0.72, 0.51, 0.37 and 0.32 cm, respectively, in compared with control treatment, which was 1.34 cm for plumile ,while radicle lengths stood at 0.36, 0.25, 0.21, 0.19 cm, respectively, in compared with control, which was 0.81 cm ,also they were most influential in reducing significantly the fresh weight for plumile and radicle of *Echinchloa.*

The results indicate that *T.h.a*. And *C.e*. isolates achieved significant differences y in germination ratio of *Echinochloa* reached 95.15, 93.55%, respectively, while it did not get any death of seedlings with these fungi, table (3), also achieved a significant increase in the plumile and radicle lengths, that was 2.85, 2.64 cm for plumile and 1.53, 1.51 cm for radicle respectively, in compared with control treatment, which amounted to 1.34 and 0.81 cm for both plumile and radicle respectively.

It also led to a significant increase in fresh and dry weight for plumile and radicle, that was 0.084, 0.072 gm for plumile and 0.063, 0.051 cm for radicle (fresh) respectively, this is due to that these fungi have the ability to provide aprotection for the seed and plants seedlings from pathogens, as well assecretion of some motivational material for the germination and growth of seedlings.

THE EFFECT OF STUDIED FUNGI IN THE GERMINATION AND GROWTH OF RICE AND ECHINOCHLOA SEEDLINGS IN PLASTIC SPOTS.

The results showed in the table (4) that there is considerable variation in the impact of the isolated fungi in seed germination and growth of rice and *Echinchloa* seedlings , the two fungi *F. o. ,F.pg*. were more shorthand for the percentage of germination of rice seeds that reached 10.34, 9.20%, respectively, in compared with control 86.25%, while in *Echinochloa*, the fungi *F.pg., R. s., F. o*. and

T.h.t. were the most influential fungi in reduced germination rate, which amounted to 8.21, 12.65, 16.24 and 20.56 %, respectively in compared with control 85.15 % ,as a result the above fungi were achieved significant reduction in the lengths of shoot and root and fresh and dry weights of rice and *Echinochloa* seedlings (SaadEddin, 1999).

Table 3. Effect of filtrates of studied fungi in the germination rate, seedlings death, lengths (cm), fresh and dry weights (gm) of plumile and radicle of rice and *Echinochloa* plants grown in petri dish

Fungi species		germination % rate	lengths (cm)		plumile weights (gm)		weights radicle (gm)	
			Plumile	radicle	fresh	dry	fresh	dry
A.niger	R	82.06	1.98	1.10	0.060	0.021	0.022	0.006
	E	71.06	1.35	0.65	0.048	0.012	0.014	0.005
A. terreus	R	65.20	1.21	0.95	0.045	0.012	0.021	0.005
	E	68.82	1.05	0.045	0.046	0.011	0.013	0.004
A. oryzae	R	62.17	1.05	0.90	0.041	0.014	0.022	0.004
	E	64.19	0.95	0.047	0.044	0.010	0.011	0.004
C globosumKunze	R	88.50	2.85	1.88	0.080	0.026	0.031	0.009
	E	87.43	2.13	1.03	0.061	0.036	0.042	0.006
C.elatum	R	93.12	2.97	1.96	0.082	0.031	0.038	0.010
	E	93.55	2.64	1.51	0.072	0.046	0.051	0.008
F.oxysporum	R	24.43	0.43	0.25	0.031	0.011	0.015	0.002
	E	14.34	0.37	0.21	0.012	0.007	0.009	0.001
F.pseudogramiarum	R	20.23	0.38	0.23	0.028	0.010	0.014	0.002
	E	09.20	0.32	0.19	0.010	0.006	0.008	0.001
P.griseofulvum	R	65.09	1.75	1.21	0.055	0.027	0.029	0.003
	E	74.89	1.27	0.73	0.031	0.017	0.020	0.002
P.oxalicum	R	91.41	1.90	1.20	0.063	0.030	0.035	0.003
	E	91.44	1.34	0.80	0.036	0.020	0.024	0.003
E.nidulans	R	90.41`	2.01	1.28	0.065	0.033	0.047	0.006
	E	90.02	1.41	0.83	0.041	0.023	0.030	0.003
T.harzianum .t.	R	93.51	3.05	1.98	0.202	0.081	0.091	0.012
	E	12.50	0.72	0.36	0.021	0.010	0.015	0.003
T.harzianum .a.	R	94.87	3.13	2.01	0.231	0.091	0.103	0.021
	E	95.15	2.85	1.53	0.084	0.052	0.063	0.009
R. solani	R	81.50	1.76	0.94	0.041	0.021	0.022	0.004
	E	17.50	0.51	0.25	0.014	0.008	0.011	0.002
Control	R	85.32	1.86	1.15	0.102	0.037	0.043	0.013
	E	85.53	1.34	0.81	0.035	0.024	0.030	0.004
L.S.D. 0.01	R	3.119	1.088	0.619	0.087	0.041	0.025	0.004
	E	43.420	1.031	0.491	000223	0.0188	0.0205	0.0021

The fungi *T.h.a., T.h.t.*, and *C.e* have achieved the highest rates in the rice seed germination that reached 94.15, 93.50 and 92.55%, respectively, while the results of the *Echinochloa* indicated that *T.h.a* And *C.e* led to a significant increase in the percentage of germination of 95.75, 92.25%, respectively. This may be attributed ,that these fungi are secreted cellulase enzyme (Decal and others, 2000), and as a result, it led to a significant increase in the lengths of shoot and root of rice and *Echinochloa* seedlings ,or to their ability to secrete some of growth regulators and catalysts for the germination and growth

of plants as wheat, rice, and these results agreed with what referred by Dewan, Musawi (2005); Dewan and others, (2007). The fungus *R.s*. achieved germination rate of rice seedlings of 77.50%, also gave increase in lengths plumile and radical that reached 17.75, 9.56 cm, respectively, as well as in fresh and dry weight, this result is inconsistent with previous studies indicating that the fungus *R.s.* characterized with the high pathogenesis ability (Diwan and Bahadli, 1985) ,as well as characterized by its ability to secrete enzymes and pathogenic toxins for plants, so this is inconsistent with Hamoudi (1999), Alwan (2005), or may be due to genetic differences between isolates (Barreto et al, 2003) Table(4).

Table 4. Effect of studied fungi (held on wheat straw) in the germination rate , seedlings death, lengths (cm), fresh and dry weights (gm) of plumile and radical of rice and *Echinochloa* plants grown in spots.

Fungi species		germination % rate	lengths (cm)		plumile weights (gm)		weights radicle (gm)	
			Plumile	radicle	fresh	dry	fresh	dry
A.niger	R	74.31	17.32	11.17	0.319	0.082	0.091	0.036
	E	76.06	9.34	3.92	0.317	0.061	0.069	0.026
A. terreus	R	67.21	16.34	10.44	0.315	0.078	0.086	0.031
	E	68.82	7.10	3.21	0.212	0.057	0.058	0.021
A. oryzae	R	63.21	16.84	10.77	0.320	0.080	0.081	0.026
	E	65.70	8.06	3.41	0.215	0.067	0.057	0.016
C globosumKunze	R	88.53	21.94	14.11	0.496	0.133	0.142	0.058
	E	86.43	13.34	6.14	0.428	0.101	0.114	0.041
C.elatum	R	92.55	22.17	14.31	0.411	0.139	0.154	0.065
	E	92.25	14.46	7.18	0.475	0.110	0. 117	0.046
F.oxysporum	R	10.34	4.61	1.55	0.165	0.051	0.059	0.012
	E	16.24	6.23	1.53	0.112	0.031	0.038	0.009
F.pseudogramiarum	R	09.20	4.54	1.22	0.151	0.035	0.042	0.011
	E	08.21	5.10	1.22	0.111	0.025	0.031	0.008
P.griseofulvum	R	72.19	18.07	11.65	0.401	0.092	0.099	0.029
	E	70.89	9.43	4.59	0.237	0.071	0.078	0.029
P.oxalicum	R	91.44	19.86	13.16	0.472	0.112	0.123	0.043
	E	90.84	12.15	5.74	0.318	0.083	0.088	0.031
E.nidulans	R	89.20	20.71	13.31	0.490	0.113	0.130	0.044
	E	91.58	12.80	5.78	0.321	0.085	0.091	0.036
T.harzianum .t.	R	93.50	22.54	14.53	0.530	0.144	0.171	0.069
	E	20.56	7.75	2.25	0.198	0.037	0.045	0.019
T.harzianum .a.	R	94.15	23.71	14.65	0.561	0.151	0.186	0.074
	E	95.75	14.85	7.75	0.481	0.131	0.142	0.054
R. solani	R	77.50	17.75	9.56	0.201	0.105	0.112	0.033
	E	12.65	4.12	1.18	0.103	0.023	0.030	0.007
Control	R	86.25	19.65	12.91	0.467	0.109	0.121	0.041
	E	85.15	12.16	5.61	0.315	0.081	0.086	0.030
L.S.D. 0.01	R	5.147	2.245	1.079	0.0275	0.0112	0.0153	0.0164
	E	6.716	2.119	1.625	0.0351	0.0180	0.0122	0.0141

REFERENCE

Abdul Aziz, M. H. A. 2001. Response to different varieties of tomatoes to infection fungus *Fusarium oxysporum* f. sp. *lycopersici*. Master. College of Agriculture University of Basrah.

Al-Helo, A.Y. 1995. Some fungi associated with the roots of tomato and its relationship to the growth of the host and seedling death caused by fungus *Fusarium oxysporium* fs.p. *lycopersici.* Master. College of Agriculture - University of Basra .P62

Al-Musawi ,A. A. I.Y.2003.Impact of agriculture soils separated in the activity of the fungus resistance bio *T harzianum* and fungi that cause wilting tomato and its relationship to the growth and productivity of tomato plants . Master. College of Agriculture. University of Kufa.

AL-Rawi ,A. A.K.B.H.1997.The impact of nitrogen and potassium and the overlap between them on the rice crop. Iraqi Journal of Agricultural Sciences.28(2)49-54.

Al-Rickabi,F.A.A.2008.Effect of extracts of vegetative growth for some bush on the pathological fungi to the roots of tomato mushroom biotic resistance *Trichoderma harzianum* Rifai. Master. Faculty of Agriculture - University of Kufa.

Alwan, S. L. 2005. Manufacturing preparation for a new bio-pesticide of the fungus *Trichoderma harzianum* Rafai to combat disease seed rot and seedling death caused by fungi *Rhizoctonia solani* and *Pythium aphanidermatum*. PhD thesis , Faculty of Education – University of Kufa .

Al -Younis,A. H. A. S.1987.Grains and legumes crops , production and improved grounds - theoretical and practical . Ministry of Higher Education and Scientific Research -Faculty Agriculture - University of Baghdad .

Al -Zobaie,I. A. I.2000.Identify sources of infection and some initial conditions predisposing to injury potatoes of genus *Fusarium* species and bio-resistance . Master. College of Agriculture. University of Baghdad .

Baltazal, A. M. and R. J. Smith. 1994. Propanil-resistance barnyard grass (*Echinochloacrus-galli* L.) control in rice (Oryza *sativa* L.). Weed Tech.8:576-581 .

Barnnett,H. L. .1965. Illustrated genera of imperfect fungi, 3rdedition .Buijess Publishing com .U.S.A .

Barreto, D.,S. Babbitt ,M. Gallyand B. A. Perez .2003. *Necteria haematococca* Causin RooRot in Olive Greenhouse Plants. Revistade la Soiciedad Argention de Horticultura 32 (1) 49-55.

Black , C.A. .1965b. Methods of soil Analysis .Part(2) Chemical and Microbiological Properties .Am.Soc.Agron .Inc.publisher, Madisoniscons in, USA.

Burgess, L. W., C.M. Liddell and B.A. Summeran1988.Laboratory manual for *Fusarium* research: Incorporation key and description of common species found in Australia .2nd Edition. Dept. of Plant Pathology and Agricultural Entomology. University of Sydney.

Carling, D.E., K.M. Kebler and R.H. Liener1986.Interaction between *Rhizoctonia solani* AG2738 plant species. Plant Dis. 70-578: 577 .

Decal , A. G.L.R. and Melgoreago,P .2000.Induced resistance by *Penicillium oxalicum* against *Fusarium oxysporum* f.sp. *lycopersici*: Histological studies of infected and induced tomato stems . Phytopathology.260:268-290.

Dewan, G. T., A. H. Al - Bahadli , S. A. Hassan and S. Jabr 1980. Disease rot Fusarium in some rice fields in Iraq. Technicia

Dewan,M.M.1989.Identity and frequency ofoccurrence of fungi in root of wheat andrye grass and their effect on take-all and host growth . Ph.D. thesis Univ. Western Austral .

Dewan, M.M., Ghisalberti, E.L., Rowland, C. and Sivasithamparam,K.1994.Reduction of symptoms of take-all of wheat and Rye-grass seedlings by the soil-borne fungus *Sordaria* fenicola. Applied soilEcology.45:1-51..

Dixon, P.B. 1993.Diseases of vegetable crops. Translated from the Prophet Mohammed Abu rich ,Saleh Mostafa Alnobarof . Arab House for publication and distribution. Pages 647.

Domsch, K.H., Gams, W. and Anderson, T.H. .2003. Compendium of soil fungi. Academic Press, London ,p894.

Dowd, P.F., J.D .Willer and R. Greenhalgh .1989 . Toxicity and interaction of some *Fusarium graminearum* metabolitic otocuterpillars. Mycologia. 81:646-650.

Evans, L.T .2002 .Crop and world food supply in crop evolution and origins of crop physiology. In: Crop Physiology. Evans, L.T. (ed.) Cambridge University Press. London1-22 .

Fischer, A., H. V.Ramírezand J. Lozano.1997Suppression of jungle rice [Echinocholoa (L.)Link] by irrigated rice cultivars in Latin America .Agro, J .89:516-521..

Ghisalberti, E.L., Narbey, M.J., Dewan, M.M. and Sivasithmparam, K. 1990. Viability among strains of *Trichoderma harzianum* in their ability and reduce take-all to produce pyrones. Plant & Soil., 121:291.

Hamoudi,A.H.M. 1999.Diagnosis of fungi present in the roots of wheat and its impact on the fungi *Rhizoctonia solani* Kuhn and *Fusaruim graminearum* Schwab. Ph.D. thesis , Faculty of Education , University of Basra .Pages221.

Harman, G.E., C.K. Hayes, M. Lorito, M., R.M. Broadway and A. Dipietro . 2000. Chitinolytic enzymes of *Trichoderma harzianum* : purification of chitobiosidase and endochitinase . Phytopathol.83:313-321.

Hayatsu, M. 1993. The lowest limit of pH for nitrification in tea soil and isolation of ammonia oxiding bacteria.39(2):219-226.

Hill, J.E., R.J. Smith and D.E. Bayer .2008 .Rice weed control: current technology and emerging issues in temperate rice. Aust. J. of Experimental Agriculture.34(7):1021-1029.

Howell, C.R., L.E. Hanson, R.D. Stipanovic, Puckhaber .2000. Inductiono Synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens* .Phytopathology 90 : 248-252 .

Inone,I.,F.Namiki and T.Tsuge 2002.Plant colonization by vascular wilt fungus *Fusarium oxysporum* requires FOW ,1ageneencodinga mitochondrial protein. The Plant Cell, American Society of Plant Biologists.14:1869-1877.

Lauren, D.R. and R .Greenhalph .1987 .Trichothecenes produced by *Fusarium* species chemistry and analysis. J. Assoc. off Anal. Chem.70:479-480..

Limon, M.C.,I.A. Pintro-Toro and T. Benitez 1999. Increased antifungal activity of Trichoderma harzianum transformants that over express a33-Kolachitinase. Phytopathology.89: 254-261.

Macnish,G.C., D.E. Carling, M.W. Sweetinghaw,A. Ogoshi and K.A. Brainard, 1995.Characterization of anostomosis group10) CAG-10 (of *Rhizctonia solani*. Australian PlantPathol.14:252-260.

Montealegre,J.R., R. Reyes, L.M. Perez, R. Herrera, P. Silva and X. Besoain 2003.Selection of bioantagonistic bacteria to be used in biological control of *Rhizoctonia solani* in tomato. Environ . Biotchnol.6:1-8.

Moubasher,A.H. and A.A. Aisha 1987. Soil fungi state of Qatar . Scientific and applied research center university of Qatar.

Moubasher, A.H. and M.B.Mazen1994.Further studies on cellulased ecomposing soil fungi in Egypt .Abhath Al– Yarmock . J.33:3-5.

Moustafa, A.f. .1982 Taxonomic studies on the fungi ofKuwait.J.univ.Kuwaitsci.9:246-250.

Slaton. N, and R. Norman,2006.DD 50 Computerized Rice Management Program. Rice Production Handbook-MP.USA.pp.192.

Tai , A.A.K.. 2000.Harvest dates in effect holds and the quality of some rice varieties . Master. Field Crops Science Department - Faculty of Agriculture / University of Baghdad . P89. .

Van Wyk, J.P.H. and M. Mohulatsi. 2003. Biodegradation of waste-paper by cellulose from *Trichoderma viride*. Bioresource Technology. Vol . 86 .P 21-23 .

Zeilinger, A.H.2003.Extraction and determination of proteins from plants residuals treated with fungi . Microbiol.55:1270-1344.

Windham, M.T., Y. Elad and R. Baker 1986.Amechanism for increased plant growth induced by *Trichoderma* spp. Phytopathology. 76:518-521. agent *Trichoderma harzianum*. Appl. Environ.Microbid.65(3):1061-1071.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

EVALUATING THE POTENTIAL PRODUCING BIOGAS FROM WHEY, A BY-PRODUCT DERIVED FROM DAIRY

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Abstract

Biogas production from cheese whey as an energetically rich product that can contain more than 50 g/ L lactose was investigated. Whey, a by-product derived from the dairy industry, represents an optimal as primary material for biogas production by anaerobic methanogenic fermentation, compatible with liquid manure. In the analysis performed, it was concluded that whey development fulfills all the conditions favorable environment bacterial anaerobic digestion. Whey, having an organic carbon content of 0,798% was used. In our study, AD batches consisting of whey, and 4% inoculum, in 5 l bench top digester were conducted. Our results indicate biogas yields over 1000 cm^3/ L fermentation medium. The concentration of main components in biogas was measured as well. Conclusion – anaerobic digestion of cheese whey is acceptable, with acidification substrate provided in the first phase.

Key words: *anaerobic digestion, biogas, methanogenic bacteria, whey.*

INTRODUCTION

Dairies are industries with high potential for environmental pollution. Pollution is the result of the waste from the process of obtaining the cheese, the main waste whey resulting from coagulation of milk. Whey is the liquid which remains after the removal of caseins from milk and is characterized by a high organic content (BOD5 approx. 50 g / l to the limit of 0.3 g / l of a normal water).
Worldwide is an absolute recipes in individual processing technologies and whey separation. Worldwide is an absolute recipes in individual processing technologies and whey separation. Only half of the whey produced worldwide is employed for human food or animal feed. The rest, by discharging to the environment, put complicated pollution problems exacerbated by the fact that a liter of whey has a biochemical oxygen demand (BOD) 50,000 mg / l, compared with 300 mg / l for affluent discharged from urban centers.
Due to the high cost of treatment whey treatment plants in some countries practice the use of whey for irrigation of agricultural land and pastures, or discharge into streams or oceans.
At first, the use of whey as a fertilizer may present some advantages, but in time, progressive mineralization of soil causes difficulties in cultivating plants. The main objective of the project is to develop, implement and promote technologies and equipment for treating whey in compact stations to reduce the environmental impact of industrial activities milk processing. Cheese production is an important part of the dairy industry in the European Union as

more than 40 % of the European Union (EU) milk is processed into cheese [1]. In the milk quota year 2008/2009 milk delivered to the dairies reached 133 621 102 tons [2]. There are a lot of varieties of cheese resulting in different cheese making technologies, but in average the final volume of whey is about 85- 90 % of the volume of the processed milk. It can be estimated that more than 45 million tons of cheese whey each year are produced in the EU. Whey has already been utilized directly as animal feed, processed for human consumption or used as field fertilizer, but usage of whey for energy production is not widespread. Although whey has sufficient biogas potential it is complicated substrate for biomethane production due to the process instability

The main components of cheese whey are: lactose (44-52 g·l-1), protein (6.1-6.6 g·l-1), fat (0.2-0.3 g·l-1) and minerals (5-7.9 g·l-1) [3]. Under anaerobic conditions lactose (main component of whey solids) is rapidly broken down into short chain fatty acids – acetic, propionic, butyric and other acids. As whey has little or no buffering capacity, pH drops dramatically inhibiting activity of methanogens what results in low gas yields with a low methane content [4; 5]. To control the optimal pH level for methanogenic bacteria several techniques have been described. Scientists of ten propose to co-ferment whey together with substrates with sufficient buffering capacities [5; 6; 7]. Ghaly and Ramkumar used a pH measurement and control system which consisted of a computer controlled pH electrode and peristaltic pump. At prescribed time intervals (30 min.) pH was automatically measured and compared with the setpoint of pH 7. If pH was lower than 6.9 the peristaltic pump added basic solution – 2.5 N NaOH [5]. Ghaly and Ramkumar reported biomethane production of 0.51 vm·vr -1·d-1 (vm – volume of methane, vr – volume of reactor, d – day) in the two stage continuous anaerobic digester with the loading rate 10 l·d-1 (hydraulic retention time (HRT) 15 days) [5]. According to Kavacik et al. codigestion of cheese whey and dairy manure in continuous fermentation with HRT of 5 days and 8 % of total solids resulted in 0.906 vm·vr -1·d-1 [6]. In the continuously stirred pilot reactor the methane yield of 2.2 vm·vr -1·d-1 of diluted poultry manure and whey mixture was reported by Gelegenis et al. [4].

As described above organic carbon is measured before inoculation and after fermentation has finished ~ 50 days.

Cominio et al. investigated biogas potential of cow manure and whey biomass mix and achieved 211.4 lm·kgvs -1 (lm – liters of methane, kgvs – kg of volatile solids) [7].

MATERIALS AND METHODS

Raw materials

The raw materials used to construct the fermentation batches are:

Whey the liquid that remains after the casein and milk fat were removed and is characterized by a high organic content (BOD5 approx. 50 g / L, compared to the limit of 0.3 g / L of an ordinary water), fresh dairy cows manure collected in the day of starting the experiment

The manure represents an important source of microorganisms for biogas fermentation.

Table1 – *Characteristics and chemical composition of cheese whey*

Characteristic	Unit	Value
COD	mg/ L	60 000
Lactose	g/ L	50
TS	g/ L	55
VS	g/ L	49
Proteins	g/ L	2.2
Phosphate	g/ L	0.6
Ca	g/ L	0.02
pH		5,5 – 6,6

1. Pretreatment of the raw material and experimental pattern

a. *Unpretreatement biomass*

The seed material was obtained from anaerobic batch reactors used for seed material cultivation in own laboratory. Diluted cow manure was used as the initial substrate. Fresh inoculum from the batch reactor, witch produced biogas with average methane content of 60 % was used for the startup procedure**.**

b. *Physicochemical pretreatment*

Whey mixed to fresh manure were pretreated by a combination of thermal and alkaline pretreatment: the biomass soaked in 2% NaOH was autoclaved 30 minutes at 121 ^{0}C. The biomass was afterwards washed with 10% H_2SO_4 until pH 6.5 and with 12 equivalent volumes of water in order to remove the inhibitors resulted during pretreatments.

c. *Determination of organic carbon* Organic carbon content in the substrate is calculated by the amount of dichromate consumed for the oxidation of anhydride. Anhydride excess dichromate is titrated with a solution of 0.2% normal Mohr salt in the presence of a redox indicator.

The indicator used in titration of the excess oxidant was orthophenanthroline whose turn from blue to red is easier to follow. The equipment used consisted of:

- Magnetic stirrer (magnet wrapped in glass or plastic)
- Porcelain crucible with a diameter of 20-25 cm
- Burette Mohr salt halfmicro

As reactive 1,485g and 0,695g orthophe-nanthroline using ferrous sulfate is bringing to volume in a 100 ml volumetric flask. The way of working is to add 5-6 drops of indicator titrating with Mohr salt solution drop by drop until the solution turn from blue-green to scarlet red. It is considered that recovery of organic carbon is done in the normal range when oxidizing reagent consumption does not exceed 75% of the added.

RESULTS AND DISCUSSIONS

In this research, the UAPB was continuously operated with HRT of 6 to 24 h.

Experiment was conducted over a period of 49 days following distinguished periods: from the beginning until day 11

Day 11 - Day29

Day 29- Day 37

Day 37- Day 49

early in the biogas production increased gradually decreasing as the microorganisms have adapted to the acidity of the whey.

The bioreactor was successfully started with HRT of 24 h, and then the removal rate was gradually increased.

While the HRT was decreased stepwise to 16 h, maximum film was built on the surface of packing. The lactose concentration at downstream drastically dropped to zero.

The COD and lactose in the effluent gradually increased as the retention time decreased stepwise

The bioreactor was successfully started with HRT of 24 h, and then the removal rate was gradually increased.

While the HRT was decreased stepwise to 16 h, maximum film was built on the surface of packing. The lactose concentration at downstream drastically dropped to zero.

The bioreactor was successfully started with HRT of 24 h, and then the removal rate was gradually increased.

CONCLUSIONS

1. Anaerobic digestion of local cheese whey in a bioreactor is acceptable.
2. Usage of pH control should be considered in anaerobic digestion of local cheese whey in a bioreactor is acceptable.
3. Smaller volume of reactor should be considered.

4. The novel anaerobic bioreactor with high performance was able to handle the high organic load.

Figure 1. Laboratory plant for biogas production

Composition of biogas production during different periods of the experiment

Period days	CH_4%	CO_2%	O_2%	H_2S ppm
0-11	56,2	16,8	4,8	83
11-29	65	22	2,9	178
29-37	53	23,3	3,8	176
37-49	62	18,6	3,4	76

ACKNOWLEDGEMENTS

This study was supported by project of doctoral studies "Technologies for biogas production from agricultural and dairy wastes".

REFERENCES

1. Communication from the Commision to the Council 2009/385/EC of Jully 2009 on Dairy Market Situation.
2. EC Directorate-General for Agricultural and Regional development Agricultural Council Quarterly Report on the Dairy Market, Brussels, 2010.
3. Fox P.F., Guinee T.P.,. Cogan T.M., McSweeney P.L.H. "Fundamentals for the Examination of Water and Wastewater 21st Ed. Washington DC, 2005 Cheese Science.
4. Gelegenis J., Georgakakis D., Angelidaki I. and Mavris V. "Optimization of biogas production by co-digesting whey with Cheese Science". Gaithersburg, Maryland: Aspen Publishers, Inc., 2000
5. Ghaly A.E., Ramkumar D.R. "Controlling the pH of Acid Cheese Whey in a Two-Stage Anaerobic Digester with Sodium.
6. Kavacik B. and Topaloglu B. "Biogas production from co-digestion of a mixture of diluted poultry manure". Renewable Energy 32 (13), 2007. pp. 2147-2160
7. Digester for biogas production from cow manure and whey mix." Bioresource Technology 100 (21), 2009. pp. 5072- 5078.
8. Apha, awwa&Wef, Standard methods for the Examination of Water and Wastwater, 21st , Ed. Washington DC
9. Vindis P, Mursec B, Janzekovic M, Cus F. The impact of mesophilic and thermophilic anaerobic digestion on biogas production. J. Ach. Mater. Manuf. Eng. 36, 192-198 (2009).
10. V. Nikolic, T. Vintila, Producerea şi utilizarea biogazului pentru obţinerea de energie, Editura Mirton Timişoara, (2009).

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

GENERAL ASPECTS REGARDING THE RECOGNITION OF MAIZE HAPLOID KERNELS ACCORDING TO THE SIZE AND INTENSITY OF ANTHOCYANIN COLORATION

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Abstract

In the last decade, the use of double haploid lines in maize breeding programmes has become a standard procedure. This has become possible due to the substantial progress achieved by using in vivo maternal haploid technology induction. Currently, there are in use haploid inducers with inducing efficiency up to 15 %, making possible utilization of double haploid (DH) technology on a large scale. The most important advantages of this system is to shorten the period for obtaining homozygous lines (only 2 years) as compared to the conventional system (5-7 years) and high recognition precision of the putative haploid kernels. The anthocyanin coloration of the aleurone can vary from a small patch to covering the entire aleurone zone, exception kernel basis. Intensity of anthocyanin can range from very poor to strong anthocyanin both in the embryo and aleurone .
According to the literature, haploids obtained from dent genotypes are more easily recognized and both anthocyanin coloration and intensity of the coloration are better expressed than in flint genotypes. Since 2013, at NARDI Fundulea, we made some observation regarding the size and intensity of the anthocyanin coloration for the dent and flint genotypes studied. Annotations done on both aleurone and embryo were assessed with scores from 0-4: 0 for the lack of pigmentation and 4 for intense pigmentation. The objective of the present study was to see what score corresponds to a good choice of the haploid kernels.

Key words: *anthocyanin coloration, doubled haploid lines, haploid inducers.*

INTRODUCTION

Every year the DH technology advances more and more, the needs of haploid plants are becoming larger, but inducers are showing disadvantages in achieving this goal. The reason might be the fact that the rate of induced putative haploid kernels (PHK) is not high enough or females express instability in the manifestation of the anthocyanin coloration based on *R1-nj* gene that is involved in the synthesis of anthocyanin and used as genetic marker (Sarmaniuc M. et al., 2013). For the above system to work, an efficient screening system for separating the PHK from non-haploid seeds was needed. The anthocyanin marker gene, *R1-nj* (Nanda and Chase, 1966; Chase, 1969; Neuffer et al., 1997; Eder and Chalyk, 2002; Röber F.K. et al., 2005) was used for this screening process. However, the expression of this gene has a strong female influence sometimes the screening of PHK might be confusing or even impossible, especially in cases when there are inhibitor genes (*C1-I*) in female genotypes (common for flint maize). Even if there were no inhibitors of the *R1-nj* gene, but the moisture of kernels during the harvesting was high, the screening of haploids might be more difficult as well (Rotarenco V. et al., 2010). Since the material to be induced is very diversified it is necessary to find a marker system that allows more precise recognition of PHK from the mass of diploid dried kernels harvested from inducing fields and thereby saving costs involved in artificial chromosomal doubling and saving greenhouse and field space and labor allocated to this process (Prasana B.M.. et al., 2012). The procedure for the production of maternal haploids allows obtaining haploids

from different genotypes on a large scale (Deimling et al. 1997; Chalyk and Rotarenco 1999; J. Eder., S. Chalyk, 2002).
The aim of this study was to determine the behavior of different female sources used (dent and flint forms) with regards to the size and intensity of anthocyanin coloration and influence of these two parameters in the precise selection of the haploid/diploid forms.

MATERIALS AND METHODS

The study was carried out at the National Institute of Research and Development Fundulea in 2013.
As female sources, 22 synthetic and F2 populations were used. Each female sources was crossed with the inducer line in the field. Grains resulted from crosses were divided in 3 categories based on the expression of the anthocyanin coloration given by *R1-nj* gene on the kernel as follows: category 1, kernels with no coloration on the aleurone and embryo; category 2, kernels with coloration in both, aleurone and embryo and category 3, PHK with purple coloration only on the aleurone. A 0-4 scale was used for visual assessment of the intensity of anthocyanin coloration on aleurone and embryo within category 2 (kernels with coloration in both aleurone and embryo): 4=intense pigmentation, 3=normal pigmentation, 2=poor pigmentation, 1=very weak pigmentation and 0=lack of pigmentation) at the level of total induced kernel bulk for each population.
The visual selection of the PHK was verified using cytological analysis. Root tips were cut from a sample of 20 PHK of each of 7 populations selected randomly.
The chromosome complement of plantlets was established by means of chromosome counts on root-tips squashed, stained by Feulgen method.

RESULTS AND DISCUSSIONS

According to the results obtained by Eder and Chalyk (2002); Kebede et al. (2011), the female sources influence the haploid rate, and those obtained by Coe (1994) demonstrated the influence of the female on the expression of the marker gene. The need to obtain haploid plants from a large spectrum of genetic backgrounds determined detailed analysis of phenotypic manifestation of anthocyanin markers. As it can be seen in table 1 and 2, the percentage of haploid varied between 0.64 and 6.68 and the scores received for the size and anthocyanin intensity varied between 1 and 4, for both embryo and aleurone.
Best percent of PHK were obtained from dent populations that received score 3 on the embryo, level that allows easy identification of PHK. Populations 3, 11 and 15 that have been scored 1-2 for anthocyanin coloration intensity on embryo, produced an induction efficiency of less than 2%. In this case the identification of haploid is possible but errors could occur due to very weak staining in the embryo. Population 3 that had an induction efficiency less than 1% (0.65%), has flint kernel and received the score 1 for anthocyanin coloration of the embryo; the anthocyanin coloration was almost inhibited in this population (table 1).

Table 1. Categories of kernels by type of coloration and efficiency of the inducer expressed as % PHKs from total kernels analysed.

Genotype	Kernel type	Total kernels analyzed	Categories of kernels by type of coloration			% PHK
			CAT. 1	CAT. 2	CAT. 3	
Population 1	D*	3932	730	3103	99	2.51
Population 2	D	4862	674	4047	141	2.90
Population 3	F**	1395	719	667	9	0.64
Population 4	D	3208	637	2416	155	4.83
Population 5	D	2826	736	1943	147	5.20
Population 6	F	789	100	667	22	2.78
Population 7	D	1852	425	1394	33	1.78
Population 8	D	2052	590	1398	64	3.11
Population 9	D	583	70	484	29	4.97
Population10	D	884	78	786	20	2.26
Population 11	D	2483	230	2207	46	1.85
Population 12	D	3728	522	3127	79	2.11
Population 13	D	1457	415	987	55	3.77
Population 14	D	938	279	616	43	4.58
Population 15	F	1497	408	1071	18	1.20
Population 16	D	4055	667	3208	180	4.43
Population 17	F	1286	221	979	86	6.68
Population 18	F	2459	447	1914	98	3.98
Population 19	D	2435	234	2147	54	2.21
Population 20	D	1718	533	1140	45	2.61
Population 21	D	2164	494	1628	42	1.94
Population 22	F	2569	274	2216	79	3.07
TOTAL		49172	9121	38145	1544	Average: 3.15

*D= Dent type
**F= Flint type

As shown in table 2, the majority of populations with dent kernel were scored 3 and 4 on embryo with one exception, the population 11, which registered a score of 2 on the embryo. In the other hand, flint kernel populations showed a lower intensity, embryo coloration being generally scored 2, but population 3 with a score of 1 and reversely population 17, that was scored 3, at the level of dent populations.

Table 2. The anthocyanin pigmentation influence of the female on embryo and endosperm

Genotype	Type of kernel	Score of intensity of anthocyanin pigmentation	
		Embryo	Aleurone
Population 1	D*	3	2
Population 2	D	4	3
Population 3	F**	1	3
Population 4	D	3	2
Population 5	D	3	3
Population 6	F	2	3
Population 7	D	3	2
Population 8	D	3	3
Population 9	D	3	3
Population 10	D	3	3
Population11	D	2	3
Population 12	D	3	3
Population 13	D	3	3
Population 14	D	4	2
Population 15	F	2	2
Population16	D	4	4
Population17	F	3	2
Population18	F	2	3
Population 19	D	3	3
Population 20	D	4	3
Population 21	D	3	3
Population 22	F	2	2

* D= Dent type
**F=Flint type

Cytological analysis (table 3; figure 1 and 2) showed that scored 3 for anthocyanin pigmentation of the embryo, conferred the best efficiency in selecting real haploid kernels. Score 2 for anthocyanin pigmentation of the embryo, the visual selecting of PHK has a lower level of confidence, a relative large number of errors can occur, as in the case of population 18 and 22.
According to the literature, haploid obtained from dent genotypes are more easily recognized and both anthocyanin coloration and intensity of the coloration on bouth aleurone and embryo are better expressed than in flint genotypes.

Table 3. Cytological analysis for 7 random sources females

Population	Kernel type	Intensity of anthocyanin pigmentation – score embryo/aleurone	Number of kernels analyzed	Number of confirmed real haploid kernels	% confirmed haploid kernels
Population 1	D*	3/2	20	15	75
Population 18	F**	2/3	20	11	55
Population 7	D	3/2	20	18	90
Population 22	F	2/2	20	7	35
Population 13	D	3/3	20	19	95
Population 16	D	4/4	20	14	70
Population 17	F	3/2	20	16	80
Total			140	100	Average=71

*D= Dent type
**F= Flint type

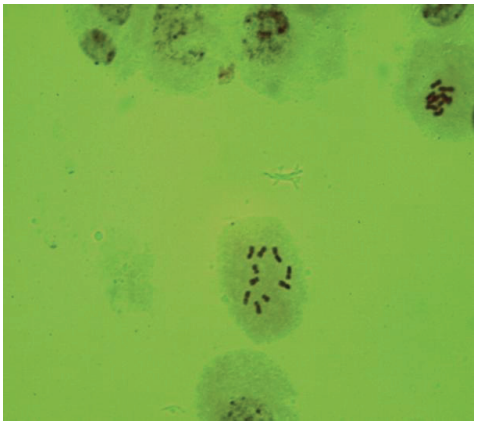

Figure 1. Mitotic metaphase; haploid plant with 10 chromosomes (photo taken during cytological analysis)

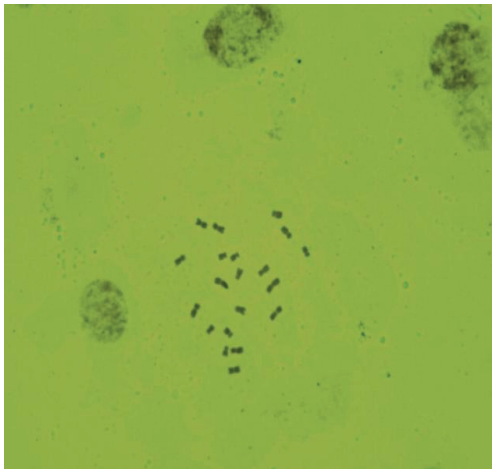

Figure 2**.** Mitotic metaphase; diploid plant with 20 chromosomes (photo taken during cytological analysis)

CONCLUSIONS

The anthocyanin coloration in both aleurone and embryo is significantly better expressed in dent populations than in flint populations; identification of haploid kernels is more confident in dent type, the real kernel percent (cytological confirmed), being over 70%.

Scores 1 and 2 for anthocyanin pigmentation of the embryo, registered generally only in flint populations resulted in a lower precise selection of PHK.

There is a stringent necessity to improve a DH procedure at NARDI-Fundulea in the very next years, by utilization of appropriate protocol to ensure a better anthocyanin pigmentation as well as to increase the inducing efficiency by testing more inductor sources.

REFERENCES

Chalyk S.T., Rotarenco V., 1999. Using maternal haploid plants in recurrent selection in maize. Maize Genet Coop Newslett 73, p. 56–57.

Chase S.S., 1969. Monoploids and monoploid derivatives of maize (Zea mays L.). Bot Rev 135, p. 117–167

Coe E., 1994. Anthocyanin genetics. In: Freeling M, Walbot V (eds) The maize handbook. Springer-Verlag, New York, p. 279–281.

Deimling S, Röber F, Geiger H.H., 1997. Methodik und Genetik der in-vivo-Haploideninduktion bei Mais. Vortr Pflanzenzüch-tung 38:203–204.

Eder J., Chalyk S., 2002. In vivo haploid induction in maize. Theor. Appl. Genet, 104, p. 703-708.

Kebede A. Z., Dhillon B. S., Schipprack W., Araus J. L., Banziger M., Semagan K., Alvarado G., Melchinger A.E., 2011. Effect of sources germolasm and season on the in vivo haploid induction rate in tropical maize. Euphytica 180, p. 219-226.

Nanda D.K., Chase S.S., 1966. An embryo marker for detecting monoploids of maize (Zea mays L.). Crop Sci 6, p. 213–215.

Neuffer M.G., Coe E., Wessler S.R., 1997. Mutants of maize. CSHL Press, New York, USA.

Prasanna B.M., Chaikan V., şi Mahuku G., 2012. Doubled Haploid Technology in Maize Breeding: Theory and Practice, D.F.: CIMMYT, Mexico.

Rotarenco V., Dicu G., State D. and Fuia S., 2010. New inducers of maternal haploids in maize. Maize Genetics Cooperation Newsletter vol. 84, p. 1-7.

Röber F.K., Gordillo G. A., Geiger H.H., 2005. *In vivo* haploid inductionin maize – Performance of new inducers and significance of doubled haploid lines in population breeding. Maydica 50, p. 275-283.

Sarmaniuc M., Mihailov M., Rusu G., 2013. Eficiența noilor inductor în obținerea haploizilor materni la porumb (Zea mays L.). Buletinul Acad. de Ştiințe a Moldovei. Ştiințele vieții, nr.2, p. 105-110.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

COMPARATIVE ANALYSIS OF POLYPHENOLIC PROFILES AND ANTIOXIDANT ACTIVITY OF *Agaricus bisporus* AND *Agaricus campestris*

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Abstract

In the context of a worldwide growing population there is a need for renewable biological resources for food and animal feed, safe and healthy, as well as materials, energy and other products. Thus, an important and sustainable use of renewable resources has become one of the major goals of bio-economic strategy at the national and European levels. Quality, food safety and human health are some of the major concerns of Romania. Due to considerable genetic resources and remarkable biological quality, mushrooms are currently considered functional foods with important nutritional and therapeutic qualities. Nationally, 90% of the mushroom production is covered by Agaricus bisporus species (champignon), being consumed mainly in urban areas. At the same time in rural areas is harvested from spontaneous mycoflora and consumed, mainly Agaricus campestris species. These two species are related, belonging to the same Agaricaeae family. Nutritional and therapeutic important properties of these species are given by their bioactive metabolites including polysaccharides, proteins, dietary fibres, polyphenolic compounds and other bio-molecules. In this paper is presented comparative qualitative (HPTLC) and quantitative (spectrophotometric) chemical composition in polyphenolic compounds and the scavenger potential of free radical DPPH of the two species. The comparative analysis is made for alcoholic and hydroalcoholic extracts of the two species. The results shows the differences and similarities in terms of composition in polyphenolic compounds, dependence between chemical composition and antioxidant potential and also the importance of the solvent used to obtain the extracts. Thus, this study contributes to understanding the importance of the valorisation of indigenous natural resources potential, respectively of the species Agaricus campestris.

Key words*: polyphenolic profiles, Agaricus campestris, Agaricus bisporus, antioxidant activity.*

INTRODUCTION

At national level, the bio-economy, benefits of the huge potential of Romanian agriculture and wild flora. Capitalization of this potential, in the context of an increasingly active local food industries and rising standards, achievements of applied research in the field and in the food and pharmaceutical industries, is a major economic target. The need for renewable biological resources for food and animal feed, safe and healthy, as well as materials, energy and other products, is increasing in the same time with the growth of population number. In Romania, the interest for quality, food safety and human health become one of the major concern at national level. Due to considerable genetic resources and remarkable biological quality, mushrooms are currently considered functional foods with important nutritional and therapeutic qualities (Perera and Li, 2011, Mizuno et al., 1995).

Rich in high-quality protein, containing a high level of dietary fiber and a high proportion of unsaturated fatty acids, rich in various vitamins and minerals, and having an acceptably low level of nucleic acids, mushrooms are suitability for daily use as a vegetable (Chang, 1999).

As therapeutic agents, mushrooms are useful in preventing diseases as hypertension, diabetes, hypercholesterolemia and cancer (Bobek and Galbavy, 1999; Bobek et al., 1995). There are also studies that have shown antioxidant, antitumor, antiviral, antithrombotic and immunomodulating properties (Mau et al., 2002; Subrata et al., 2012). With the increasing awareness of the population to food quality, nutritional and medicinal properties of them,

the mushroom demand will have a significant growth both nationally and internationally levels. Nationally, 90% of the mushroom production is covered by *Agaricus bisporus* species (champignon) (http://biotehnol. eu / mycoind/ abstract.html), being consumed mainly in urban areas. At the same time, in rural areas is harvested from spontaneous mycoflora and consumed, mainly, *Agaricus campestris* species. These two species are related, belonging to the same *Agaricaceae* family.

Nutritional and therapeutic important properties of these species are given by their bioactive metabolites including polysaccharides, proteins, dietary fibers, polyphenolic compounds and other bio-molecules (Popescu, 2006; Parvu, 1997).

In this paper is presented comparative qualitative (HPTLC- high performance thin layer chromatography) and quantitative (spectrophotometric) chemical composition in polyphenolic compounds and the scavenger potential of free radical DPPH of the two species. The comparative analysis is made for alcoholic and hydroalcoholic extracts obtained from both mushrooms. The results shows the differences and similarities in terms of composition in polyphenolic compounds, dependence between chemical composition and antioxidant potential and also the importance of the solvent used to obtain the extracts. Thus, this study contributes to understanding the importance of the valorisation of indigenous natural resources potential, respectively of the *Agaricus campestris* mushroom.

MATERIALS AND METHODS

Raw material - *Agaricus bisporus* (cultivated) sample was obtained from SC Camimar SRL (Arges County) - local producer of champignon mushroom. *Agaricus campestris* (wild) sample was obtained from University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnologies - mushroom collection.

Sample preparation: the samples was prepared by extraction with methanol or ethanol 50%(v/v) - vegetal material/ solvent rate - 1/20m/v for 2h at boiling temperature of the solvent. The solutions was filtered and frozen until analysis

Total phenol content - Total phenol content was determined with Folin - Ciocalteu method (European Pharmacopoeia 6,0). Brifly, 1 ml of extract was transferred to a 25 ml volumetric flask, 10 ml of water and 1 ml of Folin-Ciocalteu reagent was added. The volume was made to 25 ml with 5% sodium carbonate (w/v). The blend was left at room temperature for 30 minutes. Then the absorbance of the samples was read at 760 nm with a UV/VIS spectrophotometer (Helios λ, Thermo Electron Corporation). Distilled water was used as a blank. Total phenol content was determined from the extrapolation of the calibration curve (*$y=1,474x-0,438$, $R_2 = 0.992$)*, which was obtained for gallic acid (Sigma Chemical Co., St. Louis, USA) The results was expressed as miligrams of gallic acid equivalents (GAE) per gram of dried raw material.

(HP)TLC Analysis for phenols:

The densitometric analysis (HPTLC) was made according to TLC Atlas - Plant Drug Analyses (Wagner H Balt S. 1997). The characteristic fingerprint profile for phenolic compounds was determined. 3-3.5µl of the samples and 1-3µl of references substances (10^{-3} M rutin, hyperoside, chlorogenic acid, caffeic acid- Sigma-Aldrich) were loaded as 10 mm band length in the 20 x 10 Silica gel 60F254 TLC plate using Hamilton- Bonaduz, Schweiz syringe and CAMAG LINOMAT 5 instrument. Ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v) was the mobile phase. After development, plates were dried and derivatized in NP-PEG (Natural Product - Polyethylene glycol) reagent. The fingerprints were evaluated at 366nm in fluorescence mode with a WinCats and VideoScan software.

*Free radical scavenging assay- w*as evaluated using the Sanchez-Moreno et al. (1998) assay. The extracts concentration were 1%, 0.1%, 0.01%, 0,.01% in methanol. 50µl aliquots of the extract were mixed with 2950µl of the DPPH methanolic solution (0.025g/l). The radical scavenging activity of the extracts against 2,2-diphenyl-1-picryl hydrazyl radical (Sigma-Aldrich) was determined by measuring UV absorbance at 517nm. A blank solution was prepared containing the same amount of methanol and DPPH, and measured after standing at room temperature 30 minutes. The

radical scavenging activity (RSA) was calculated using the following formula:
% inhibition = {(AB – AA)/AB} x 100.
Where AB is the absorption of blank sample and AA is the absorption of tested extract solution. IC50 (the half maximal inhibitory concentration) was calculated from the graph of RSA percentage against extract concentration and expressed as mg/ml.

RESULTS AND DISCUSSIONS

Total phenol content

Agaricus campestris and *Agaricus bisporus* are both species of *Agariaceae* family.
An important study report that antioxidant activity of natural vegetal extracts are well correlated with their content in phenolic compounds (Velioglu et al., 1998).
Agaricus campestris - commonly known as field or meadow mushroom is white, wild and edible. Is can be seen from spring to autumn on manure or in well fertilized places on meadows, pastures and gardens. (Parvu, 1997).
Agaricus bisporus - also known as button mushroom is white or brown, cultivated and edible. At the present is one of the most cultivated mushrooms in the world.
Both species are reported regarding the content phenolic compounds (Woldegiorgis, et al 2014; Jun et al, 2013).
Therefore, is important to investigated the antioxidant activity of phenolic compounds of mushrooms methanolic and hydroalcoholic extracts. Table 1 shows the total phenol content of the four extracts expressed as gallic (GAE) acid equivalents per g of raw material.

Table 1. Total phenol content of mushroom extracts

No	Extract	mg (GAE)/g
1	Agaricus campestris (methanolic)	4.96
2	Agaricus bisporus (methanolic)	4.63
3	Agaricus campestris (hydroalcoholic)	12.19
4	Agaricus bisporus (hydroalcoholic)	5.2

In the present study the total phenol content in the methanolic extracts is similar in *Agaricus campestris* (4.96 mg (GAE)/g) and *Agaricus bisporus* extract (4.63 mg (GAE)/g). For *Agaricus bisporus* methanolic extracts the total phenol content obtained in other studies are 3.4 mg GAE/g dw (Palacios et al, 2011) and 4.5 mg GAE/g dw, from Spain (Ramirez-Anguiano et al., 2007), as for ethanolic extracts - 6.18 mg GAE/g dw, from China (Liu et al, 2013) and 8.0 mg GAE/g dw, from the United States (Dubost et al., 2007). For *Agaricus campestris* the total phenol content obtained by us 12.19 mg(GAE)/g for hydroalcoholic extract is similar to other results – 10.2 mg/g (Schaffer et al., 2004) and 14.6 mg GAE/g dw (Woldegiorgis et al., 2014).

(HP)TLC Analysis for phenols

Figure 1 shows the (HP)TLC phenolic profiles of the extracts: tracks - T2, T3 *Agaricus campestris* hydroacoholic extract (duplicate sample), track T4 - *Agaricus campestris* methanolic extract, tracks T5, T6 - *Agaricus bisporus* hydroalcoholic extract (duplicate sample) track T7 - *Agaricus bisporus* methanolic extract, tracks T1, T8 - references substances (rutin, chlorogenic acid, hyperoside and caffeic acid - duplicate sample).
The present HPTLC study have revealed that there are no differences between the methanolic and hidroalcoholic extracts (for both species).
For *Agaricus campestris* HPLC-DAD study have shown the presence of p-coumaric, acid ferulic acid, gallic acid p-hydroxybenzoic acid and myricetin (Woldegiorgis et al., 2014). In our study the phenolic profile (HP)TLC of *Agaricus campestris* have 7 major phenolic spots, that are phenolic acid derivate (caffeic acid Rf~0.97 and gallic acid Rf~0.92), according to Wagner and Bladt, (1996) and based on the relationship spot color - Rf.
For *Agaricus bisporus* caffeic acid (Rf~0.97) and gallic acid (Rf~0.92) are detected.

Free radical scavenging assay

When DPPH (2,2 - diphenyl -1-picrylhydrazil) radical accepts an electron from other compounds became very stable and the color of intensive violet disappears (becoming yellow). This action is considered radical scavenging properties (Brighente et al., 2007, Sharma et al., 2009, Ionita, 2005; Huang et al., 2005). Free radicals are chemical species associated with unpaired electron. They are highly reactive species and can be formed when oxygen interacts with certain molecules. Free radicals cause cell damage leading to various helth problems such as cancer, atherosclerosis,

malaria, and rheumatoid arthritis and neurodegenerative diseases. Antioxidant are capable to trap free radicals preventing oxidative damage (Shiv Kumar, 2011; Pham-Huy et al, 2008).

Table 2 presents IC50 value obtained for concentration between 1-0.001% of the extracts (results are presented as mean ± SD (n = 3).

The results indicated that all the extracts have antioxidant activity in a concentration-dependent manner. Hydroalcoholic extracts show the best DPPH scavenger activity for *Agaricus campestris* (IC50 – 0.13 mg/ml) and *Agaricus bisporus* (IC50 – 0.86 mg/ml).

Table 2. IC50 value of the extracts.

No	Extract	IC50 (mg/ml)	R^2
1	Agaricus campestris (methanolic)	1.48	0.997
2	Agaricus bisporus (methanolic)	2.00	0.997
3	Agaricus campestris (hydroalcoholic)	0.13	0.980
4	Agaricus bisporus (hydroalcoholic)	0.86	0.994

Our results are similar to the DPPH scavenger activity obtained in other study that are comprised between 0.18 µg/ml and 9.61 mg/ml for *Agaricus bisporus,* depending on the extraction solvent (Elmastas et al., 2007; Barros et al., 2008).

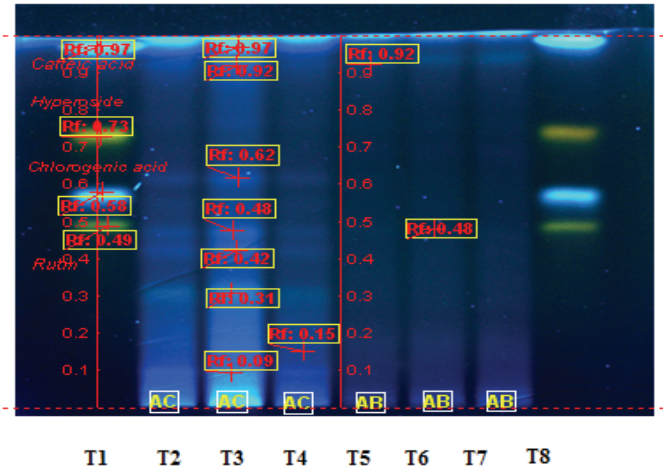

Figure 1. Phenolic profiles of the extracts of *Agaricus campestris* (AC) and *Agaricus bisporus* (AB) comparative with references substances

CONCLUSIONS

Given the fact that mushrooms are considered functional food, having both nutritional and therapeutic properties is important to take into consideration the wild species. Nutritional properties of meadow mushroom are known for many years.

Our results shows that the both species proved to have antioxidant properties, namely radical scavenging activity. The solvent used influences directly the content and bioactivity of the extracts.

For both species, hydroalcoholic extracts have shown both a higher content in total phenolic compounds and a stronger antioxidant activity than methanolic extracts.

Presenting the lowest IC50 value in DPPH assay, *Agaricus campestris* hydroalcoholic extract was the most efficient, having the antioxidant activity correlated with the total phenol content.

Agaricus bisporus hydroalcoholic extract have a lower antioxidant activity comparative with *Agaricus campestris* hydroalcoholic extract, but not inconsiderable.

In the same time, the chromatographic profile shows 7 major compounds for meadow mushroom, comparative with champignon mushroom that have three phenolic derivates, but there was no difference between the extracts (regarding to the extraction solvent).

As mushrooms are important natural resources, these extracts represent considerable source of

compounds that can be used in preparation of bioproducts with therapeutic value. Such extracts can be useful in preventing disease caused by free radical.

Our results shows that *Agaricus campestris* can be taken into consideration for the introduction in controlled culture, which can be a first step in superior valorification of this wild species.

ACKNOWLEDGEMENTS

This research work was financed from Project PCCA 174/2014 (UEFISCDI - Ministry of National Education).

REFERENCES

Barros, L., Falcão, S., Baptista, P., Freire, C., Vilas-Boas, M., Ferreira, I.C.F.R., 2008. Antioxidant activity of Agaricus sp. mushrooms by chemical, biochemical and electrochemical assays. Food Chem. 111, 61–66.

Brighente I.M.C., Dias M., Verdi L.G. and. Pizzolatti M.G, 2007. Antioxidant Activity and Total Phenolic Content of Some Brazilian Species Pharmaceutical Biology 45, 156 .

Bobek P, Galbavy S. 1999. Hypocholesterolemic and Antiatherogenic Effect of Oyster Mushroom (Pleurotus ostreatus) in Rabbit. Nahrung; 43: 339-342;

Bobek P, Ozdyn L, Kuniak L, 1995. The Effect of Oyster (Pleurotus ostreatus) Ethanolic Extracts and Extraction Residues on Cholesterol Levels in Serum Lipoproteins and Liver of Rat ; Nahrung 39: 98-99)

Chang S.T., 1999, Global impact of edible and medicinal mushrooms on human welfare in the 21st century nongreen revolution, Int. J. Med. Mushrooms, 1, 1-7

Dubost, N.J., Ou, B., Beelman, R.B., 2007. Quantification of polyphenols and ergothioneine in cultivated mushrooms and correlation to total antioxidant capacity. Food Chem. 105, 727–735.

Elmastas, M., Isildaka, O., Turkekulb, I., Temura, N., 2007. Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. J. Food Compos. Anal. 20, 337–345;

European Pharmacopoeia 6,0

Huang DJ, Ou BX, Prior R L. 2005 The chemistry behind antioxidant capacity assays. J Agric Food Chem;53:1841-1856.

Ionita P., 2005, Is DPPH Stable Free Radical a Good Scavenger for Oxygen Active Species? Chem. Pap. 59 (1) 11-16

Kumar Shiv 2011, Free Radicals and Antioxidants: Human and Food System Advances in Applied Science Research, 2 (1): 129-135;

Liu Jun, Jia Liang, Kan Juan, Jin Chang-Hai. 2013 In vitro and in vivo antioxidant activity of ethanolic extract of white button mushroom (Agaricus bisporus) Food and Chemical Toxicology 51 310–316).

Mau LL, Lim HC, Chen CC. 2002. Antioxidant Properties of Several Medicinal Mushrooms, J. Agric. Food Chem 50: 6072 6077;

Mizuno Takashi, Sakai Tadamoto, Chihara Goro 1995 (published online 2009) Health foods and medicinal usages of mushrooms Food Reviews International, Volume 11, Issue 1, Special Issue: Mushrooms: The versatile Fungus-Food and Medicinal Properties

Palacios, I., Lozano, M., Moro, C., D'Arrigo, M., Rostagno, M.A., Martínez, J.A., García-Lafuente, A., Guillamón, E., Villares, A., 2011. Antioxidant properties of phenolic compounds occurring in edible mushrooms. Food Chem. 128, 674–678.

Pathirage Kamal Perera, Yunman Li -2011, Mushrooms as a functional food mediator in Preventing and ameliorating diabetes Functional Foods in Health and Disease; 4:161-171, review

Parvu C. 1997– Universul plantelor Mica enciclopedie – Ed. Enciclopedica , Bucuresti).

Pham-Huy, L. A., He, H., & Pham-Huy, C. 2008. Free Radicals, Antioxidants in Disease and Health. *International Journal of Biomedical Science : IJBS*, *4*(2), 89–96).

Popescu Maria Lidia - 2006, Ciuperci. Potential terapeutic si toxicologic. Editura Tehnoplast. Company S.R.L., Bucuresti,ISBN 973-8932-08-4, ISBN 978-973-8932-08-1;

Ramirez-Anguiano, A.C., Santoyo, S., Reglero, G., Soler-Rivas, C., 2007. Radical scavenging activities, endogenous oxidative enzymes and total phenols in edible mushrooms commonly consumed in Europe. J. Sci. Food Agric. 87, 2272– 2278.

Sebastian Schaffer, Gunter P. Eckert, Walter E. Müller, Rafael Llorach Diego Riverab, Simona Grande, Claudio Galli, and Francesco Visioli. 2004. Hypochlorous Acid Scavenging Properties of Local Mediterranean Plant Foods Lipids, Vol. 39, no. 12

Sanchez-Moreno C., Larrauri J.A., Saura-Calixto, F., 1998 A procedure to measure the antiradical efficiency of polyphenols. Journal of Agricultural and Food Chemistry 76, 270-276.

Sharma Om P. Tej. K Bhat, 2009, DPPH antioxidant assay revisited Food Chemistry, Volime 113, Issue 4, 15 April 2009, Pages 1202–1205

Subrata Giri, Gunjan Biswas, Prakash Pradhan Subhash C. Mandal, Krishnendu Acharya1 2012. Antimicrobial Activities Of Basidiocarps Of Wild Edible Mushrooms Of West Bengal, India International Journal of PharmTech Research Vol.4, No.4, pp 1554-1560, ISSN : 0974-4304;

Velioglu, Y. S., Mazza, G., Gao, L., & Oomah, B. D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables grain. Journal of Agricultural and Food Chemistry, 46, 413–417.

Wagner H., Bladt S., 1996 - Plant Drug Analysis, Second Edition, Springer.

Woldegiorgis Ashagrie Z., Abate Dawit, Haki Gulelat D., Ziegler Gregory R., 2014. Antioxidant property of edible mushrooms collected from Ethiopia, Food Chemistry 157, 30–36;

http://biotehnol. eu / mycoind/ abstract.html

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PROTOCOL FOR EFFICIENT *IN VITRO* MULTIPLICATION OF *LYCIUM BARBARUM* L. (GOJI) BY DIRECT ORGANOGENESIS

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Abstract

The plant known scientifically as Lycium barbarum L., and commonly in the West as the wolfberry, or simply as "goji", is considered by many authors as the most nutrient rich plant on earth. It has been used for thousands of years by Chinese and Tibetan therapists as a source of health. The plant Lycium (Goji) is a shrub of the family Solanaceae is a true national treasure for China used in traditional medicine for over 2000 years.Thus, according to information published in various specialized articles and those taken from traditional beliefs, goji fruit is considered to be an important antioxidant, antidiabetic, and a natural source with excellent effects on the cardiovascular system and in decreasing the level of cholesterol in the human body. Given the properties of this imported super fruit the initiative to develop a propagation protocol to be widely used in Romania too is therefore considered to be one relevant for this plant species. The direct organogenesis protocol used in this study yields explants and microcuttings from meristematic apexes consisting of 2-3 node fragments detached from 30 day old goji seedlings germinated in in vitro conditions. The average was 89-95% for morphogenetic culture; for the offshoots rooted in a liquid culture supplemented with 1 mg / l IBA, it was 100% .In vitro goji rooted plantlets were successfully acclimatized (average survival rate of 90-98%) in a peat substrate mixed with sand. After being placed under ex vitro conditions, the vitroplantlets increased rapidly and developed well. Over a period of one month, they formed new branched roots and many axillary shoots with healthy leaves.

Key words: *Lycium barbarum* L., direct organogenesis, goji.

INTRODUCTION

Lycium barbarum L. known as goji, has been recognized as one of the most valuable medicines. Goji berry has a long history of medicinal use, especially among tribes in China. Sorted from various literatures and from traditional beliefs, goji is considered to have an important antioxidant, antidiabetic effect (Nurliyana, et al., 2012; Osman, et al., 2012 Thomson, 2010), repair epidermal damage (Zhao, et al.,2005) and in providing excellent effects on the cardiovascular and cholesterol levels 1-6 (Deli M.A., et al., 2012; Nedelchev, et al., 2012).

Ancient Chinese medical texts celebrated wolfberries for their many uses, including hardening of the body's vital force due to multiple mineral and organic compounds that they contain fruits and seeds vitamins (B1, B6, A, C and E) 18 amino acids (8 of them essential to life), 21 minerals (including significant amounts of Zn, Fe, Cu, Ca, Se, P and others) essential fatty acids (needed for hormone production and normal operation brain and nervous system) and the current results of researchers confirm these properties. (Peteros, et al., 2012; Potterat O.,2010; Jing and Yin. 2010; Luo, et al.,2004; Feng, et al.,2001).

Considered a superfood by specialists, Goji fruit bush has been adopted by Romanian farmers (Tarcza, 2012). A manufacturer in Ciuperceni village (15 kilometers away from Satu Mare, in northern Romania) has set up the first organic goji plantation in Europe, on an area of 2 hectares. He did so after receiving 50 thousand seeds from North America four years ago. Even if at first he did not know much about how to cultivate this plant, he was thinking of future investments.

It was encouraged by the recognized effects of goji fruit, used to treat diabetes or even cancer (www. https://ro.stiri.yahoo.com).

Micropropagation techniques are used as biotechnological tools that allow the production of a large number of plants in small pieces taken from a mother plant in a relatively short period of time (Cristea, 2010; Stănică, et al., 2002; Roşu, 1999; Cachiță-Cosma D., 1987).

Numerous scientific articles have reported using tissue culture techniques, with many researchers successfully applying these to the goji shrub (Osman, et al., 2013b, 2012; Hu et al.,2001) and by those coming from research centers or universities in Romania (Fira et al.,2012, Fira et al.,2011).

By employing tissue culture techniques, *Lycium barbarum* L. (goji) has been propagated through direct organogenesis (Osman, et al., 2013b; Fira et al.,2011, Hu et al.,2001) and indirect organogenesis (Osman, et al., 2013a; Hu et al.,2008). Different explant sources have been utilized for *in vitro* propagation of goji shrub through direct organogenesis. Researchers Fira A. and Clapa D. (2011) from Fruit Research Station Cluj (Cluj-Napoca town, Romania) reported in their study the use of shoot tip and nodal segments for efficient micro-propagation protocol, whereas Osman, et al., (2013b) and Hu Z.,with collaborators (2001) used leaf, stem axillary buds and also root for *in vitro* multiplication of *Lycium barbarum* L. (goji) plants. In most cases, shoot proliferation was achieved by axillary bud growth from nodal explants.

In this context, the paper presents a new protocol for efficient *in vitro* multiplication of *Lycium barbarum* L. (goji), in order to highlight the importance of explants in inducing the direct organogenesis process.

MATERIALS AND METHODS

The seeds taken from fresh ripe berries of goji (commercial market sources) were surface sterilized in two versions with a product undiluted commercial bleach (ACE) containing 4.85% sodium hypochlorite for 10 minutes (V1) and a dilute bleach product concentration trading 4.50% sodium hypochlorite for 20 minutes (V2), followed by three washes with sterile distilled water.

Aseptic, sterilized seeds were placed individually in glass tubes with a capacity of 19 x 110 mm containing 10 ml of culture medium for germination test. The culture medium was prepared with a composition diluted to the half of the formulation of basal Murashige-Skoog (MS) (Murashige and Skoog, 1962) without hormones, to which were added 20 g /l sucrose, 7 g /l agar and pH 5.8.

In subsequent work steps basal MS (1968) recipe consisting of salts with vitamins normal concentration was used but was supplemented with auxin type phytohormones (IAA, NAA, IBA) cytokinins (BAP and Kin) and gibberellins (GA3) in different concentrattii (0.0022 to 2 25 mg /l) for triggering proliferation and morphogenetic processes caulogenesis and rootedness. Cultures were incubated at 25 ± 2°C under fluorescent light 16 hours of light photoperiod.

RESULTS AND DISCUSSIONS

Inoculation and incubation of goji explants

Goji explants were obtained in advance from in vitro plantlets developed through the process of seed germination in aseptic culture media without additional plant hormones.

After five weeks, leaves, apexes and nodal parts from the seedlings obtained in vitro were detached and used as explants for future culture leaves, apexes and nodal parts from the seedlings obtained *in vitro. L. barbarum* explants were excised at about 0.5-1.0 cm length and grown on basal medium Muraschige & Schoog (1962) MS in six variants of of exogenous plant hormones IAA, NAA, IBA, GA3, BAP Kin and with different concentrations (from 0.0022 to 2.25 mg /l), sucrose 3% and agar 0.8% with pH adjusted to 5.8, according to Table 1.

Inoculation of explants consisted of placing variants of the culture medium chosen for experimentation, distributed in the culture dish sterile operations performed in a laminar flow hood (Stănicã, et al., 2002; Roşu, 1999; Cachiță-Cosma, 1987).

After inoculation vitro cultures were transferred into the growth chamber, air conditioner, thus exposing them to an ecophysiological regime required by the nature and type inoculants induced morphogenetic processes.

Table 1. Variants of recipes used to induce experimental organogenesis at goji *(Lycium barbarum* L.)

Medium Variant	*Hormonal Balance*	*Othercomponent*	**Organogenous processes induced**
Var I	***MS(1962)+0,5mg/l BAP+0,2mg/l NAA***	***8 gr./l agar+ 30 gr./l*** sucrose	initiation of adventitious organogenesis
Var II	***MS(1962)+0,5 mg/ l BAP+0,5mg/l NAA***	***8 gr./l agar+ 30 gr./l*** sucrose	initiation of adventitious organogenesis
Var III	***MS(1962)+ 1mg/l BAP +0,5 mg/l GA3+0,1mg/l IBA***	***8 gr./l agar+ 30 gr./l*** sucrose	caulogenesis proliferation
Var IV	***MS(1962)+ 2,25 mg/l BAP +0,18mg/l IAA***	***8 gr./l agar+ 30 gr./l*** sucrose	caulogenesis proliferation
Var V	***MS(1962)+ 1,8mg/l IAA+0,022 mg/l Kin***	***8 gr./l agar+ 30 gr./l*** sucrose	rootedness process
Var VI	***MS (1962)+ 1mg/l IBA***	***+ 30 gr./l*** sucrose without ***agar***	rootedness process
Var 0	***MS(1962)-*** without hormones	***7 gr./l agar+ 20 gr./l*** sucrose	***Control seed germination***

Legend*:* MS - Murashige and Skoog Media (Murashige*;* BAP- 6-benzylaminopurine*;* NAA –naftyl-acetic acid*;* IBA - indolyl butyric acid*;* IAA –indolyl acetic acid*; Kin – chinetina;GA3 – giberelic* acid

Establishing long-term morphogenetic cultures.

In regeneration systems *"in vitro"* caulogenesis express themselves by developing unipolar structure represented shoots and directly shoots structure from explant or via callus, cytokinins having an essential role in inducing this kind of morfogenesis; by posting strains develop *"in vitro"* and rooting their on special rhyzogene media can be regenerated independent plants, a process that is sitting at the base of the nonconventional technology vegetativ way .

During the initial phase of establishing the cultures morphogenetic included in the first 6-8 weeks of culture, the leaf explants developed callus nodules and small roots while the nodal explants were formed by direct organogenesis leaf primordia and shoots.

After 8 weeks of culture goji plantlets regenerated *in vitro* on induction medium (VarI and VarII) variable subcultivations were performed on fresh MS basal culture medium of variants VarIII VarIV for induced proliferation and morphogenetic crops processes.

After 14 weeks of culture, a thorough assessment at explants (Goji) of *Lycium barbarum* on *in vitro* regeneration was performed and recorded (Table 2). Referring to the data obtained, it showed us that treatment with 0.5 mg /l NAA and 0.5 mg /l BAP (variable) used in the cultivation of leafy explants page is the best combination to induce initiation of *in vitro* adventitious organogenesis initiation to *L. barbarum* species.

Table 2. Influence variants used to induce experimental recipes from goji organogenesis, after 14 weeks of culture; (average values)

Medium Variant	Inoculated explant type	Elongated adventitious shoots/explant	
		No.	Length Sprouts (cm)
Var III	leaf	5	5,80
	apexes	3	2,2
	nodal fragments	15	7,1
Var IV	leaf	5	4,7
	apexes	5	2,5
	nodal fragments	10	5,5

The advantage of the culture *"in vitro"* apical or axillary buds situated at the explants used as inocula (apexes and parts nodal) is due miniaturized indicated at the tip of the shoot growth, such as regenerating an independent plants require only induce elongation and rootedness process of the new obtained vitroplants. In the case of leaf explant inoculated on to the VarIII the hormone balance was made up of 1 mg /l BAP 0.5 mg /l GA3 + 0.1 mg /l IBA morphogenetic cultures was produced concurrently with the proliferation of multiple shoots and the formation of adventitious roots in vitro conditions (Figure 1).

Figure 1. Morphogenetic crops from goji (*Lycium barbarum*) initiated from nodal explants *in vitro* conditions.

Induction rootedness process

Subcultivation were made at intervals of 3 to 4 weeks in culture medium variant used in initiation, because it has proven so effective in stimulating the development of multiple shoots and in terms of their elongation. On the occasion of each elongated subcultivations shoots over 2 cm in size were detached and transferred on two (VarV and VarVI) rizogenetic media (Figure 2).

Figure 2. *Lycium barbarum* elongated shoots (Goji), transfer on the rizogenetic culture media variants: VarV- (with agar) and VarVI- (without agar)

The first root primordia were formed after 3 weeks and efficient root system to develop after 6 weeks of incubation under these conditions (Figure 3).

Figure 3. Goji *vitro*-plantlets with well developed roots system, suitable for acclimatization after 3-4 maintenance in liquid rooting medium

CONCLUSIONS

This study was conducted to determine the best protocol subculturing and hormonal compositions suitable for *in vitro* regeneration of *Lycium* plant. They were also identified age and body seedlings *in vitro* with an optimal level of proliferation. For the *in vitro* regeneration of leaves and nodes were used as transferable items on regeneration medium and cultivation in presence of various concentrations of hormone combinations of- α naphthalenacetic acid (NAA) and 6-benzyl amino purine (BAP).

Nodal explants were identified as being more receptive than leaf explants, since the latter took them two weeks to produce adventitious buds.

The yield of morphogenetic crops from goji meristematic apexes explants detached from seedlings germinated *in vitro* conditions was 89-95% and the rooting of shoots in the liquid culture medium supplemented with 1 mg /l IBA it was 100%. Goji rooted plantlets in *in vitro* conditions were successfully acclimatized (median survival rate of 90-98%) in a peat substrate mixed with sand. *Lycium* plantlets placed *in vivo* have grown rapidly and have developed well branched adventitious roots and the numerous side shoots with leaves healthy over a period of one month (Figure 4).

Figure 4. Goji vitroplants transferred to pots after 6 to 8 weeks of acclimation period

REFERENCES

Cachiță-Cosma D. 1987. Metode *in vitro* la plantele de cultură. Editura Ceres,Bucureşti.

Clapa D., A. Fira, N. Joshee. 2013. An Efficient ex Vitro Rooting and Acclimatization Method for Horticultural Plants Using Float Hydroculture, Hortscience, 48: 1159-1167.

Cristea V. 2010. Culturi *in vitro* fotoautotrofe la specii de *Dianthus* endemice şi periclitate din România. Editura Todesco, Cluj-Napoca.

Deli MA, Yang D, Li S-Y, et al., 2012. *Lycium barbarum* extracts protect the brain from blood–brain barrier disruption and cerebral edema in experimental stroke. Plos One 7(3):e33596.

Feng H. J., Y. C. Jie, P. L. Yun & Z. L. Gui. 2001. The Effect of *Lycium barbarum* (Wolfberry) on Antioxidant Activity in the Retina of Diabetic Rats. Retrieved June 3,2010 from http://www.richnature.com.

Fira A., Clapa D., Vescan L.A., 2012. Direct *ex Vitro* Rooting and Acclimation in blackberry cultivar 'Loch Ness', Bulletin USAMV Animal Sciences and Biotechnologies, 69(1-2)/2012, Print ISSN 1843-5262; Electronic ISSN 1843-536X, p.247- 254;

Fira A., Clapa D., 2011. Results Regarding *In Vitro* Proliferation in Goji (*Lycium barbarum*). Bulletin UASVM Horticulture, 68(1)/2011, Print ISSN1843-5254;Electronic ISSN 1843-5394; pp. 503;

Funayama S., G.-R. Zhang and S. Nozoe. 1995. Kukoamine B, a Spermine Alkaloid from *Lycium* Chinense, Phytochemistry vol. 38, No. 6: 1529-1531.

Hu Z., Y. Hu, H. H. Gao, X.Q. Guan and D.H. Zhuan. 2008. Callus production, somatic embryogenesis and plant regeneration of *Lycium barbarum* root explants, Biologia Plantarum, 52 (1): 93-96.

Hu Z., G.-Q. Guo, D.-L. Zhao, L.-H. Li, G.-C. Zheng. 2001. Shoot Regeneration from Cultured Leaf Explants of *Lycium barbarum* and *Agrobacterium*-Mediated Transformation, Russian Journal of Plant Physiology, 48 (4): 453–458, din Fiziologiya Rastenii, 48 (4): 529–535.

Jing L. & L. Yin. 2010. Antihyperglycemic activity of polysaccharide from *Lycium barbarum.* Journal of Medicinal Plants Research. 4(1): 23–26.

Luo Q., Y. Cai, J. Yan, M. Sun & H. Corke. 2004. Hypoglycemic and Hypolipidemic Effects and Antioxidant Activity of Fruit Extracts from *Lycium Barbarum*. Life Sci. 76(2): 137–149.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum, 15: 473-497.

Nedelcheva Anely, 2012. Traditional knowledge and modern trends for Asian medicinal plants in Bulgaria from an ethnobotanical view; EurAsian Journal of BioSciences, vol.6, 60-69;

Nissen S. J. and Sutter E.G., 1990, Stability of IAA and IBA in Nutrient Medium to Several Tissue Culture Procedures, HORTSCIENCE 25(7):800-802*;*

Nurliyana R., I. Syed-Zahir, K. Mustapha-Suleiman, M. R. 'Aisyah, & K. Kamarul-Rahim. 2010. Antioxidant Study of Pulps and Peels of Dragon Fruits: A Comparative Study. International Food Research Journal. 2010(17): 367–375.

Osman N.I., Awal A., Sidik N.J. and Abdullah S., 2013a. Callus Induction and Somatic Embryogenesis from Leaf and Nodal Explants of *Lycium barbarum* L. (Goji). Biotechnology, 12(1): 36-45;

Osman N.I., Awal A., Sidik N.J. and Abdullah S., 2013b. *In Vitro* Regeneration and Antioxidant Properties of *Lycium barbarum L.* (Goji) Jurnal Teknologi (Sciences & Engineering) *62:2 35–38.*

Osman N.I., Awal A., Sidik N.J. and Abdullah S., 2012. Antioxidant activities of *in vitro* seedlings of *Lycium barbarum* (Goji) by diphenyl picrylhydrazyl (DPPH) assay, International Journal of Pharmacy & Pharmaceutical Sciences; Oct2012 Supplement, Vol. 4, p.137-141;

Peteros P. & M. M. Uy. 2010. Antioxidant and Cytotoxic Activities and Phytochemical Screening of Four Philippine Medicinal Plants. J Med Plants Res*,* 4(5): 407–414.

Potterat O.,2010. Goji (*Lycium barbarum* and *L. chinense*): Phytochemistry, pharmacology and safety in the perspective of traditional uses and recent popularity. Planta Med. 2010 Jan;76(1):7-19.

Roşu A., 1999. Elemente de Biotehnologii Vegetale - Aplicații în ameliorare. Ed. Ametist, Bucureşti.

Stănică F. & colab. 2002. Înmulțirea plantelor lemnoase, Editura CERES, Bucureşti.

Tarcza, T. M., 2012. A theoretical spotlight over the Romanian agricultural products; Annals of the University of Oradea, Economic Science Series;2012, Vol. 21 Issue 1, p1221;

Thomson G. E.. 2010. Further Consideration of Asian Medicinal Plants in Treating Common Chronic Diseases in the West. Journal of Medicinal Plants Research. 4(2): 125–130.

Zhao H., Alexeev A., Chang E., Greenburg G. & Bojanowski K., 2005. *Lycium barbarum* Glycoconjugates: Effect on Human Skin and Cultured Dermal Fibroblasts. Phytomedicine. 12: 131–137.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PLANT GROWTH REGULATORS A KEY FACTORS IN WHEAT – MAIZE CROSSES FOR HAPLOID PRODUCTION IN WHEAT

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Abstract

The paper highlights the role and importance of plant growth regulators (PGR) application before or post pollination with regard to embryo formation and haploid plant regeneration in wheat-maize hybridization. The efficiency of some PGR combinations (doses, procedures, time of application) and genetic influence on some parameters characterizing the efficiency of haploid production at NARDI Fundulea are briefly described. Taking into account the decreasing efficiency, in the last period, the need for new and different variants of PGR are discussed.

Key words: haploidy, doubled haploid, Zea system, PGR.

INTRODUCTION

Today, the haploidy is a topic of great interest in both genetics and breeding researches, and a very useful tool for developing of mapping populations and in shortening breeding programs by rapid homozygosity in a single generation.

Although, the haploid condition in wheat can be attained by using several procedures such as androgenesis, gynogenesis, microsporogenesis, the most effective ones is based on sexual hybridization of wheat by maize and spontaneous elimination of maize chromosomes in the early cycles of zygote cell division.

However, in postpollination events after elimination of male parent chromosomes the formed zygotes are immediately aborted due to the lack of endosperm formation. This conspicuous barrier was however overcome by using *in vitro* culture of pollinated spikelet or ovules on artificial medium that contains auxins (Laurie and Bennett, 1986, 1988; Comeau et al., 1988). Soon after that, Suenaga and Nakajima (1989) described a simplified method wich included a tiller injection with 2,4-D (2,4-dichlorophenoxyacetic acid) and Riera-Lizarazu and Mujeeb-Kazi (1990) tested a 2,4-D floret spray treatment and detached spike culture method. The regulatory effects of auxin treatments on the embryo formation by *in vivo* treatments were also proved using different pseudogamous species or lines of the *Poaceae* (Matzk, 1991). In barley, by using barley-Bulbosum hybridization system, Mihailescu et al (1994), Pickering and Walace (1994) have reported a positive influence on seed set, embryo formation and embryo differentiation after *in vivo* application of PGR solution containing GA3 (gibberellic acid) and 2,4-D.

These first reports emphasized that haploid embryos could be obtained and haploid plants certainly regenerated by *in vivo* treatments with auxins.

Further improvements and refined procedures concerning mode and time of application, PGR composition and doses, make the wheat - maize crosses the most efficient procedure for haploid production in common wheat.

At NARDI- Fundulea the wheat maize hybridization program was initiated in 1991, firstly by testing cross-compatibility between several maize early hybrids and some old and new released wheat cultivars (Giura, 1994).

Afterwards, working protocol was improved and particularly adapted to the relative limitative logistical supports, especially those for handling and growing parental plants in a greenhouse provided with heating facilities during winter time and supplemental illumination by incandescent bulbs. After several attempts have become obviously that in

such conditions wheat-maize crosses could be successfully done within a single crossing cycle of 30-45 days in March-April period when interaction of environmental factors especially temperature and atmospheric humidity proved to be more favorable.

During 1991- 1995 many PGR treatment variants were tested for their effects upon embryo formation, differentiation, germination and haploid plant recovery. The best ones were again tested in 1996 and two PGR variants (A_2 : 2,4-D 25 ppm, GA3 75 ppm and C_1: 2,4-D 18 ppm, Dicamba 9 ppm, BA 2 ppm) were noticed as having significant superior values for the parameters characterizing the *Zea* system efficiency in common wheat haploid production (Table1).

Table 1. The effects of hormonal treatment variants applied in vivo in cross breeding wheat x maize - Fundulea 1996-

Treatment	Parameters			
	QK/F	E/F	E/DK	E/Sp
A (control)	49.7	13.7	27.5	2.9
A1	49.7	13.8	27.8	3.2
A2	**60.0**	**21.4**	**35.6**	**5.3**
A3	62.5	12.1	19.4	3.2
B2	71.2	12.4	18.1	3.0
C	73.9	17.4	23.5	4.4
C1	**68.6**	**21.4**	**31.2**	**5.4**
C2	74.3	6.0	8.0	1.4

(After Giura and Mihailescu, 2000)

QK / F - quality kernels / 100 pollinated flowers
E / F - embryos formed / 100 pollinated flowers
E / DK - embryos / 100 dissected kernels
E / Sp - embryos / spike

Since then, the PGR variant A_2 was preferential used. Nevertheless, in the last years, in a new greenhouse, this PGR variant proved to be less efficient probably due to specific environmental conditions inside greenhouse provided with heating system based on methane gas turbo-blower that might decrease the humidity and increase CO_2 concentration.

Therefore it has become necessary a comparative reassessment of the PGR variants A_2 (2,4-D 25 ppm, GA3 75 ppm) and C_1 (2,4-D 18 ppm, Dicamba 9 ppm, BA 2 ppm).

MATERIALS AND METHODS

A number of 21 winter wheat F_1's genotypes from the wheat breeding program and sweet maize hybrid "Delicios" were cultivated in the same greenhouse compartment in 2014. A complex fertilizer (N:P:K; 20:15:10) was administrated by broadcasting before soil tillage. From the mid of December to the mid of February maize parent were sown in rows at several planting times, once per week to ensure a continuous pollen window during wheat - maize crosses in March – April. Wheat plantlets previously vernalized for 45 days were planted in large pots at the mid-January: three plantlets/pot. For tiller development and to reach on optimum physiological state at the time of pollination, wheat plants were supplementary watered before spike emergence with a complex nutritive solution of macro and micro nutrients.

Spike of wheat plants were hand emasculated leaving 18-20 flowers per spike. The upper spikelets and central florets of each spikelet were removed but one or two basal spikelets were left to prevent in some way spike desiccation. At the predicted day of anthesis, spikes previously emasculated were pollinated with freshly collected maize pollen. The hormonal treatments with A_2 (2,4-D 25 ppm, GA3 75 ppm) and respectively C_1 (2,4-D 18 ppm, Dicamba 9 ppm, BA 2 ppm) solutions (variants) were administrated by spraying on the spikes at 24 hours after pollination.

At 13-14 days after pollination the caryopses were extracted from the spikes and sterilized in 90% ethyl alcohol for a half minute, then in a 7% sodium hypochlorite solution for 5 minutes and rinsed subsequently 5 times in sterile water. The accidentally resulted self-pollinated seeds are very distinctly after size with solid endosperm and can be easily eliminated. The immature embryos were aseptically excised and cultured in vials containing modified B5 medium (Jensen, 1975) supplemented with 2% sucrose and 0,8% agar. The number of developed caryopses, cultured embryos and haploid plants regenerated were recorded for each spike and genotype.

RESULTS AND DISCUSSIONS

The two PGR variants A_2 and C_1 were applied after pollination on 1032 spikes. Following embryo rescue operation in aseptically condition under binocular microscope in a laminar flowhood a number of 1630 haploid embryos were transferred on artificial medium for regeneration.

Parameters haploid embryo/spike (He/Sp) and haploid plant regeneration capacity / cultured embryos (Hp/Ce) on each treatment variant were considered as the most appropriate to reevaluate the efficiency of the two PGR variants.

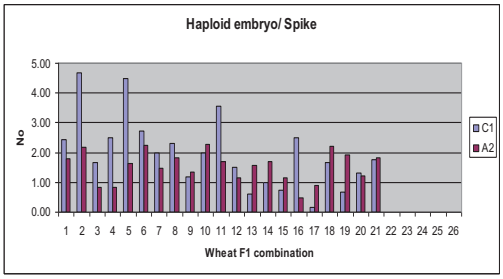

Figure 1: Haploid embryo/spike for C_1 and A_2 treatment variants

From the chart presented in Figure 1 it is obvious that under variant C_1 treatment the F_1's genotypes produced more embryos per spike compared to A_2 variant. However, some F_1's genotypes presented a higher average values for He/Sp when treated with A_2 variant. This fact could be explained if we be take into consideration a possible influence of genotype. A such phenomenon was also reported by Inagaki and Tahir (1990).Although the quality of embryos was not quantified, the regeneration capacity on B5-modified medium was in average higher with A_2 variant as compared to C_1 variant (Figure 2).

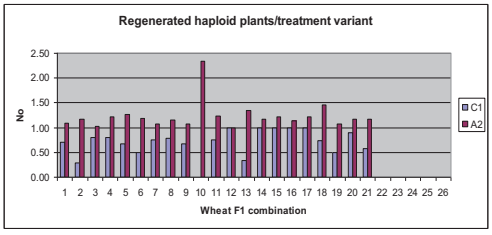

Figure 2: Haploid plants regenerated / cultured embryos for C_1 and A_2 treatment variant

Because the embryo development is a key point for *in vitro* culture response, the differences in embryo size might be closely related to the success of embryo growth response and plant regeneration ability (Giura, 1994). We could admit that A_2 treatment version might provide the necessary development conditions for a better regeneration rate. Moreover, the beneficial effects of A_2 variant on regeneration capacity were registered for all F_1's genotypes without any exception. Similar results were also registered in 1996 experiment when A_2 and C_1 variants over yielding significantly the control and other PGR variants by higher values, especially for haploid embryos per spike (He/Sp) parameter (Giura and Mihailescu, 2000).

However, when we evaluate and compare the results registered for 1996 and 2014 a decreasing efficiency of both PGR variants is evident (Table 2).

Table 2. Efficacy of growth hormone treatment expressed by the ratio embryos / spike

Year	Treatament A_2	Treatment C_1
1996	5.3	5.4
2014	1.5	2.0

Therefore, the influence of genotype could not be ignored, each year we received from breeders new and different series of F_1's genotypes, the experimental conditions inside greenhouse could remain an essential factor since haploid production efficiency is strongly influenced by temperature during the period of *in vivo* development up to the harvest caryopses. (Giura and Mihailescu, 2000).

CONCLUSIONS

Both growth hormone treatments gave satisfactory results, but it is however necessary to reevaluate the influence of different factors that operate inside greenhouse and to establish new combination/doses of growth hormones that can lead to better results.

It may be considered that new and different PGR variants may be further tested for increasing the haploid production efficiency mainly by sustain a better embryos development in the absence of endosperm as well as embryo regeneration capacity *in vitro* conditions.

REFERENCES

Comeau, A., Plondre, A., St. Pierre, C.A., Nadeau, P., 1988 – Production of doubled haploid wheat lines by wheat x maize hybridization. Genome 30 (Suppl. 1): 482, (Abstract)

Giura, A., 1994 – Preliminary results on wheat haploid production using wheat x maize crosses. Rom. Agric. Research vol. 1: 1-4

Giura, A., 1994 – Progress in wheat haploid production. In:Proc. 8th Int. Wheat Genet. Symp., Beijing, China, I, 741-745

Giura, A., Mihailescu, A., 2000 – Metode moderne de reducere a duratei programelor de ameliorare la grâu și orz prin homozigotare rapidă (haploidie și dihaploidie), În: Metode de Cercetare în Cultura Plantelor, Editura Agris, București, 17-36

Laurie, D.A., Benneet, M.D., 1986 – Wheat and maize hybridization. Can. J. Genet. Cytol. 28:313-316

Laurie, D.A., Benneet, M.D., 1988 – The production of haploid wheat plants from wheat x maize crosses, Theor Appl Genet 76:393-397

Matzk, F., 1991 – A novel approach to differentiated embryos in the absence of endosperm. Sexual Plant Reproduction 4:88-94

Mihailescu, A., Giura, A., Bude, Al., 1994 – Increasing efficiency of barley haploid production by combined treatments with exogenons plant growth regulators applied in vivo, Romanian Agricultural Research 2: 25-33

Pickering, R.A., Wallace, A.R., 1994, - Gibberelic acid +2,4-D improves seed quality in *Hordeum vulgare* L. x *Hordeum bulbosum* L. crosses. Plant Breed. 113: 174-176

Suenaga, K., Nakajima, K., 1989 – Efficient production of haploid wheat (*Triticum aestivum*) through crosses between Japanese wheat and maize (*Zea mays*). Plant Cell Reports. 8:263-266

Riera-Lizarazu, O, Mujeeb-Kazi, A., 1990 – Maize (*Zea mays* L.) mediated wheat (*Triticum aestivum* L.) polyhaploid production using various crossing methods. Cereal Res. Comm. 18: 339 – 345

Inagaki, M.N., Tahir, M., 1990 – Comparison of haploid production frequencies in wheat varieties crossed with Hordeum bulbosum L. and maize.

Jensen, C.J., 1975 – Barley monoplid and double monoploids: tehniques and experience. In: Barley Genetics III, Proc. 3 Int. Barley Genet. Symp., Garching:316-34

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

LEATHER HYDROLYSATE EVALUATED AS ORGANIC NITROGEN SOIL INPUT

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Abstract

The leather manufacturing process produces annual tons of misspends being considered very harmful for nature. Solid waste generated in leather industry contains protein as main component. The biotechnological sector allows us to use this worthless material as microbial substrate for enzyme production. Those enzymes have multiple uses and can cover numerous industrial needs.
This paper covers some potential applications in the agriculture sector as fertilizer. Leather hydrolysate results from bacterial conversion of leather proteins. The specific bacteria where obtained through isolation of the compostation of leather and incubated 48 hours into a minimal media. During the optimization process the maximum proteinase production was 1.5 U/ml and it was achieved after 120 hours of incubation at 35°C, using a minimal media and 0.6 shredded leather. Leather debris containing proteins and amino acids which can be applied as bio-growth increase by environmental secure technologies, and eventually in organic output production.
The leather hydrolysate resulting from the microbial conversion of hide protein can be manipulated as organic nitrogen soil input.

Key words: *leather degradation, bacterial isolation, proteinase, fertilizer.*

INTRODUCTION

Low commercial value proteinase waste of animal origin such as skins and fur are generated in huge amount in skins and fur industry and are hard-to-degrade. Currently this waste is disposed by incineration which can lead to serious pollution of the environment.

An alternative to incineration is the microbial degradation, through this process obtaining proteinase enzyme, which can be applied as agricultural fertilizer, being rich in nitrogen and also inexpensive.

MATERIALS AND METHODS

Three isolated bacteria from compostation on fur and skins were used on 3000 mL minimal media (MM)(g/L - 1.0g $NaCl_2$, 0.05g $CaCl_2$, 0.7g KH_2PO_4, 0.9g $MgSO_4$, 2.38g K_2HPO_4, 3,0g sucrose, 0.6g skin and fur, pH 7,2), sterilized at 121°C, 20 min, incubated at 35°C, 135 rpm, 120 hours.

ISOLATION OF PROTEIN HYDROLYZING BACTERIUM

Skins and fur degrading bacteria was isolated from compostation of skin and fur soil for 90 days.

Upon incubation 1cm^3 of soil was prepared in suspension in 40 mL minimal media for 48 h, at 35^{o}C and 135 rpm. After incubation the isolated colonies were spread on PCA plates and incubated 24 h at 35^{o}C.

Individual colonies exhibit clear halo around them, resulting from proteinase hydrolysis, were picked and purified by repeated sub-culturing on Luria Bertani agar plates. The pure colonies were screened for their ability to degrade fur and skins on minimal media at 600nm (Habib et al.,2012)

PROFILE OF PROTEASES PRODUCTION BY ISOLATES

The 500 mL Erlenmeyer flasks containing 0.6% (w/v) skins and fur were inoculated with isolated cultured on MM to obtain an initial cultured density (OD_{600NM}). They were incubated 120 h at 35^{o}C, 135 rpm. The turbidity was measured at 24 h, 36 h, 48 h, 72 h, 96 h and 120 h of incubation and monitored for growth and protease activity.

CONCENTRATION OF ENZYMES

The fermentation broth was harvested after 120 hours of incubation and it was centrifuged at 4^{o}C and 9000xg for 20 minutes. The supernatant was collected and concentrated ten times using the rotary evaporator at 60^{o}C.

USE OF SKINS AND FUR AS BIOACTIVE AGRICULTURAL FERTILIZER (6 DAYS PLANT GROWTH)

The study was carried out in different concentration(1%;1,5%;2%;2,5%;3%;3,5%;10%; 15%;20%;25%;20%;35%) and in dilution of 1:10 mL in 100 mL plastic pots with 40 g of soil and 4 g of grain seeds (276 mL of skins hydrolysates/ 4320g soil)

All the pots were watered regularly with tap water to reduce the evaporation loss. After 6 days of sowing all plants were uprooted and washed. The growth parameters (plant height and root length) were recorded.

ANALYTICAL PROCEDURE: PROTEINASE ACTIVITY

Proteolytic activity was spectrophotometric measured at 578nm. The reaction mix contained 0.5mL enzymatic solution and 1 mL casein 1% in phosphate buffer 0.2M (pH 7), incubated at 37^{o}C for 10 min. Enzymatic reaction was stopped with 2 ml of trichloroacetic acid 5%. The reaction mix was kept 30 min at room's temperature and then it was filtrated. For every 0.5mL filtrate were added 0.5mL HCl 0.2N, 2mL NaOH 0.5N and 0.6 mL Folin-Ciocalteu 1:2. After 30 min at room's temperature the extinction was measured.

One unit of proteases activity is defined as the amount of enzyme that releases 1µmol tyrosine per minute, under analysis condition.

RESULTS AND DISCUSSIONS

ISOLATION OF PROTEOLYTIC HYDROLYZING BACTERIA

Three proteolytic bacteria strains (DA 7, DA 10 DA 13) isolated from the compotation of skins and fur were screened for skin degradation on PDA indicated their ability to use fur and skins protein as a sole carbon source for growth.

MEDIA COMPONENTS OPTIMIZATION FOR PROTEASE PRODUCTION

The isolates produced maximum proteolytic enzyme (1,213 U/mL) in the presence of 3 g of glucose and fur as substrate in a MM, a phenomenon observed for biosynthesis of bacterial proteases.

Table 1. Proteolytic screening of the isolated strains (DA 7, DA 10, DA 13) at 578 NM after 120 h incubation at 135rpm and 35°C.

Samples	Dilution	24 h	48 h	72 h	96 h	120 h	Media
DA 7	1:10	0.231	0.415	0.586	0.938	0.993	0.633
DA 10	1:10	0.556	0.630	0.815	0.906	0.987	0.779
DA 13	1:10	0.183	0.139	0.426	0.506	0.616	0.374

THE APPLICATION OF SKINS AND FUR FERMENTATION AS FERTILIZER IN AGRICULTURAL FIELD

The content of proteinase hydrolysates resulting from the conversion of fur and skins proteins by isolates was 1,213 U/mL.

Accordingly the skins and fur digest was concentrated 10 times and evaluated for its use as an organic fertilizer on the growth of *Hordeum vulgare L.* It was observed that the added skins hydrolysates exerted a beneficial effect on the germination. Plant height and root length was increased by 79, 86 and 73 % in the treated soil over the untreated soil. (Similar effect has been reported by Kim et al. (2005), A. Bose et al. (2013) and Vasileva-Tonkova et al. (2009) but on feather hydrolysate as nitrogen input)

The skins and fur hydrolysate obtained upon decomposition of skins and fur by isolates, holds potential be applied as agricultural fertilizer.

Figure 1 Wheat after 6 days of growth, concentration 35% against the blank

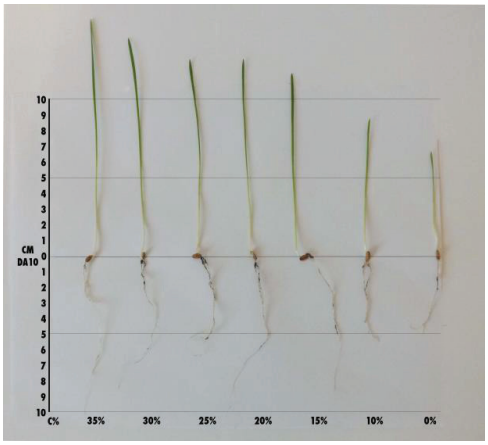

Figure 2 Wheat fertilized with DA10 strain after 6 days of growth at concentrations varying between 35% (left) and 10% (right)

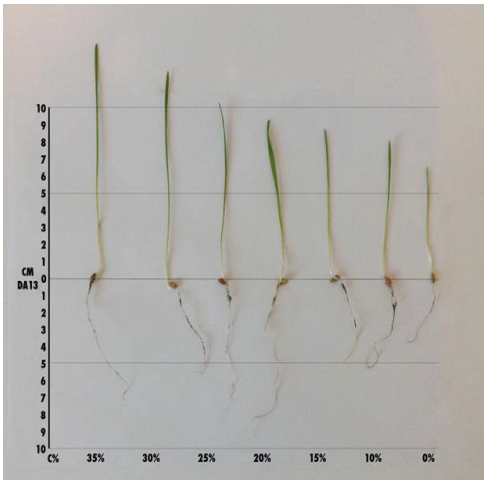

Figure 3 DA13 strain at different concentration between 35% (left) and 10% (right), after 6 days of growth. On the right we have the blank for comparison.

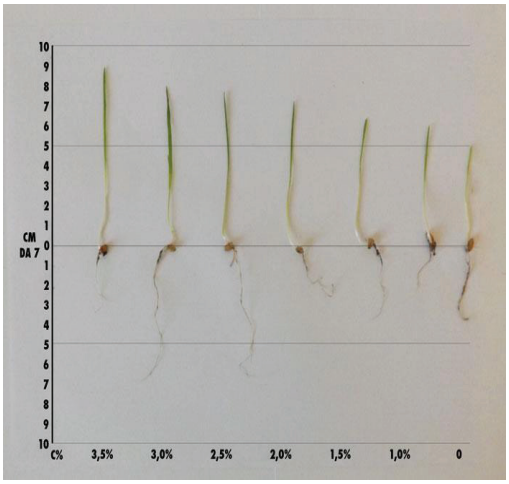

Figure 4 The wheat after 6 days of growth, after adding concentrated enzyme produced by DA7 strain, 3,5% to 1,5 % concentration from left to right against the blank

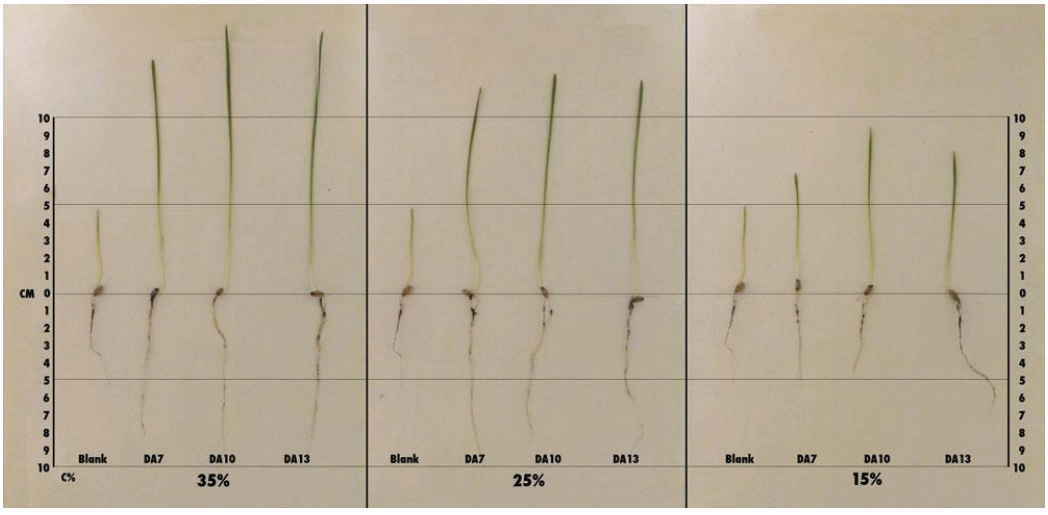

Figure 5 Compared wheat growth after 6 days with added concentrated enzymatic solution (35%, 25%, 15% from left to right) produced by the 3 isolated strains DA7, DA10, DA13 against the blank.

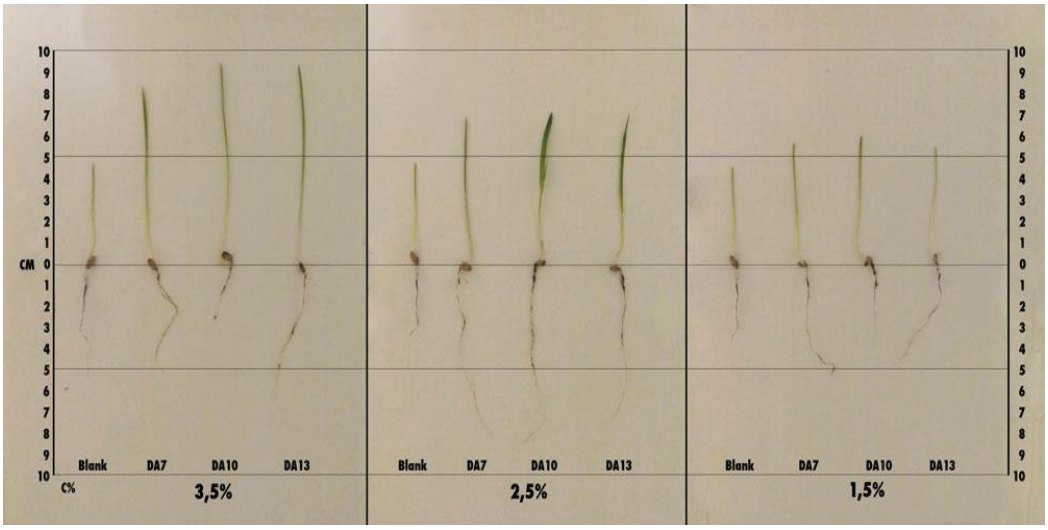

Figure 6 The wheat growth after 6 days at different enzymatic concentrations (3,5%, 2,5%,1,5%, from left to right) of the 3 isolated strains DA7, DA10, DA13 compared with the blank

CONCLUSIONS

In the present study the strains isolated from the compostation of the skins and fur were found to efficiently degrade fur in 120 h of incubation, at 135 rpm, 35°C. The strains ability to degrade makes it a potential tool for the development of suitable processes for conversion of fur and skins into fertilizer.

The best growth was obtained using 35% enzymatic concentration produced by DA10 strain.

The enzyme production was characterized and used as fertilizer soil input which proved from the evaluation as beneficial for plant growth.

ACKNOWLEDGEMENTS

The research was supported by BIOFUR E 5770/2012, project funded by UEFISCDI.

BIBLIOGRAPHY

Bose A., Pathan S., Pathak K., Keharia H., 2013, Keratinolytic proteases production by *Bacillus amyloliquefaciens* 6B using feather meal as substrate and application of feather hydrolysate as organic nitrogen input for agricultural soil, Waste Biomass Valor

Habib S.M.A., Fakhruddin A.N.M., Begum S., Ahmed M.M., 2012, Isolation and screening of thermostable extracellular alkaline protease producing bacteria from tannery effluents. J. Sci. Res. 4: 515-522

Kim J.M., Choi Y.M., Suh H.J., 2005, Preparation of feather digests as fertilizer with *B. pumilus* KHS-1. J. Microbiol. Biotechnol. 15, 472-476

Vasileva-Tonkova E., Gousterova A., Neshev G. 2009, Ecologically safe method for improved feather wastes biodegradation. Int. Biodeterior. Biodegradation 63, 1008-1012

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INFLUENCE OF DIFFERENT TEMPERATURES AND RELATIVE HUMIDITIES ON *IN VITRO* GERMINATION OF THREE ENTOMOPATHOGENIC FUNGAL STRAINS OF *BEAUVERIA BRONGNIARTII*

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Abstract

The entomopathogenic fungus Beauveria brongniartii Sacc. (Petch) *is the most important natural enemy of Melolontha melolontha L. Three strains of B. brongniartii isolated from natural diseased M. melolontha larvae were tested in order to assess the effects of temperature and relative humidity on conidial germination, the most important factors during the initialization of infection process. Conidial germination was examined at temperatures ranging from 4° to 33°C, on PDA medium. The effect of relative humidity was tested using the method described by Beyer et al. (2004) with slight changes. RHs tested were 29.8% $CaCl_2$, 52.6% $Ca(NO_3)_2$, 75.3% NaCl,, 84.7% KCl,, 92.7% KNO_3 and 100% deionized water. The results show that no strain germinated at 4 and 10°C. At 22°C all isolates exceeded 95% germination after 24 h. Temperatures over 25°C had a negative influence on the rate of spore germination. Incubation of conidia at low humidity (RH 29%) suppressed germination for all strains. All relative humidities over 29.8% were found favorable for germination of all this strains.*

Key words: *Beauveria brongniartii, temperature, humidity, germination.*

INTRODUCTION

The European cockchafer larvae (chafer grubs) cause serious damage in Romanian forest nurseries. The entomopathogenic fungus *Beauveria brongniartii* Sacc. (Petch) is the most important natural enemy of *Melolontha melolontha*. *Beauveria* species attack their host insect generally percutaneously. Temperature can affect an entomopathogen in different ways by influencing the germination, growth and viability of the fungus on and in the host insect and in the environment. Humidity is a very important environmental factor affecting the efficacy and survival of entomopathogens (Zimmerman, 2007). The importance of temperature and humidity on infection process was demonstrated by studies since 1965 (Clerk and Madelin), then the research carried out by Benz (1987), Daoust and Pereira (1986) proved that the temperature and the humidity are the most important factors that affect the conidial germination rates. James et al. (1998) consider that the effect of humidity is the greatest during the initiation of infection, because it is primarily a requirement for conidial germination.

A review recently published (Jaronski, 2009) also emphasizes the importance of temperature and humidity, as abiotic factors affecting the fungal efficacy in foliar and soil applications. In vitro results on conidial germination are presented by Lazzarini et al. (2006).

In this paper are presented the results of laboratory tests performed with three indigenous *B. brongniartii* isolates, in order to assess the effects of temperature and relative humidity on *in vitro* conidial germination.

MATERIALS AND METHODS

Source of conidia

Three isolates of *B. brongniartii* (ICDPP#2-4, re-named here 1Bbg, 2Bbg, 3Bbg respectively) used in this study were from Culture

Collection of Entomopathogenic Fungi maintained in the Department of Useful Organisms, (Research - Development Institute for Plant Protection, Bucharest). The identity of the isolates as *B. brongniartii* was confirmed by polymerase chain reaction (PCR) (http://www.icdpp.ro, 2010). The strains were initially isolated from natural diseased *Melolontha melolontha* grubs.

B. brongniartii conidia were obtained from four months old sporulating cultures grown at 25°C on PDA in tubes. Conidia were harvested from the surface of these cultures by washing, using sterile distilled water with 0.1% Tween® 80. Conidial concentrations were determinates by counting in haemocytometer.

Germination at various temperatures

Conidial germination was examined at temperatures of 4°, 10°, 15.5°, 20°, 22°, 25°, 28°, 30°, 33°C on PDA medium. Spore suspension was calibrated at a concentration of 10^7 conidia/ml diluted in sterile distilled water to which was added Tween 80 (0.01%). Petri dishes (96 mm) were inoculated with 0.5 ml of conidial suspensions for each isolate, spread with a sterile Drigalski spatula and incubated under saturated atmosphere in total darkness. Petri dishes were examined after 8, 16, 24, 32 h post-inoculation using a microscope under 400 × magnifications. Germination process was stopped by dripping a lacto phenol cotton-blue solution. Conidia were considered germinated when germinative tube length exceeded 1 to 1.5 times their length. For each plate, four areas of at least one hundred conidia were counted.

Germination at various relative humidities

The effect of relative humidity was tested using the method described by Beyer et al. (2004) with slight changes. Briefly, the inner side of Petri dish lids was coated with PDA and inoculated as was described before. After droplet drying (1 hour), the dishes were filled with constant humidity solutions (Wexler, 1995) or deionized water and incubated for 24 h at 25° C. Subsequently, the dishes were closed and sealed with parafilm, such that the spores attached to the lid were incubated above constant humidity solutions or pure water. RHs tested were 29.8% /$CaCl_2$, 52.6% /$Ca(NO_3)_2$, 75.3% /NaCl, 84.7% /KCl, 92.7% /KNO_3 and 100% /deionized water.

Data were analysed using the Kuskal-Wallis nonparametric test of the AnalistSoft Inc., BioStat v2009.

RESULTS AND DISCUSSIONS

Influence of temperature on conidial germination

The temperature parameter influences the germination of tested strains. The period of time required until over 95% of spore to germinate was 24 h for all strains at 22°C (Table 1, 2, 3).

No strain germinated at 4 and 10°C even after 32 h, germination started from 15.5°C. At this temperature all isolates germinated after 24 h post–inoculation, 2Bbg strain being distinguishable by the highest percentage of germination, both after 24 and after 32 h (Table 2). At 20°C the germination started after 16 h just in case of 2Bbg strain, reaching the maximum rate after 32 h.

At 22°C all isolates exceeded 95% germination after 24 h, strain 2Bbg standing out at a rate over 98%. The speed of germination is at a higher percentage at 22°C than at 25°C for each isolate. No germination was observed after an incubation of 8 h at all temperatures.

Table 1. Effect of temperature on conidial germination of 1Bbg strain under saturated atmosphere

Temperature (°C)	Germination rates after specified number of hours (means+SD) (%)			
	8	16	24	32
4	0	0	0	0
10	0	0	0	0
15.5	0	0	2.3±0.5	21.6±1.2
20	0	0	9.7±0.4	†
22	0	3.0±0.4	94.5±0.3	†
25	0	2.5±0.2	95±0.7	†
28	0	0	0	0
30	0	0	0.9±0.5	0
33	0	0	0	0

† Germination rates over 99%

Table 2. Effect of temperature on conidial germination of 2Bbg strain under saturated atmosphere

Temperature (°C)	Germination rates after specified number of hours (means+SD) (%)			
	8	16	24	32
4	0	0	0	0
10	0	0	0	0
15.5	0	0	14.6±0.5	67.5±0.8
20	0	4.5±0.2	53.4±0.7	†
22	0	22.9±0.5	98.0±0.2	†
25	0	20.5±0.7	†	†
28	0	0	0	0
30	0	1.3±0.7	1.76±0.8	0.2±0.3
33	0	0.9±0.9	0.17±0.2	0.1± 0.9

† Germination rates over 99%

Table 3. Effect of temperature on conidial germination of 3Bbg strain under saturated atmosphere

Temperature (°C)	Germination rates after specified number of hours (means+SD) (%)			
	8	16	24	32
4	0	0	0	0
10	0	0	0	0
15.5	0	0	2.7±0.4	48.8±0.4
20	0	0	21.9±0.4	†
22	0	10.0±1.2	96.5±0.9	†
25	0	5.6±0.3	95.6±0.3	†
28	0	0	0	0
30	0	0	0	0
33	0	0	0	0

† Germination rates over 99%

Temperatures over 25°C had a negative influence on the rate of spore germination, decreasing under 1.7% for 2Bbg strain (Table 2) and being almost not observed for 1Bbg and 3Bbg strains.

Kruskal-Wallis ANOVA showed no significant differences between strains regarding the influence of temperature, with all this, the strain 2Bbg has a faster speed of germination than the two other strains, at all temperatures.

The optimum temperature for germination was 22°C for all this isolates, in accordance with literature findings (Zimmerman, 2007). Although no germination occurred at 4 and 8°C after 32 h, during four months of preservation at 4±1°C, vegetative development was observed only for 2Bbg strain. Müller-Kögler (1960) found that the conidia of *B. brongniartii* (syn. *B. tenella*) have somewhat higher degree of germination than those of *B. bassiana* after three or four months on artificial media (Steinhaus, 1964).

Effect of relative humidity on conidial germination

Incubation of conidia at low humidity (RH 29%) suppressed germination for all strains (Table 4). All relative humidities over 29.8% were found favorable for germination of all this isolates. Over this percent, there is no variability between the relative humidity and percent of germination. The highest rate of germination was registered at 92.7 and 100% RH for 2Bbg and 3Bbg strains.

Table 4. Effect of relative humidity on conidial germination of *B. brongniartii* isolates after 24 hours of incubation at 25° C

Relative Humidity (%)	Germination rates after 24 hours (means+SD) (%)		
	1Bbg	2Bbg	3Bbg
29.8	0	0	0
52.6	76.2±1.3	92.13±1.5	94.3±2.3
75.3	96.9±2.3	98.5±2.2	96.7±1.7
84.7	92.2±1.8	62.7±3.4	93.6±1.6
92.7	96.1±0.8	†	†
100	95.8±3.4	†	†

† Germination rates over 99%

Our results showed that RH between 52.6 and 100% is not a limiting factor for germination of *B. brongniartii* isolates, which is in contrast with findings of Luz and Fargues (1997) on humidity requirements over 90% for *B. bassiana*. Also Gillespies and Crawford (1986) noted that for optimal development of most entomopathogenic hyphomycetes, the relative humidity should be above 97%. The present study is in accordance to Piatti et al. (1995) who observed that humidity requirement for fungal growth of *B. brongniartii* in soil was 57%. Also Padmini and Padmaja (2010) reported that vegetative growth and sporulation were excellent at 60% RH and 30°C temperature for twenty isolates of *Beauveria* species. Ferron (1977) found that the infection and incubation phases are not affected by relative humidity.

CONCLUSIONS

Our studies on temperature and humidity requirements for conidial germination of three *B. brongniartii* Romanian isolates, pathogenic for *Melolontha melolontha* revealed that the optimum temperature for conidial germination is 22°C; the relative humidity between 52.6 and 100% does not influence negatively the *in vitro* germination of this strains.

REFERENCES

Benz G., 1987. Environment. In J.R. Fuxa and Y. Tanada (eds.). Epizootiology of insects diseases.. Wiley, New York,177-214.

Beyer, M., Röding S., Ludewig A., Verreet J-A., 2004. Germination and survival of *Fusarium graminearum* macroconidia as affected by environmental factors. Journal of Phytopathology 152: 92-97.

Clerk G.C., Madelin M.F., 1965. The longevity of conidia of three insect-parasiting hyphomycetes. Trans. Brit. Mycol. Soc. 48, 193-209.

Daoust R.A., Pereira R.M., 1986. Survival of *Beauveria bassiana* (Deuteromycetes, Moniliales) conidia on cadavers of cowpea pests stored outdoors and in the laboratory in Brazil. Environ. Entomol. 15, 642-647.

Ferron P., 1977. Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana (Fungi Imperfecti, Moniliales*) in imagines of *Acanthoscelides obtectus* (Col.: Bruchidae). Entomophaga22, 393-396.

Gillespie A.T., Crawford E., 1986. Effect of water activity on conidial germination and mycelial growth of *Beauveria bassiana, Metarhizium anisopliae, Paecilomyces* spp., and *Verticillium lecanii*. In Samson RA, Vlak JM, Peters D eds. Fundamental and Applied Aspects of Invertebrate Pathology , Found IVth Intern Coll Invertebr Pathol, Wageningen, 244.

James R.R., Croft B.A., Shaffer B.T., Lighthart B. 1998. Impact of temperature and humidity on host-pathogen interactions between *Beauveria bassiana* and a Coccinellid. Environ. Entomol. 27 (6), 1506-1513.

Lazzarini G.M.J., Rocha L.F.N., Luz C., 2006. Impact of moisture on in vitro germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. Mycol. Res. 110 (4), 485-492.

Luz, C. & J. Fargues. 1997. Temperature and moisture requirements for conidial germination of an isolate of *Beauveria bassiana*, pathogenic to *Rhodnius prolixus*. Mycopathologia 138: 117-125.

Padmini P.P.C. and Padmaja V., 2010. Impact of different relative humidities on in vitro growth and sporulation of entomopathogenic fungal isolates of *Beauveria* species. International journal of Pharmaceutical& Biological Archives;1(4): 355-359.

Müller-Kögler, 1960. In Biological control of insect pests and weeds (1964); DeBach P.(ed.) Chapman and Hall, London, 515-547.

Wexler A.,1995. Constant humidity solutions. In: Lide D.R. (ed.), Handbook of Chemistry and Physics, 15-23, CRC Press, Boca Raton, FL.

Zimmermann (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and Beauveria brongniartii, Biocontrol Science and Technology, 17:6, 553-596.

http://www.icdpp.ro/ro/cercetare/combaterea-biologica?searched=bilateral&advsearch=oneword&highlight=ajaxSearch_highlight+ajaxSearch_highlight1

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INITIATION AND SELECTION OF CALLUS CULTURES FROM FUMARIA ROSTELLATA KNAF. AS POTENTIAL PRODUCERS OF ISOQUINOLINE ALKALOIDS

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Abstract

The aim of the study was development of protocols for obtaining callus cultures from Fumaria rostellata Knaf. For the purpose leaves, stems and flowers of the investigated plant were used as explants. Callus was initiated on a Murashige and Skoog nutrient medium, supplemented with 30 g/l sucrose, 5.5 g/l "Plant" agar and various concentrations (0.2; 0.5; 1.0; 2.0; 3.0 mg/l) of auxin (2,4-dichlorophenoxyacetic acid) and cytokine (6-benzylaminopurine). The best results for initiation of calli were observed at a low concentration of 2,4-D (0.2 mg/l or 0.5 mg/l) and a high concentration of BAP (2.0 mg/l or 3.0 mg/l). It is well known that Fumaria plants are rich sources of isoquinoline alkaloids and obtained in vitro cultures from F. rostellata could be used as potential technological matrixes for development of a commercial process for protopine production.

Key words*: Fumaria rostellata, callus, in vitro, 2,4-D, BAP.*

INTRODUCTION

Higher plants are sources of natural products used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides (Balandrin and Klocke, 1988). The search for new plant derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Phillipson, 1990). In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as an alternative to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Rao and Ravishankar, 2002, Steingroewer et al., 2013).

Plants of the genus *Fumaria* have been used in traditional medicine as anti-hypertensives, diuretics, hepatoprotectants and laxatives (to treat gastrointestinal disorders), as well as in the treatment of some skin diseases (rashes or conjunctivitis) (Martindale, 1996; Stubing and Peris, 1998; Suau et al., 2002).

The biological activity of *Fumaria* spp. is mostly associated with the presence of isoquinoline alkaloids, the most important of which is protopine. This alkaloid possess hepatoprotective activity (Rathi et al., 2008), inhibits histamine H1 receptors and platelet aggregation (Saeed et al., 1997), inhibits serotonin and noradrenaline transporters and has an antidepressant effect (Xu et al., 2006), as well as antimicrobial, antiviral (Orhana et al., 2007) and anti-inflammatory activities (Saeed et al., 1997).

Fumaria rostellata Knaf. belongs to the genus *Fumaria* (Fumariaceae), which consists of 60 species widely distributed all over the world

(Suau et al., 2002; Jaberian et al., 2013). The plant has branched stems, alternate and petiole leaves with small zygomorphic purplish pink flowers. Given the morphological similarities between species, the identification of different taxons is difficult and based on several specific morphological characters: presence of sepals, their length, the shape of the fruit, the length of the fruit pedicel and the length of the fruit pedicel bracteole. *F. rostellata* presents well developed calyx and corolla with sepal length between 3.5-4 mm and petal length between 8.5-9.5 mm. The plant spreads in the hills or lowland areas, through cultures, ditches and ruderal places (Paltinean et al., 2013). There are scanty data available about potential of *Fumaria rostellata* as a producer of protopine and therefore investigation of its alkaloid metabolism is of high importans.

During the last two decades *in vitro* systems of medicinal plants have been considered as a valuable alternative to intact plants as a source of bioactive substances (Pavlov A., 2014). However, only one report is currently available about *Fumaria in vitro* cultures. Tanahashi and Zeng (1985) were investigated presence of isoquinoline alkaloids in cell suspension cultures of *F. capreolata.* This way assessment of the potential of *in vitro* cultures of *Fumaria* spp. is of high interest.

The aim of the present study was development of protocols for callus obtaining from *F. rostellata* and evaluation of the possibilities for protopine production.

MATERIALS AND METHODS

Plant material

Plants of *F. rostellata* used in this study were collected in May 2013 from their natural habitats near Blagoevgrad, Bulgaria. Identification of the plant species was made through the references deposed in Herbarium of the Institute of Biodiversity and Ecosystem Research in Sofia and Herbarium of Sofia University.

Callus induction

Leaves, flowers and stems of the collected plants were washed with tap water and sterilized by using 70 % ethanol for 20 sec and 7 % (w/v) sodium hypochlorite for 20 min. The explants were triple-washed in sterile water, dried on filter paper, and transferred on callus induction media. Various combinations of induction media were used, all based on Murashige and Skoog (MS) medium, supplemented with 30.0 g/l sucrose, 5.5 g/l ‘‘Plant agar’’ (Duchefa, The Netherlands) and different concentrations (0.2; 0.5; 1.0; 2.0; 3.0 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4D, Sigma, USA) and 6-benzylamynopurine (BAP, Duchefa). Half of the explants were cultivated under illumination (16 h light/8 h dark) with light intensity of 110 $\mu mol/m^{-2}.s^{-1}$ (SYLVANIA Gro-Lux fluorescent lamps, F18W/GRO-LUX), and the other half was grown in darkness at 26ºC. The formed calli were separated from explants and transferred for self-growth in petri dishes under the same conditions with sub-culturing period of 21 days.

Extraction of alkaloids

Lyophilized biomass (0.15 g) was triple extracted with 5 ml ethanol in ultrasonic bath for 15 min. The combined extracts were concentrated under vacuum and dissolved in 3% sulfuric acid (2 × 2 ml). The neutral compounds were removed by extraction (three times) with diethyl ether. The alkaloids were fractionated after basification of the extracts with 1 ml of 25 % ammonia and extraction with chloroform (3×5 ml). The chloroform extracts were then dried over anhydrous sodium sulfate and evaporated to dryness.

TLC-method

For TLC qualitative determination of protopine chloroform – ethyl acetate – methanol – ammonium hydroxide (80:80:40:0.05, v/v/v/v) as a mobile phase was used. The protopine standard was dissolved in water in concentration 1 mg/ml and dried extracts of *F. rostellata* calli were disovled in 1 solution of 1N HCl in methanol (1.0 ml). The samples were spotted onto silica gel aluminium plates (ALUGRAM SIL G, 20x20), (Macherey-Nagel, Germany). The alkaloids were visualized by triplicate spraying with Dragendorff reagent.

RESULTS AND DISCUSSIONS

The species of genus *Fumaria*, including *F. rostellata* grow in grassy areas around crops

as a weed in degraded areas along roads and fences across up to 1000 m altitude. Sterilization procedure of explants from *F.rostellata* was consistent with the places from which plant material was picked. For the purpose explants were treated with 7% solution of sodium hypochlorite for 20 min., after presterilization with 70% EtOH for 15 sec. At these conditions explants remained alive and about 80 % of them were sterile.

Beside sterilization procedure the size of the explants was the second variable that influenced their viability. Smaller explants (up to 10 mm) did not survive sterilization procedure, probably due to tissue damage upon excision and treatment with EtOH and sodium hypochlorite during the process of sterilization of the explants (Goswami et al., 2013). It was proved that the size of 10 mm to 30mm were optimal for our purposes.

Sterilized leaves, stems and flowers of selected plant were transferred on Murashige and Skoog nutrient media, supplemented with different concentrations of growth regulators as it is described in the section Material and Methods. Explants were incubated at 26°C under illumination and in darkness. The first callus structures were observed 10 days after transferring explants on the selected media. Callogenesis of different explants (leaves, stems, flowers) of *F. rostellata* is shown in Figure 1.

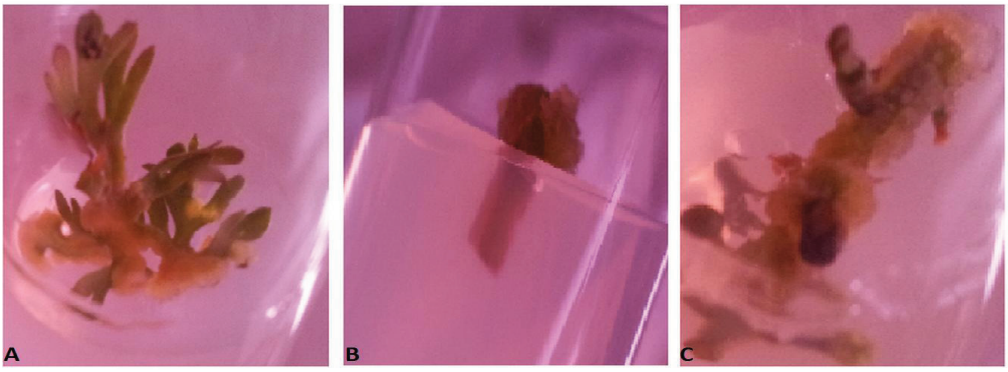

Figure 1 Callogenesis of different explants of *F. rostellata* after 10 days of cultivation: A) leaves; B) stems; C) flower stems

During incubation were contaminated with bacteria or fungi 36 % of all cultivated explants. It was observed, that the explants cultivated under illumination showed a high degree of survival (between 60 and 100 %). The lowest survival degree was observed at the cultivation on medium supplemented with 1.0 mg/l 2,4-D and 3.0 mg/l BAP) (Figure 2A). As a whole, the explants cultivated in darkness showed a lower survival degree. The best combinations of growth regulators in this case were 0.2 mg/l 2,4-D and 2.0 mg/l BAP and 1.0 mg/l 2,4-D and 1.0 mg/l BAP (Figure 2B). The new formed calli were transferred to fresh MS media and cultivated under illumination or in darkness at 26ºC. After 3–4 sub cultivation periods three morphology types of calli were observed. There was not any relationship between type of explants and calli types observed. Newly formed calli were of three morphological types- very compact and hard callus; soft and friable callus; a mixture of compact and friable callus. The type of formed callus strongly depended on used combination of plant growth regulators.

As it was expectable, the calli cultivated under illumination had green colour, while those cultivated on dark possessed yellow colouring

All survived explants cultivated under illumination induced callus (100% induction), while in darkness this value decreased to 87% (Figure 3).

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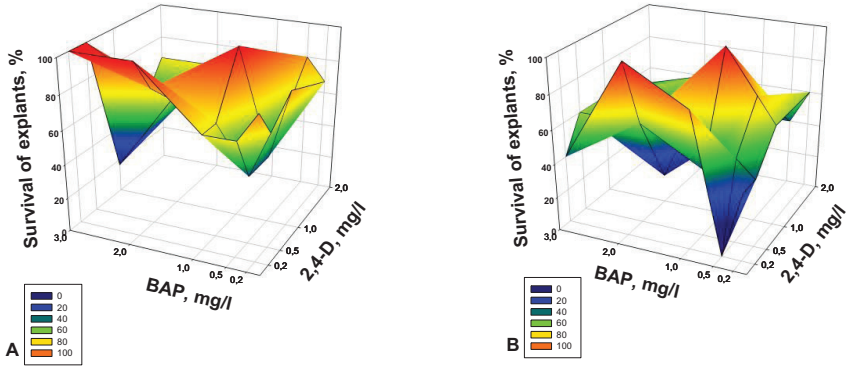

Figure 2 Survival of explants of *F rostellata* calculated as % of cultivated explants: A) under illumination; B) in darkness

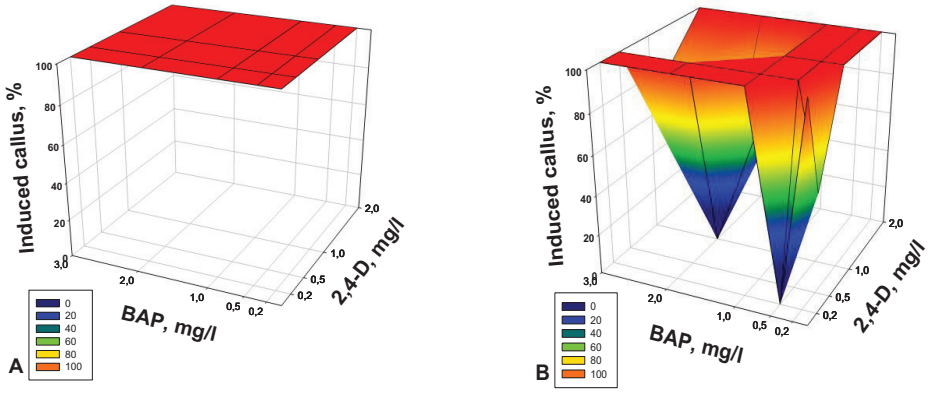

Figure 3 Induced callus of *F rostellata* calculated as % of survived explants: A) under illumination; B) in darkness

When explants were cultivated in darkness 0.2 mg/l 2,4-D and 0.5 mg/l BAP; 0.5 mg/l 2,4-D and 0.2 mg/l BAP; 1.0 mg/l 2,4-D and 2.0 mg/l BAP and 1.0 mg/l 2,4-D and 3.0 mg/l BAP were not appropriate combinations of growth regulators for development of calli with appropriate morphology.

As concequence of callus induction experiments, more than 100 lines were obtained Based on assessment of callus friability and growth 8 lines were selected for further experiments – 5FRL14 (0.2 mg/l 2,4-D and 2.0 mg/l BAP), 6FRL20 (0.2 mg/l 2,4-D and 3.0 mg/l BAP), 18FRL56 (1.0 mg/l 2,4-D and 1.0 mg/l BAP) and 23FRL60 (2.0 mg/l 2,4-D and 0.2 mg/l BAP) cultivated under illumination and lines 11FRL106 (0.5 mg/l 2,4-D and 1.0 mg/l BAP), 17FRL119 (1.0 mg/l 2,4-D and 0.5 mg/l BAP), 23FRL129 (2.0 mg/l 2,4-D and 0.2 mg/l BAP) and 23FRL130 (2.0 mg/l 2,4-D and 0.2mg/l BAP) cultivated in darkness.

TLC qualitative method for fast screening of protopine-synthesizing callus cutures was used (Figure 4). The alkaloid extracts of selected calli lines were spotted in volume of 50 µl (with an unknown concentration of protopine) and the protopine standard was spotted in a concentration of 20 µg/g. Obtained results showed that *Fumaria in vitro* cultures are prospective for further development of biosynthetic process. However for the more detaled assessment quantification by HPLC and/or GC should be performed.

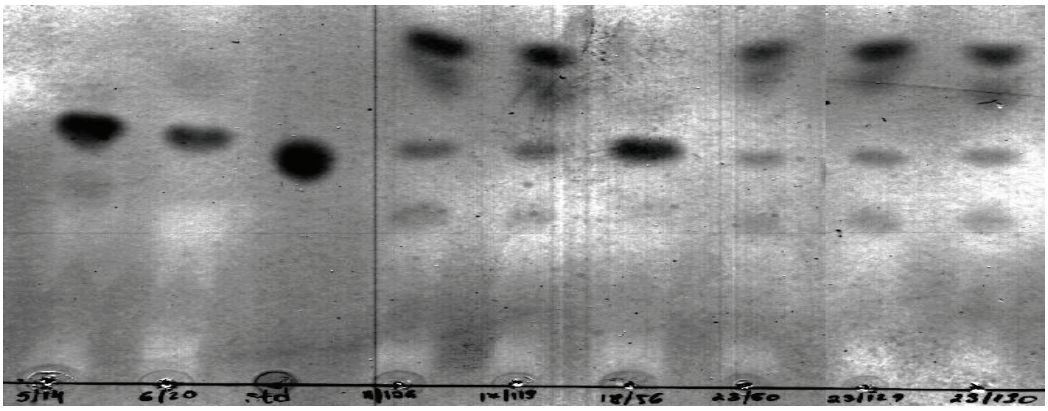

Figure 4 TLC of alkaloid extracts of selected callus lines of *F rostellata*

CONCLUSIONS

In this work, we have established for the fist time protocol callus induction of *Fumaria rostellata* Knaf. The best morphological characteristics and growth behavior were observed for callus lines cultivated on MS media supplemented with low concentration of 2,4-D (0.2 or 0.5 mg/l) and high concentration of BAP (1.0, 2.0 or 3.0 mg/l). Pimary analyses of protopine content showed that the *F. rostelata* in vitro cultures are prospective producers of this pharmacologically important alkaloid.

ACKNOWLEDGEMENTS

The authors thank for the financial support of this research by the Bulgarian Science Foundation, Bulgarian Ministry of Education and Science under contract number: DMU 03/77 – 2011.

REFERENCES

Balandrin J., Klocke J., 1988. Medicinal, aromatic and industrial materials from plants. In Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plant, Vol. 4. Springer-Verlag, Berlin, Heidelberg, 1–36.

Goswami N., Handique P., 2013. Explants size response to in vitro propagation of Musa (Aaa Group) "Amritsagar" Musa (Aab Group) "Malbhog" and Musa (Aab group) "Chenichampa" Banana. Indian Journal of Applied Research, 3(8): 40–43.

Jaberian H., Piri K., Nazari J., 2013. Phytochemical composition and in vitro antimicrobial and antioxidant activities of some medicinal plants. Food Chemistry, 136(1): 237–244.

Martindale W., 1996. Martindale: The Extra Pharmacopoeia, 31st edn. Pharmaceutical Press, London

Orhana I., Özçelik B., Karaoğlu T., Sener B., 2007. Antiviral and antimicrobial profiles of selected isoquinoline alkaloids from *Fumaria* and *Corydalis* species, Zeitschrift für Naturforschung C, 62(1-2): 19–26

Paltinean R., Wauters N. J., Tits M., Frederich M., Angelot L., Tamas M., Crisan G., 2013. Comparative morphological studies on some species of the genus *Fumaria*. Farmacia, 6(2): 371–377.

Pavlov A., 2014. Plant cells and algae in bioreactors II. Engineering in Life Sciences. in press, 14(6): 548–549.

Phillipson J.D., 1990. Plants as source of valuable products. In B.V. Charlwood, and M.J.C. Rhodes (eds.), Secondary Products from Plant Tissue Culture. Oxford: Clarendon Press, 1–21.

Ramachandra Rao S., Ravishankar G., 2002. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology Advances, 20: 101–153.

Rathi A., Srivastava K., Shirwaikar A., Rawat S., Mehrotra S., 2008. Hepatoprotective potential of *Fumaria indica* Pugsley whole plant extracts, fractions and an isolated alkaloid protopine. Phytomedicine, 15: 470–477.

Saeed A., Gilani H., Majoo U., Shah H., 1997. Anti-thrombotic and anti-inflammatory activities of protopine. Pharmacological research, 36(1): 1–7.

Steingroewer J., Bley Th., Georgiev V., Ivanov I., Lenk F., Marchev A., Pavlov A., 2013. Bioprocessing of differentiated plant in vitro systems. Engineering in Life Sciences, 13(1): 26–38.

Stubing G., Peris B., 1998. Plantas Madicinales de la Comunidad Valenciana. Generalitat Valenciana, Valencia.

Suau R., Cabezudo B., Rico R., Nájera F., López-Romero M., 2002a. Direct determination of alkaloid contents in *Fumaria* species by GC-MS. Phytochemical Analysis, 13: 363–367.

Suau R., Cabezudo B., Rico,R., López-Romero. M., Nájera F., 2002b. Alkaloids from *Fumaria sepium*

and *Fumaria agrarian*. Biochemical Systematics and Ecology, 30: 263–265.

Tanahashi T., Zenk N. M., 1985. Isoquinoline alkaloids from cell suspension cultures of *Fumaria capreolata*. Plant Cell Reports, 4: 96–99.

Xu F., Chu J., Qing Y., Li S., Wang S., Qing W., Fei J., Guo H., 2006. Protopine inhibits serotonin transporter and noradrenaline transporter and has the antidepressant-like effect in mice models. Neuropharmacology, 50: 934–940.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

STAKEHOLDERS PERCEPTION AND ACCEPTANCE TOWARDS APPLICATION OF BIOTECHNOLOGY IN AGRICULTURE BASED INDUSTRIES IN MALAYSIA

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Abstract

Biotechnology and agriculture based sectors have been identified as important drivers of economic development in the State of Johor. These sectors are regarded as the new-wave of wealth creation and economic activity characterized by high economic value addition. The progress of biotechnology development is in the state of Johor is highlighted in this report. In addition the study provides a robust strategic direction and action plan for creating a strong biotechnology ecosystem. This study used stakeholders based approach to investigate public perception on application of agricultural biotechnology and stakeholder interest in Malaysia particularly in State of Johor. The results suggested that agriculture based industries strongly believed the creation of vibrant biotechnology ecosystem is needed to jump-start the biotechnology industry. Moreover, most of the companies are looking forward for new technology to improve their business by reducing their operating cost and increasing their productivity. Instead, they claimed the state government should reclaim stewardship of the industry and proactively synergise with the current players to place Johor as the leading biotechnology state in Malaysia. The paper mentioned that state of Johor has become a leader in prospering biotechnology and bioeconomy activities while Iskandar Malaysia, most developed region in Southern Peninsular Malaysia that is expected to act as a prime growth catalyst and complement Johor's overall economic contribution to the Malaysian economy constitutes 45.6% of the Johor population and half of total employment. Biotechnology awareness need to be revitalized to a new level through encouraging the adoption of biotechnology innovation and establishing a synergistic framework for acceleration of agro based industrial development towards sustainable market driven, commercially oriented and environmentally friendly.

Key words*:Malaysia, Agricultural biotechnology awareness, stakeholders perception, framework, ecosystem.*

INTRODUCTION

Biotechnology has been advanced significantly in the past ten year. Current advancement in biotechnology has focused towards economy driven by biotechnology based activities that expected to reach US$262 billion, with an average annual increased rate of 11.0% over the past five years. Globally, agriculture has become a more market-driven sector, with a slow but gradual economic recovery to an average growth rate of 1.9%. Growth prospects for the OECD (Organisation for Economic Co-operation and Development) area in the short term are assumed to remain relatively weak, with an overall growth rate of 1.38% in 2013 just above the low rate of 1.3% in 2012. EU15 members, as a group, are expected to exhibit minimal growth averaging 0.3% in 2013. However, in the medium term, prospects appeared to be better growth (OECD-FAO, 2013).

The term agricultural biotechnology encompasses a variety of technologies used in food and agriculture, for a range of different purposes such as the genetic improvement of plant varieties and animal; genetic characterization and conservation of genetic resources; plant or animal disease diagnosis; vaccine development; and improvement of feeds (FAO, 2009).

Globally, factors such as pest, poor soil quality and unfavorable condition (drought, flooding etc.) reduced crop yields up to 30%, but the impact can be much higher in developing countries because the climatic conditions favor the survival and breeding of insect pests and disease vectors. Therefore, development of high-yielding genetically engineered varieties that are resistant to abiotic and biotic stress can help to increase crop yield. Therefore, it could help stabilize crop production and significantly contribute to food security and economic prosperity.

The agriculture biotechnology is the second largest contributor to the overall biotechnology sectors revenues after the healthcare segment, accounting for 11.5% or around USD 14 billion in 2007. There are an increasing number of biotechnology companies growing globally. Asia Pacific alone is expected to house 57 % of the total number of biotechnology companies by 2014 (Frost and Sullivan, 2009).

Malaysia has targeted biotechnology as a national priority to enhance productivity and sustainability, as well as build wealth and economic growth by 2020 contributing 5% of the nation's GDP. Under this National Biotechnology Plan, agriculture biotechnology, R&D acquisition and financial infrastructure development are given priorities. It is worth noting that FRIM (Forest Research Institute of Malaysia) has been studying jungle plants that potentially could be used as medicine. IBD UTM (Institute of Bioproduct Development) also is looking forward to venture a new dimension in herbal processing and downstream to uplift the national herbal industry.

The biotechnology development in Johor is further boosted by the establishment of Bio-Xcell biotechnology park and ecosystem facility in the new developing region of Iskandar Malaysia (IM). It has attracted a number of key investments from companies including Biocon, Stelis Biopharma, GlycosBio and Metabolic Explorer. In addition, the state government has alienated 10,000 acres of land for Bio-Desaru Organic Food Valley initiative to facilitate the creation of an environment that leads to the industry to flourish in Agriculture Biotechnology.

Although Malaysia has identified biotechnology and agriculture as key economic drivers, the commercialization of local grown technology is still at infancy. Scientists are striving to translate their bench work into dollars and cents, however the success rate of the technology transfer to local entrepreneurs and industry need to be improved. Hence, there is a real need for all those involved in this industry to address the challenges in order to enhance the biotechnological growth in the Malaysia, particularly in agricultural biotechnology (Ismail, 2012).

Industrialized countries seemly have been well versed on public discussion on agricultural biotechnology that ultimately aimed to improve the livelihood of developing countries. However, the discussion regarding advent of biotechnology are less explained probably due to the risks in threatened life rather than potential long term hazard of new advancement (Gaskell et al., 2004).

The objective of this study is to access the current status of biotechnology development in the state of Johor. In addition, to investigate the perception of the stakeholders involved particularly the agriculture based industries about adoption of biotechnological tools in their R&D and manufacturing activities. Furthermore, the study provides a robust strategic direction and action plan for creating a strong biotechnology ecosystem.

METHODOLOGY AND SURVEY DESIGN

2.1 Description of study area

Johor is located in the southern peninsular of Malaysia known as the "Southern Gateway" being south of Malacca, Negeri Sembilan and Pahang and north of Singapore from which it is separated by the Straits of Johor. Johor is the second largest state (19,984 km^2) and also one of the most developed state in Peninsular Malaysia (1.4872^0 N, 103.7811^0 E). Johor has a population of 3.5 million. An increased economic activities and expanding population is expected to become reality when IM was established in 2008. Iskandar Malaysia (IM) is the third largest metropolis (2,217 km^2) and the most developed region in the state of Johor with five flagships zones: Zone A (JB city centre), Zone B (Nusajaya), Zone C (Western Gate Development, Zone D (Earstern Gate Development), and Zone E (Senai-Skudai).

In Johor, the agriculture sector has been identified as the third engine of growth after manufacturing and service sectors. This sector experienced a moderate growth of performance of 12.3% in 2012 in which the Gross Domestic Product (GDP) increased from RM8.14 billion in 2011 to RM8.45 billion (Statistic Malaysia, 2013). The Johor state's GDP size of RM53.2bn is the third-biggest contributor to Malaysia's GDP (9.3-9.8% each year) with 60% coming from the Johor Bahru area. The state's economy expanded 3.7% p.a. in 2005-10, moderately below the national real economic growth of 4.4% p.a. In line with this, Johor is set to lead in agriculture research and development (R&D) activities in the country, with agriculture poised to become one of the main revenue earners for the state.

From this sense, this study was designed as the targeted population in this region meets the requirement of the diverse background (R&D institutions, biotechnology related industries) to access the needs and industries response on the development of biotechnology in Johor.

2.2 Survey Data Collection

This study used the stakeholders-based approach suggested by Aerni (2005) to investigate public perception and relevant stakeholder interest. All the sample selection was all agricultural based industries. Twenty two stakeholders were invited to participate in this questionnaire. Sample selection was covered the three main sectors which was crops, livestock and fisheries industries. Participants were selected from relevant stakeholders that were mainly active and contribute to the development of biotechnology in Johor with the assistance of Johor Biotechnology and Biodiversity Corporation (J-BIOTECH). This selection of participants was accordingly matched with Laumann and Knoke methodology (Laumann and Knoke, 1987). The selected stakeholders were contacted personally. After agreeing for being as one of respondents by telling them the purpose of the survey, field and site visit were conducted to access them. The stakeholder representatives were given an early introduction of current development of biotechnology and examples of latest achievement in Johor State Government.

This approach was recommended by Kelley (1995) to reduce measurement errors and provision of adequate information prior to survey. They were also assured that their views and opinions would be kept confidential. The selected stakeholders have their right either to accept or reject to participate or preferred to a replacement with same size of their business operation.

The selected stakeholder representatives were asked based on the questionnaires provided that contained of eleven items. The participants were asked about the basic information and history of their operational business since establishment. The data were analyzed using Microsoft Office Excel statistical package.

RESULTS AND DISCUSSIONS

3.1 Background analysis

22 respondents from different respective organizations completed the questionnaire. The majority of industries in this survey are operating small business activities (50%), followed by micro (22%), large (17%) and medium business activities with 11% (based on SME Corp. classification SME Corp, 2013).

The respondents were categorized into the following institutional groups:

Government agencies: four respondents from research institutions and government state agencies related to agriculture, livestock and fishery.

Industries: 17 respondents from local companies (fields of agriculture, aquaculture, agrotourism businesses, producer of organic farming food, herbal based product manufacturing, plant tissue culture, oil palm plantation and bio-based fertilizer manufacturing).

The first part of the questionnaire began with the general information of particular industries regarding on their identification of geographical scope based on R&D, manufacturing and marketing. This included the accreditation status secured, raw material supply, total average annual R&D investment and capability of their product to penetrate the market.

The second part was about the supported institutional network collaboration, selection of business location, availability of skillful human resources, business climate and technology provision. This part had to be assessed on a scale ranging from one to five. The third part contained the future plan and their recommendation for the improvement of the development of biotechnology in state of Johor as well as for whole nation Malaysia.

3.2 Descriptive analysis

Most of stakeholders participated in the survey involved in manufacturing activities. These companies are accredited under Malaysia Good Agriculture Practice (MyGAP), ISOs (9001: 2000, 9008, 17025, 2200) and Competency of Oil Palm Nursery (COPN) for their agriculture business. Of all companies surveyed, seven are awarded with Bionexus status by Biotech Corp. All companies acquire 100% of their raw material locally thus restricting their manufacturing capacity. Acquisition of semen from abroad has guaranteed the good quality potential for goat breeding purpose. The artificial insemination technique was utilized to produce high quality of progeny with the controlled environment. In other aspect for consumer preference towards organic food, some organic vegetable producers have been gone beyond a leap abound by accreditation from Malaysia Organic Scheme (SOM), National Association for Sustainable Agriculture Australia (NASAA) and International Organic Standard (IFOAM). These accreditations are included under good agriculture practice to ensure their products remain competitive internationally (Salleh et al., 2005). Livestock based industries improved the milk station facilities for the bulk fresh milk production to capture the regional market demand. Most of the stakeholders voluntarily adopt a secured accreditation status to avoid any economic loss from foodborne illness and improved market accessibility 20 out of 22

respondents conduct their own R&D activities to add value of their products. However, some of the government agencies still preferred to practice conventional methods. Their approach is basically to help smallholder farmers in solving agronomic problems with new technologies and services. Engagement in collaborative research with other research institutes and universities has promoted technological innovation and transfer into certain industries for the betterment of long term benefit. Moreover, most of the stakeholder representatives are looking forward for new technology to improve their business by reducing their operating cost and increasing their productivity.

The strategy to have a network collaboration is encouraged to embrace and enhance synergy between the four key mainstays – Government, youth, institutes of higher learning (IHL) or public research institutes (PRI) and industry. The ISAAA (International Service for the Acquisition of Agribiotech Application) and USAID (United State Agency for International Development) are the examples of smart partnership of participation of NGO's that facilitate the acquisition of agricultural biotechnology application in pursuit of enhanced food security of developing countries (Horsch and Montgomery, 2004). Even, a prominent private company such as Monsanto is committed to contribute for the benefit of innovative agriculture in farming worldwide.

The awareness level of biotech companies on J-BIOTECH and BIOTECHCORP presence is at 86.4% (19 out of 22) and 68.2% (15 out of 22) respectively. 26.3% (5 out of 19) and 46.7% (7 out of 15) of these companies engaged J-BIOTECH and BIOTECHCORP in research and development activity respectively. However, only one company engaged with local university in carry out research activities. Stakeholders acknowledged that the Malaysian biotechnology industry is "only in its infancy," and BIOTECHCORP is needed to nurture a supportive environment and build capability in this area. It is intended to be the principle agency coordinating institutional support that includes tax incentives, research and technology parks (e.g., Bio-Xcell), talent immigration visas, venture capital financing, and industry trade representation. This "institutional strategy" embraced by Malaysia has been adopted previously by many developing countries where institutional instruments tend to play a significant and central role at the firm and national levels of innovation. This strategy has been shown to confer positive effects such as enhanced focus, inter-firm coordination and efficiency.

Previously, In 2005 Johor proposed a visionary ten-year masterplan for the development of niche biotechnological area in the state while biotechnology was still in its infancy in Malaysia. This ground breaking effort even preceded the national agenda and the inception of BIOTECHCORP. It was recommended that Johor focused on the multi-trillion global market of wellness products, such as herbal, nutraceutical, and phytopharmaceutical products. In JMP (Johor Master Plan) 2005, a bioproduct validation centre, first of its kind in Malaysia that was proposed to add value to local bioproducts did not materialized. Therefore, the masterplan suggested that agriculture based industries strongly believed the creation of vibrant biotechnology ecosystem is needed to jump-start the biotechnology industry.

In the JMP 2005, the main focus was on agrobiotechnology. However, with the rapid development in Johor particularly the establishment of Iskandar Malaysia (IM) and Bio-Xcell, the Johor biotechnology industry is now more diverse and has included pharmaceutical and biomedical biotechnology. The entry of Biocon Ltd. with an investment of RM500 million at Bio-Xcell, Nusajaya is a testament to this development. A number of Johor-based biomedical companies are now producing pharmaceuticals as well as traditional medicines. However, not many of the respondents know about the implementation

of this masterplan alongside the current achievement of biotechnology in this region. This indicates that ongoing discussion and workshop on this appropriate matter continues to be an issue.

All respondents selected Johor as their business location due to the lower land cost in comparison to other state in Malaysia, the availability of skilled workers and vicinity to the international port, as well as the accessibility of raw materials. In addition, Johor is a strategic business location by the presence of Iskandar Malaysia (IM) and neighbouring Singapore. The proximity of Johor region to Singapore has been exploited to improve in bilateral ties and the inception of Iskandar Malaysia (IM) plans to leverage on each country's strengths and resources. This concern of business location encouraged the respondents to have active network collaboration among the related technology providers and key players within state of Johor as this region offered many opportunities for long term investment.

In addition, Iskandar Malaysia (IM) has offered various incentives through agencies to attract investors to come to Iskandar Malaysia (IM). This myriad of opportunities requires a focal agency to conduct local facilitation. This is the respondents' hope for any state agencies specially J-BIOTECH to facilitate to become a one stop center for all information regarding this matter. Johor state government through J-BIOTECH should promote a more enterprise-friendly culture that supports entrepreneurs, not only financially but also through networks, training, and business information systems. This also could create the availability of support services and networks deemed critical in developing a sustainable biotechnology industry with the capacity to start and manage IP-based ventures with specialist skills such as patent attorneys, venture capitalists, and contract research organizations.

A major issue highlighted by agriculture related industries is the availability of general workers in Johor. 45.5% (10 out of 22) of the companies prefer to employ local staff whilst 54.5% (12 out of 22) of the companies hire foreign workers. The processing time for work permit for foreign workers is between three to six months and most of the companies are satisfied with the service provided by the immigration department.

There is high availability of management staffs category for the agricultural biotechnology related industries. However, the employment of appropriate R&D staff category is very limited that directly impacts the growth of the companies. Malaysia exhibited a low ratio of R&D personnel to the general population compared to Singapore with 15:10,000 and 83:10,000 respectively although there is increment in pattern from year to year (MASTIC, 2004).

Higher education has a strong presence in Malaysia particularly in state of Johor. Studies have shown that the lack of human capital and essential skill sets was the major barrier for the biotechnology industry that is knowledge driven to grow in Malaysia (Abuduxike et al., 2012). The state of Johor should take a proactive step in assisting the engagement between IHL and biotechnology and bioeconomy industry within the unique context of IM and Johor. The engagement should generate inputs from industry that are applicable through immersion basis throughout the years of study in IHL. The respondents also tended to agree that state government should provide a platform to the young graduate in order to nurture them with biotechnology skill and create a sustainable pool for human capital for Johor sate. This skilled talent could be channeled to the required companies to ensure the needs of human capital in every single of biotechnology level are fulfilled.

From the survey, 50% (11 out of 22) of the respondents stated that it is challenge to obtain government funding to support their business activities. Their view on the current government funding procedures is complicated and cumbersome paperwork. Some of the companies also are not aware of what type of funding provided by the government. On the other hand 27.3% (6 out

of 22) of the respondents stated that they have no problem with funding as they have adequate capital. Significant funding for R&D activities in Malaysia especially in Johor is required to promote the cutting edge technological innovation in the country. Majority of the respondents are categorized as small industries and struggle to secure funding. They do not have the expertise and the technical know-how to apply for the grant. Large companies do not have this problem as they are funded privately. In China, a substantial financial capacity from USD10.5 million (1995) to USD38.9 million (2000) had been allocated to create a competitive biotechnology research, indicating this effort becomes one of the priorities area in promoting human wellbeing (Huang and Wang, 2002).

The current regulation on minimum wages, the product grading system and complex protocol for export hinders the company activities. For instance, one of the companies stated that, to export pineapple flour from Malaysia, Korea's government insists the products to go through food radiation treatment. Higher regulation compliance is important if the company decides to export their product. The regulations that have an impact are the minimum wage, accreditation status food radiation treatment, product grading system and detailed protocol for export purposes. Majority of respondents also agreed on these regulations to be followed.

From this sense, the recommendation for the selected stakeholders is to create the synergistic ecosystem for all agricultural biotechnology key players and stakeholders to form this integrated biotic farming system. Main activities of each companies is inter related from upstream level to optimize the downstream process for mass production from biodiversity resources (Ronald and Adamchak, 2008). Agriculture biomass waste could be converted to produce high value product such as biofuel through fermentation and bioconversion towards sustainable economic development (Hautea and Escaler, 2004). They claimed the state government should reclaim stewardship of the industry and proactively synergise with the current players to place Johor as the leading biotechnology state in Malaysia.

CONCLUSIONS

The research findings provide as a basis database for understanding public acceptance of biotechnology in the developing countries. The involvement of Johor stakeholders in biotechnology is positive and could be improved and many of them are aware of biotechnological tools for enhancement of their performance. Respondents also are pleasant towards acceptance of biotechnology in order to place Johor as the leading biotechnology state in Malaysia. Stakeholders also consider biotechnology in agriculture sector is strategically significant tool for achieving national food security. Biotechnology awareness need to be revitalized to a new level through encouraging the adoption of biotechnology innovation and establishing a synergistic framework for acceleration of agro based industrial development towards sustainable market driven, commercially oriented and environmentally friendly policy outcomes.

ACKNOWLEDGEMENTS

The authors wish to acknowledge for all the agriculture based industries and selected stakeholders for participating this survey.

REFERENCES

Abuduxike G., Aljunid S.M., Sulong S., 2012. Main challenges in developing biotechnology industry in Malaysia: perspectives from the innovative biotechnology firms. BMC Public Health, 12: A25.

Aerni P., 2005. Stakeholder attitudes towards the risk and benefits of genetically modified crops in South Africa. Envoironemntal Science & Policy, 8: 464-476.

FAO, 2009 Background document to ABDC-09. Biotechnology applications in crops in developing countries. When finalized, available at www.fao.org/biotech/abdc/ backdocs/en/.

Frost & Sullivan, 2009. Malaysian Biotechnology Sector: The Impact of the Global Economic Crisis http://www.frost.com/prod/servlet/press-release.pag?docid=165356648&txst=FcmCtx1&txht=FcmCtx2&ctxhl=FcmCtx3&ctxixpLink=FcmCtx3&ctxixpLabel=FcmCtx4

Gaskell G., Ten Eyck T., Jackson J., 2004. Seeds, food and trade wars: public opinion and policy responses in the US and Europe. Journal Commercial Biotechnology, 10 (3), 258-267.

GDP by State National Account 2005-2012, Statistic Malaysia, 2013.

Hautea R.A., Escaler M., 2004. Plant biotechnology in Asia. Journal of Agrobiotechnology Management & Economics, 7: 2-8.

Horsch R., Montgomery J., 2004. Why we partner: collaborations between the private and public sectors for food security and poverty allevation through agricultural biotechnology. Journal of Agrobiotechnology Management & Economics, 7: 80-83.

Huang J., Q Wang., 2002. Agricultural biotechnology development and policy in China. Journal of Agrobiotechnology Management & Economic, 5(4): 122-135.

Ismail S., 2012. Malaysian Agricultural Biotechnology: An Outlook on Recent Developments, Regulatory Framework and Impediments. Australian Journal of Basic and Applied Sciences, 6(11): 342–353.

Kelley J. 1995. Public perceptions of genetic engineering: Australia, 1994, Final report to the Department of Industry, Science and Technology, http://www.international-survey.org./Kelley_1995_Pub_Percept_Genetic_Engineering2.pdf.

Laumann E.O., Knoke D., 1987. The organizational state. In: Social Choice in National Policy Domains, The University of Winconsin Press, Madison.

Malaysian Science and Technology Information Centre (MASTIC), 2004. Malaysian Science and Technology Indicators 2004 report, pp40.

OECD-FAO, 2013. Agricultural Outlook 2013-2022: Highlight, OECD/FAO

Ronald P., Adamchak R.W., 2008. Tomorrow's table. New York: OXFORD University Press.

Salleh M.M., Yunus H., Osman N., 2007. Status and perspectives on good agricultural practices in Malaysia. Proceedings of the International Seminar on Technology Development for Good Agricultural Practice in Asia and Oceania, 45-52.

SME Corp, "Guideline for new SME definition," Issued by: SME Corp Malaysia Secretariat to the National SME Development Council, October 2013. [Online]. Available: www.smecorp.gov.my/vn2/sites/default/files/guideline_0.pdf. [Accessed: 21-Mar-2014].

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

CAPSAICINOIDS EXTRACTION FROM SEVERAL *CAPSICUM* SPECIES CULTIVATED IN ROMANIA

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Abstract

The latest world trends in scientific research are directed towards the production and application of secondary metabolites. The plant of the genus Capsicum produces a fruit (chilli pepper) with unique bioactive compounds. Pepper fruits are a rich source of metabolites with potential health-promoting properties, for example carotenoids (provitamin A), ascorbic acid (vitamin C), tocopherols (vitamin E), capsaicinoids and flavonoids.
Capsaicinoids are the compounds responsible for the hot, spicy flavour presented by many varieties of peppers. There were identified over 20 compounds, capsaicin analogues, of which most important (>95%, w/w) are: capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin. Capsaicinoid extraction from peppers is typically performed using organic solvents, however, the extraction efficiencies can vary with peppers, their parts and pre-extraction processing. A major component of this group is capsaicin.
This study involves extraction of capsacinoids from three varieties cultivated in Romania. Capsaicinoids were identified in all extracts with concentration ranging from 0.5 to 0.8% (dry weight) and the best results were obtained with 96% ethanol as solvent.

Key words*: capsaicinoids, extraction, Capsicum sp., ethanol.*

INTRODUCTION

Secondary plant metabolites represent a significant economic group used in different areas such as production of food additives, pigments, pharmaceuticals and biopesticides. Around the world is known five varieties of *Capsicum sp.*, which are *C. annuum, C. frutescens, C. chinense, C. baccatum* and *C. pubescens* (Dhaliwal et al, 2014).
The most important components in the group of secondary metabolites are derived from the biologically active components of the species *Capsicum annum L.*. (Tilahun et al., 2013). The pungent metabolites in the fruits of *Capsicum* species are called capsaicinoids, and among the most abundant of these components are capsaicin (CAP) and dihydrocapsaicin (DHC), which are responsible for about 90% of total pungency (Islam et al., 2015; Amruthraj et al., 2013; Anwar et al., 2014).
In addition to capsaicin and dihydrocapsaicin, many less abundant capsaicinoids have been detected in *Capsicum* extracts, including nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin (Mozsiksi et al., 2009).
Capsaicinoids are alkaloids produced by a condensation reaction between an aromatic moiety and a C9-C11 branched-chain fatty acid and are synthesized exclusively in the epidermal cells of the placenta of *Capsicum* fruits (Zaki et al., 2013).
Capsicum is the only genus known to produce capsaicinoids (Cisneros-Pineda et al., 2007), and capsaicin is the major pungent lipophilic alkaloid of the *Capsicum* fruits (Chen et Kang, 2013).
The amount of capsaicinoids in peppers is dependent on the genetic makeup of the plant, developmental stage, and the environment where it is grown, such as geographical origin and temperature, nutrients soil, light, water stress (Zewdie and Bosland, 2000; Bosland and Baral, 2007; González-Zamora et al., 2013; Sganzerla et al., 2014).
Jurenitsch et al. (1979) found considerable differences in total capsaicinoids within cultivars grown in greenhouses, laboratory field

studies, and regular plantations, highlighting the effect of environmental conditions on capsaicinoid content.

Capsaicin is currently used for the treatment of diabetic neuropathy, osteoarthritis, post-herpetic neuralgia, and psoriasis, as well as there are many patents on insecticides, insect or animal repellents, and pesticides containing capsaicinoids (Dang et al., 2014).

Therefore, the present study was undertaken to determine the capsaicinoids content of three varieties of Capsicum cultivated in Romania during 2014, using different organic solvents for extraction (ethanol, methanol, acetone and acetonitrile).

MATERIALS AND METHODS

1. Standards and Chemical Reagents

The solvents, such as methanol, acetonitrile, acetone and ethanol used for extraction of capsaicinoids were purchased from Merck (Germany) and pure capsaicin obtained from Sigma Aldrich was used as reference standards. HPLC grade chemicals were used for the mobile phase and all the other reagents used in the analysis were analytical grade.

1.1. Plant material

Dried fruits of three varieties of hot peppers, such as *Pintea* (SP1), *Habanero rouge* (SP2) and *De Cayenne* genotypes (SP3) were used for extraction of capsaicinoids. Peppers used in this study were harvested from a local farm in September 2014 at maturity stage and at full fruit size.

1.2. Capsaicinoids extraction

Capsaicinoids are soluble in various solvents such as chloroform, acetone, ethyl ether, ethyl acetate, methyl chloride, ethanol, and 2-propanol, methanol, acetonitrile, among others (Santamaria R.I. et al., 2000).

First fully ripe fruits samples were allowed to dry at 50°C for 24-48 hours, with a moisture content ranging between 3-4%, and crushed into powder before the extraction. So, 25 g of powder was dissolved in 300 mL organic solvents, using Soxhlet extraction, for 5 hours, and the temperature was adjusted to obtain at least 40 cycles (Figure 1). Then, the solvent was removed after extraction using a rotary evaporator, and the vacuum level was adjusted so the temperature to be less than 40 degrees Celsius.

Figure 1: Soxhlet extraction

The capsaicinoids contents, such as capsaicin, dihydrocapsaicin and nordihydrocapsaicin were determined using High performance liquid chromatography. It was used a standard solution of capsaicin with a concentration of 0.05%, 0.025% nordihydrocapsaicin, and 0.025% dihydrocapsaicin in methanol. Also, a phenylsilicagel column (5 µm), l = 0.250 m, Ø = 4.6 mm, temperature = 30 °C it was used. Mobile phase was acetonitrile R, 1g/L solution of phosphoric acid R (40:62 V/V), flow rate: 1.0 mL/min, detection: spectrophotometer at 225 nm, injection:10µL and the elution order: nordihydrocaspsaicin, nonivamide, capsaicin, dihydrocapsaicin.

RESULTS AND DISCUSSIONS

From all three *Capsicum* varieties, the best results were obtained with Habanero rouge genotype (SP2), while the lowest results were obtained with Pintea genotype (SP1) (Table 1)

Table 1. The percentage of capsaicinoids content from ethanolic extracts of *Capsicum sp.*/ dry weight

Genotype / Capsaicinoids	Pintea	Habanero rouge	De Cayenne
Nordihydrocapsaicin (%)	0.086	0.201	0.102
Capsaicin (%)	0.241	0.417	0.359
Dihydrocapsaicin (%)	0.173	0.182	0.194
Total (%)	0.500	0.800	0.655

As we can see from the data presented in Table 1 the amount of capsaicin and dihydrocapsaicin concentrations ranges between 75%-85%. (Figure 2).

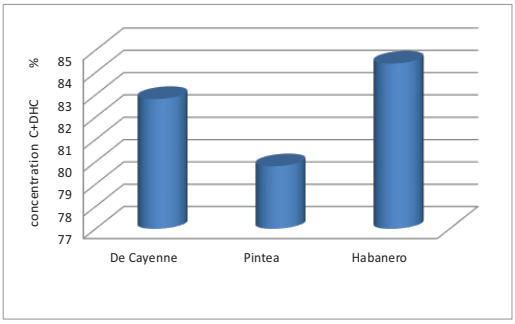

Figure 2: Comparative chart on the capsaicinoids concentrations of *Capsicum sp.*

Comparing the efficiency extraction of capsaicinoids from three varieties of *Capsicum* sp. with the four solvents tested, it can be observe that ethanol showed the higher content of capsaicinoids (0.8%), and methanol have the lowest content of capsaicinoids (0.599%) (Table 2).

Table 2. The percentage of capsaicinoids content obtained with organic solvents from *Capsicum sp.*/dry weight

Solvents / Capsaicinoids	EtOH	MeOH	Me_2Co	Acetonitrile
Nordihydrocapsaicin (%)	0.201	0.155	0.199	0.194
Capsaicin (%)	0.417	0.272	0.405	0.366
Dihydrocapsaicin (%)	0.182	0.172	0.177	0.199
Total (%)	0.800	0.599	0.776	0.759

So, the organic solvents influence the total amount of capsaicinoids, but not their ratio in extract (Table 3, Figure 3).

Table 3: The influence of solvents on total amount of capsaicinoids

Solvents / Capsaicinoids	EtOH	MeOH	Acetone	Acetonitrile
Capsaicin (%)	52.12	45.41	52.19	48.22
CAP (%) + DHC(%)	74.88	74.12	75.0	74.44

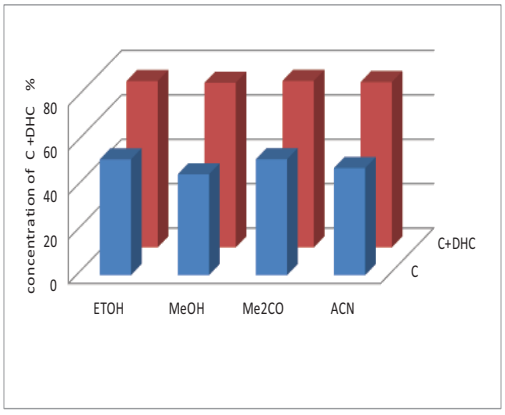

Figure 3 : Comparative chart on the influence of solvents on concentrations of capsaicinoids

CONCLUSIONS

This study showed the influence of genotype on capsaicinoids extraction from three varieties of peppers. Among four solvents tested, ethanol showed the higher content of capsaicinoids comparing with methanol, which showed the smallest amount of capsaicinoids.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

REFERENCES

Amruthraj Nagoth Joseph, Raj J.P. Preetam, Lebel L. Antoine, 2013. Polar aprotic extraction of capsaicinnoids from Capsicum Chinense Bhut Jolokia for Antimicrobial activity. International Journal of Biological & Pharmaceutical Research, 4, (12), 959-964

Anwar Effionora, Ramadon Delly, Harmita, 2014. Formulation and evaluation of gel and emulgel of chili extract (Capsicum frutescens L.) as topical dosage forms. International Journal of Pharmacy and Pharmaceutical Sciences, 6, (3), 13-16

Bosland Paul W., Baral Jit B., 2007. 'Bhut Jolokia' - The World's Hottest Known Chile Pepper is a Putative Naturally Occurring Interspecific Hybrid. HortScience, 42,(2), 222-224

Cisneros-Pineda Olga, Torres-Tapia Luis W., Gutierrez-Pacheco Luis Carlos, Contreras-Martın Fernando, Gonzalez-Estrada Tomas, Peraza-Sanchez Sergi R., 2007. Capsaicinoids quantification in chili peppers

cultivated in the state of Yucatan, Mexico. Food Chemistry, 104, 1755-1760

Dang Yan-Yan, Zhang Hua, Xiu Zhi-Long, 2014. Three-liquid-phase Extraction and Separation of Capsanthin and Capsaicin from Capsicum annum L.. Czech J. Food Sci., 32, (1), 109-114

Dhaliwal M.S., Abhay Yadav, Jindal S.K., 2014. Molecular characterization and diversity analysis in chilli pepper using simple sequence repeats (SSR) markers. African Journal of Biotechnology, 13, (31), 3137-3143

González-Zamora Alberto, Sierra-Campos Erick, Luna-Ortega J. Guadalupe, Pérez-Morales Rebeca, Ortiz - Juan Carlos Rodríguez, García-Hernández José L., 2013.Characterization of Different Capsicum Varieties by Evaluation of Their Capsaicinoids Content by High Performance Liquid Chromatography, Determination of Pungency and Effect of High Temperature. Molecules, 18, 13471-13486

Islam Md Aminul, Sharma Shyam Sundar, Sinha Pratima, Negi Madan Singh, Neog Bijoy, Tripathi Shashi Bhushan, 2015. Variability in capsaicinoid content in different landraces of Capsicum cultivated in north-eastern India. Scientia Horticulturae, 183, 66-71

Jurenitsch J., M. David, Heresch G., Kubelka M., 1979. Detection and identification of new pungent compounds in Capsicum fruits. Planta Med., 36, 61-65.

Lei Chen, Young-Hwa Kang, 2013. Anti-inflammatory and antioxidant activities of red pepper (Capsicum annuum L.) stalk extracts: Comparison of pericarp and placenta extracts. Journal of Functional Foods, 5, 1724-1731

Materska Malgorzata, 2014. Bioactive phenolics of fresh and freeze-dried sweet and semi-spicy pepper fruits (Capsicum annuum L.). Journal of Functional Foods, 7, 269-277

Mozsik Gyula, Past Tibor, Salam Omar M. E. Abdel, Kuzma Monika, Perjesi Pal, 2009. Interdisciplinary review for correlation between the plant origin capsaicinoids, non-steroidal antiinflammatory drugs, gastrointestinal mucosal damage and prevention in animals and human beings. Inflammopharmacol., 17, 113-150

Naima Zaki, Aziz Hasib, Abdelmalek Hakmaoui, Fatima Dehbi, Aaziz Ouatmane, 2013. Assessment of Color, Capsaicinoids, Carotenoids and Fatty Acids Composition of Paprika Produced from Moroccan Pepper Cultivars (Capsicum Annuum L.). Journal of Natural Sciences Research,3, (7).

Santamaria R.I., Reyes-Duarte M.D., Barzana E., Fernando D., Gama F.M., Mota M., Lopez-Munguia A., 2000. Selective Enzyme-Mediated Extraction of Capsaicinoids and Carotenoids from Chili Guajillo Puya (Capsicum annuum L.) Using Ethanol as Solvent. Journal of Agricultural Food Chemistry, 48, 3063-3067

Sganzerla Marla, Janclei Pereira Coutinho, Arlete Marchi Tavares de Melo, Helena Teixeira Godoy, 2014. Fast method for capsaicinoids analysis from Capsicum chinense fruits. Food Research International, 64,718–725

Tepić Aleksandra N., Dimić Gordana R., Vujičić, Žarko S. Kevrešan Biserka L., Varga Marika , Šumić Zdravko M., 2008. Quality of Commercial Ground Paprika and its Oleoresins. Apteff, 39, 1-212

Tilahun Samuel, Paramaguru Pandiyan, Rajamani Kandhasamy, 2013. Capsaicin and ascorbic acid variability in chilli and paprika cultivars as revealed by HPLC analysis. Journal of Plant Breeding and Genetics, 01, (02), 85-89.

Zewdie Y., Bosland P.W., 2000. Evaluation of genotype, environment, and genotype-by environment interaction for capsaicinoids in Capsicum annuum L.. Euphytica, 111, 185–190

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE EFFECT OF UV IRRADIATION ON *IN VITRO* CULTURES DEVELOPMENT OF GOLDEN ROOT – ENDANGERED MEDICINAL PLANT

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Abstract

Increasing production of secondary metabolites from medicinal plants is highly important for food and pharmacology. The plant cell and tissue culture methods offer an integrated approach for valuable substances production. Biosynthesis could be enhanced by different chemical and physical stimuli including UV radiation. Some investigations demonstrated increase of metabolite components in cultivated plant cells and tissues after their irradiation, particularly with UV-B (280-315 nm) and UV-C (200-280 nm) However, in vitro cultures potential to produce secondary metabolites after UV exposure is not extensively studied giving a perspective to enlarge the investigations on the role of UV light as elicitor. In this respect medicinal plants are promising. Golden root is a wildly used medicinal plant, containing a range of antioxidant compounds. The aim of this work was to study the effect of low doses UV radiation on the process of callus formation and in vitro growth.
Plants, calli and explants cultivated in vitro on different nutrient media were irradiated with low doses UV-B (280-315 nm) and UV-C (200-280 nm). Irradiation exposure to UV-B light was for 3, 5 and 7 minutes and to UV-C light for 1 and 2 minutes.
Irradiation caused changes in callus growth, structure and color without influencing calli and plants viability. Lethal effect was observed only for leaf explants exposed to UV-C for 2 min and UV-B for 7 min.

Key words*: medicinal plant, golden root, calli; UV-B; UV-C.*

INTRODUCTION

The use of medicinal plants and their biologically active compounds is increasing worldwide. The plant cell and tissue culture methods offer an integrated approach for valuable substances production. Biosynthesis could be enhanced by different chemical and physical stimuli including UV radiation.
Ultraviolet radiation (UV) is a fraction of sunlight reaching the Earth's surface haring the character of abiotic stress factor. It is arbitrarily divided into UV-A with wave length λ 315-400 nm, UV-B (280-315 nm) and UV-C (200-280 nm). Every type UV radiation affects plant growth and development, such as damage to gradually increase with decreasing wave length. UV-A radiation is poorly absorbed by the ozone because it's the smallest harm, and have not a great interest for ecology, physiology and genetics of plants. Independently of the magnitude and specificity of the impact each type UV radiation changes the growth and metabolic processes.
It is known that the duration of exposure and the dose-response are essential to metabolic reactions. In the natural conditions, UV-A radiation influences sustained at low doses on the plants. The reduction of the stratospheric ozone layer caused by anthropogenic factors leads to a systematic increase in UV-B radiation in the last decades (McKenzie et al., 2007). UV-C rays are absorbed by ozone and oxygen and do not reach the earth's surface, except in the high mountain areas (Häder et al., 2007). Despite the ecological insignificance of UV-C radiation on plants growing in natural conditions their use under controlled conditions is essential for various experimental models. Photons of UV-C radiation are high energy and cause rapid damage to the plant metabolism. However the low doses of UV-C can delay

aging as well as to inhibit the development of phytopathogens which is of practical interest to storage. On the other hand, the effects of prolonged exposure of plants to low doses of UV-B and UV-C have not been well studied, especially those of the UV-C. Some authors presented UV-B and UV-C radiation like a stress agent.

Some investigations demonstrated increase of metabolic components in cultivated plant cells and tissues after irradiation, particularly with small doses UV-B (280-315 nm) and UV-C (200-280 nm) (Schreiner et al., 2014). Mutants of *Arabidopsis* lacking flavonoids are hypersensitive to UV radiation whereas another type of *Arabidopsis* mutant possessing constitutive elevated accumulation of flavonoids and other phenolics is tolerant to lethal UV doses (Bieza and Lois, 2001). Flavonoids strongly absorb light in the range of 220-380 nm and are known to be photo-stable (Stapleton and Walbot, 1994; Ghanati et al., 2013). However, *in vitro* cultures potential to produce secondary metabolites after UV exposure is not extensively studied and giving a perspective to enlarge the investigations on the role of UV light as elicitor. In this respect medicinal plants are promising. Sometimes the antioxidant concentration in a plant production might be dependent on the time of evaluation too, like analyzing immediately after the radiation treatment or after a certain period of time duration (Alothman at al., 2009).

Rhodiola rosea is an indigenous plant grown in the cold regions and high mountains area about 1800 m altitude. The species is protected by Low in Bulgaria and other countries due to is it poor germination and over exploitation. Golden root is a wildly used medicinal plant, containing a range of antioxidant compounds. The stimulating and adaptogenic properties of *Rhodiola rosea* are attributed to p-tyrosol, salidroside, rhodioniside, rhodiolin, rosin, rosavin, rosarin, and rosiridin. The plant extracts have a positive influence on memory, nervous system and brain. They have anti-stress and anti-depressant properties, and increase antitumor activity of the body etc.

The aim of this work was to study the effect of low doses UV radiation on some *in vitro* process like callus formation, plant *in vitro* growth and development.

MATERIALS AND METHODS

In vitro culture. In vitro propagated plants *Rhodiola rosea* were used in the experiments (Tasheva and Kosturkova, 2010, 2012a, 2013), leaf explants obtained from these plants and calli. Calli was obtained from leaf explants of *in vitro* propagated plants and cultivated on solidified Murashige and Skoog medium (1962) with added different combination and concentration of growth regulators N^6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid (BAP and 2.4-D) and casein hydrolysate, pH 5.8 (Tasheva and Kosturkova, 2012a, b). The contain of nutrient media for callus induction was as follows:

Variant 1 - BAP (1.0 mg/l) and 2.4-D (1.0 mg/l); Variant 2 – BAP (1.0 mg/l) and 2.4-D (0.5 mg/l); Variant 3 – BAP (0.5 mg/l) and 2.4 –D (1.0 mg/l); Variant 4 – BAP (1.0 mg/l), 2.4-D (1.0 mg/l) and casein hydrolisate 1000 mg/l

However, content of sucrose was 20 g/l, content of agar-agar was constant 6.0 g/l. Culture media for plant propagation was MS medium containing zeatin (2.0 mg/l) and IAA (0.2 mg/l). Culture media were autoclaved at 1.1 $kg.cm^{-2}$, 121^o C for 20 min.

Irradiation of the explants, calli and *in vitro* propagated plants were held 7 days after their transfer and cultivation on the fresh nutrient medium.

Irradiation by UV light/rays. Effect of UV on the growth of *Rhodiola* cells and tissues was monitored by comparing percent of survival of treated with the control ones. As a source of UV rays **(**280-315nm) were used UV-B and UV-C lamps.

Parameters of lamps. As a source of UV-B (200-280nm) radiation was used mercury lamp (Philips TL 20W/12 UV-B c λmax=312±25nm, 2*20W=40W) and UV-C lamps (STYLO STY 115, GE Lighting, c λmax 254 nm, 15 W). The distance between UV lamps and irradiated tissues were 0.31±0.01 m. The irradiation by UV-B light were applied for 1, 5 and 7 minute, and with UV-C for 1 and 2 minutes. The UV-C is a most power and is expected to have stronger effect on plant material.

Characteristics of in vitro cultures. During the study have been monitored morphological characteristics, such as development, shape, texture and pigmentation of callus tissue.

Control observations were conducted on the 3rd, 7th, 15th, 20th day. For the main signs were evaluated qualitative and quantitative characteristics.The physiological characteristics such as period of time needed to induce dedifferentiation in tissues in the formation of callus and time to form non differentiated and differentiated structures such as buds and roots in regenerants, etc. were monitored.

In vitro cultivation condition. Callus cultures were induced and maintained in cultivation room at temperature of 23-24°C and dim light (20 $\mu Mm^{-2}s^{-1}$). The *in vitro* plants were cultivated in a cultivation room at temperature of 23-24°C and 40 $\mu Mm^{-2}s^{-1}$ light intensity. Response of 30 to 50 explants was examined for each variant of media and UV – irradiation. Sigma Plot 11.1 was applied for statistics.

RESULTS AND DISCUSSIONS

In vitro cultures. In vitro propagated plants were used like a source for the leaf explants, reported in our previously study (Tasheva and Kosturkova, 2010).

Growth and characteristics of the callus. Calli were obtained from leaf on *in vitro* propagated plants and cultivated on different nutrient medium. Callus growth rate and tissue characteristics varied depending on the culture media composition (Tasheva and Kosturkova, 2012a, 2013). Characteristics of callus tissues used in the experiments was as follows:

Calli variant 1: soft calli pale in colour, 20% loosy calli with liquid like texture and brownish color;

Calli variant 2: grain, compact calli pale in color;

Calli variant 3: 62 % more compact calli; 38 % loose, liquid like calli

Calli variant 4: compact, grain calli, pale, yellowish-beige in color.

The type of calli tissue affects their survival after irradiation.

Irradiation by UV light/rays. Callus tissue was irradiated to stimulate the growth and biomass accumulation, followed by biologically active substances production. The calli and explants cultivated for callus induction were obtained on various nutrient media. Plants regenerants grown in *in vitro* conditions were irradiated, too. The irradiation was with different UV light (B and C) and time of duration.

On the 3rd day after irradiation leaf explants, calli and *in vitro* propagated plants survived 100 % with exception of calli variant 1 with survival rate 60 %. The necrosis in plant material began to occur later.

Observation on the 15th day after irradiation showed the following changes: high survival rate was observed in the calli tissues and in *in vitro* propagated plants in all case of UV irradiations.

On the 15th day after irradiation with low doses UV-B for 3 minute and UV-C for 1 minute the percentage of survived calli is almost from 70.0% to 95.4 % for UV-B and 64.4 % to 100% for UV-C, respectively (Table 1).

Table 1. Survival rate (%) on explants, calli and plants on 15th and 20 days after UV irradiation.

UV irradiation	Survival [%]											
	15 day						20 day					
	Leaf expl	*In vitro* plants	Calli				Leaf expl	*In vitro* plants	Calli			
			var. 1	var. 2	var. 3	var. 4			var. 1	var. 2	var. 3	var 4
UV – B												
3 minute	42.2	75.5	70.0	82.2	95.4	84.4	0	31.1	62.5	46.6	59.1	72.7
5 minute	26.6	71.1	42.5	75.5	81.8	6.6	0	0	0	48.8	40.9	0
7 minute	0	0	23.0	65.9	48.8	4.0	0	0	0	11.1	0	0
UV – C												
1 minute	17.8	48.8	64.4	100 green 20 %	93.3	96.0	0	12.0	40.0	73.3	42.2	66.0
2 minute	2.0	0	22.2	0	0	0	0	0	0	0	0	0
Control	100	100	100	100	100	100	100	100	100	100	100	100

The control variant were calli, explant and plants cultivated on the same medium without irradiation.

On the 20th day after UV-B irradiation the survival rate of the calli was almost equally. Three minute UV-B irradiation caused survival rate from 46.6 % (variant 2) to 72.7 % (variant 4), while 5 min irradiation resulted 0 % for variant 1 and 4 and 48.8 % survival rate for variant 3. UV-B irradiation for 7 min caused lethal effect on calli variant 1, 2 and 4, while variant 3 hat survival rate 11.1 %. Irradiation with UV-C for 1 min resulted survival rate from 40.0 % (variant 1) to 66 % for variant 4, while 2 min UV-C irradiation caused 100 % necrosis in all type of calli tissue.

Leaf explants were more sensitive to UV irradiation in comparison to *in vitro* propagated plants and calli. On the 15th day their survival rate after 3 and 5 min of UV-B irradiation was 42.2 % and 26.6 %, respectively, while 7 min irradiation caused 100 % lethal effect. One minute irradiation with UV-C resulted in 17 % survival of explants, while irradiation for 2 min caused extremely low survival – 2 %. Lethal effect on the explants was observed on the 20th day after irradiation with any of the different doses UV-B and UV-C light (Table 1).

The survival rate of *in vitro* propagated plants varied depending on the type of UV rays. UV-C irradiation of plants with the minimal time of 1 minute reduced twice survival rate (48 %), while in the case of UV-B longer irradiation for 3 min and 5 min caused less damages (survival of 75.5 % and 71.1 %, respectively). On the 20th day necrosis was observed in the case with UV-B irradiation for 5 and 7 min and UV-C for 2 min, while 3 min UV-B irradiation and 1 min UV-C caused survival plants 31.1 % and 12.0%, respectively. There was not obtained the changes in the structure and texture at the all of the irradiated plant material (Figure 1 – 5).

The survived cultures were transferred on the fresh nutrient medium for mass propagation, followed by the analysis of the biologically active complex.

Exposure to UV adversely affected the growth of *Rhodiola* cells and tissue and this was more pronounced in UV-C treatments. Similarly viability of the cells significantly decreased after exposure to UV-B and UV-C, compared to the control cells. The most adverse effect of UV on cell viability was observed in prolonged exposure periods. Decrease of the viability of the cells was more pronounced under UV C treatments.

The high doses of UV-C and UV-B radiation leads to destructive changes in the organism, as a part of them are similar, like inhibition of growth, decrease the content of nonspecific (protective) substances, etc. From the other side Schreiner et al. (2014) reported that often the treatment with UV light, particularly from the UV-B range (280–320 nm), is an example for effective elicitor application.

Some authors showed that the UV-B radiation had negatively affected the growth of the bean plants, and reduced total biomass with 61.6% (Singh et al., 2011). The short –term irradiation of *Pisum sativum, Triticum aestivum* and *Hordeum vulgare* with 49 kJ m-2d-1 UV-B increased the prolin quality and UV absorbed substances (Alexieva et al., 2001; Fedina et al., 2007).

Figure 1. *In vitro* propagated plants on the 3-th day after UV-B irradiation for 3 minutes.

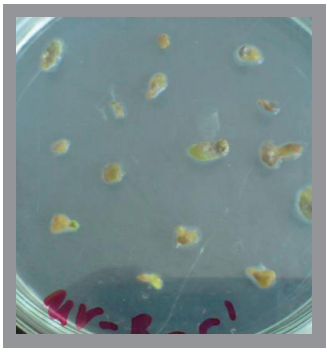

Figure 2. Leaf explants irradiated on the 3th day with UV-B for 5 minutes

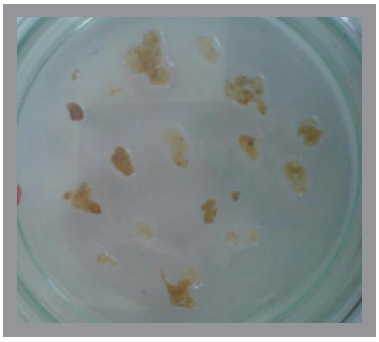

Figure 3. Calli Variant 1 on the 3th day after UV-B irradiation for 3 minutes

Change in biomass accumulation is an important measure to assess s UV-B sensitivity, since this parameter reflects the cumulative effect of many small disruptions in plant function. UV-B radiation exclusion studies have also indicated that UV-B radiation reduced biomass accumulation in cucumber (Krizek et al., 1997), spinach (Mishra and Agrawal, 2006) and mung bean (Agrawal et al., 2006).

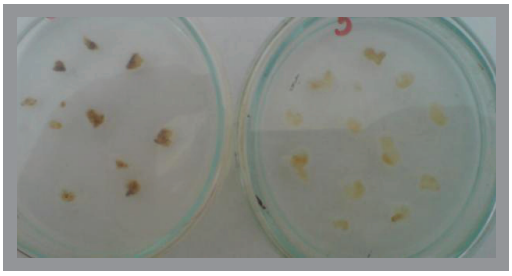

Figure 4. Calli (Variant 1 (left) and Varint 3 (right)) on the 3th day after UV-B irradiation for 5 minute

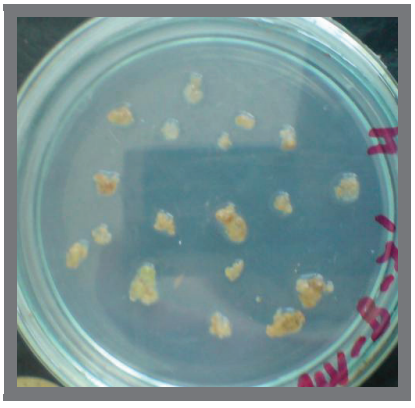

Figure 5. Calli (Variant 2) on the 3th day after UV-B irradiation for 7 minute.

CONCLUSIONS

Obtained results contributed to deeper understanding of influence of UV - rays on plant growth, development and metabolism.The experiments can serve as a basis for future research in the field of increase biologically active substances production using UV irradiation. This would also allow synthesis of valuable substances in laboratory conditions for protection of species from overharvest from their natural habitats.

ACKNOWLEDGEMENTS

Research was supported by National Science Fund of Bulgaria – Project for Junior Scientists DMU 03/55 (leader Dr. K. Tasheva).

REFERENCES

Agrawal S.B., Rathore D. and Singh A., 2006. Combined effects of enhanced ultraviolet-B radiation and mineral nutrients on growth, biomass accumulation and yield characteristics of two cultivars of Vigna radiate L. J. Environ. Biol., vol. 27, 55-60.

Alexieva V., Sergiev I., Mapelli S., Karanov E., 2001.The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell and Environment, 24, 1337–1344

Alothman M., Bhat R., Karim A.A., 2009. Effects of radiation processing on phytochemicals and antioxidants in plant produce. Trends in Food Science and Technology, vol. 20, 201 – 212.

Bieza K. and Lois R., 2001. An *Arabidopsis* mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics'. *Plant Physiology*, vol. 126, 1105-1115.

Fedina I., M. Velitchkova, K. Georgieva, K. Demirevska and L. Simova. 2007. UV-B response of green and etiolated barley seedlings. Biol. Plant. 51(4):699-706.

Ghanati F., Khatami F., Bemani E., 2013. Effects of UV B and UV C radiation on viability, growth, and major natural compounds of *Malve neglecta* L. cells. Iranian Journal of Plant Physiology, vol 4(1), 881 – 887.

Häder D.P., Kumar H.D., Smith R.C., and Worrest R.C., 2007. Effects of solar UV radiation on aquatic ecosystems and interactions with climate change. Photochem, Photobiol Sci. vol. 6, 267 – 285.

Krizek D.T., R.M. Mirecki and Britz S.J., 1997. Inhibitory effects of ambient levels of solar UV-A and UV-B radiation on growth of cucumber. Physiol. Plant, vol. 100, 886-893.

McKenzie R.L., Aucamp P.J., Bais A.F., Bjorn L.O. and Ilyas M., 2007. Changes in biologically-active ultraviolet radiation reaching the earth's surface, Photochem. Photobiol. Sci., vol. 6, 218-231.

Mishra S. and Agrawal S.B., 2006. Interactive effects between supplemental ultraviolet-B radiation and heavy metals on the growth and biochemical characteristics of Spinacia oleracea. Braz. J. Plant Physiol.,vol. 18, 307-314.

Singh R., Singh S., Tripathi R., Agraval S.B., 2011. Supplemental UV-B radiation induced changes in growth, pigments and antioxidant pool of bean (Dolichos lablab) under field conditions. Journal of Environmental Biology, vol. 32, 139 – 145.

Schreiner M., Martinez-Abaigar J., Glaab J., Jammsen M., 2014. UV-B induced secondary plant metabolites. Optik&Photon, vol. 2, 34 – 37.

Stapleton, AE. and Walbot V., 1994. Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. Plant Physiol., vol. 105, 881-889.

Tasheva K. and Kosturkova G., 2010. Bulgarian golden root *in vitro* cultures for micropropagation and reintroduction. Central European Journal of Biology, vol. 5(6), 853-863.

Tasheva K. and Kosturkova G. 2012a. Establishment of callus cultures of *Rhodiola rosea* Bulgarian ecotype. Acta Horticulture, vol. 955, 129-136.

Tasheva K. and G. Kosturkova, 2012b. The role of biotechnology for conservation and biologically active substances production of *Rhodiola rosea* – endangered medicinal species. The Scientific World Journal, 2012, 1-13, doi:10.1100/2012/274942

Tasheva K. and G. Kosturkova, 2013. Chapter 11: Role of Biotechnology for Protection of Endangered Medicinal Plants. In: Marian Petre (Ed.), Environmental Biotechnology – New Approaches and Perspective Applications, Chap. 11, 235 – 286, InTech Publisher, Croatia.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE MOLECULAR ASSESSMENT OF GENETIC DIVERSITY OF EGGPLANT CULTIVARS FROM NORTHERN KARNATAKA IN INDIA USING RANDOMLY AMPLIFIED POLYMORPHIC DNA MARKERS

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Abstract

This is the most comprehensive study to present the genetic diversity available in populations of eggplant cultivars using molecular markers. An attempt was made to analyze genetic variability among twelve eggplant cultivars by Randomly Amplified Polymorphic DNA (RAPD) techniques using twenty random primers. Among the twenty RAPD primers used, OPA-04, OPA-07, OPA-14 and OPA-20 primers gave the best positive results with all twelve egg plant cultivars and bands generated showed a higher level of polymorphism. This reveals a very narrow genetic base of the different collections and the results also indicate that all the populations show more than 80% similarity irrespective of their flower colour and other morphological features. POPGENE software was used for the population genetic analysis, each band produced was treated as a locus and variations among the alleles were calculated. The RAPD markers used in the present study were able to differentiate the genetic diversity in the eggplant cultivars. The genetic diversity of eggplant cultivars was revealed by percentage of polymorphic loci. Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated in the present study shows the segregation of twelve cultivars of eggplant into three main clusters: five entries were grouped into cluster 1, four entries were grouped into cluster 2 and remaining three entries were grouped into cluster 3. The results of RAPD showed that there appears a need to take individuals from more different populations so as to preserve their diversity for the future. The study also confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of different accessions of an important vegetable plant like eggplant.

Key words*: eggplant, genetic variability, RAPD, northern karnataka, molecular markers.*

INTRODUCTION

Biodiversity may be defined as the variation present in all species of all life forms that exist in nature, their genetic material and the ecosystems in which they occur. Diversity can occur at three levels: genetic diversity, species diversity and ecosystem diversity. The importance of biodiversity for human beings has been well recognized in the recent decades and many would argue that diversity is essential for allowing sustainable development of various human activities. Biodiversity enables us to develop social and economic systems that allow the poorest to fulfil their food and nutritional needs and retain the ethnic diversity of countries all over the world (Shiva, 1994). The bioresources of each nation are important for the sustainable development of that nation, but only few countries are endowed, and coordination among countries is required for successful conservation and sustainable use of our biodiversity.

Genetic diversity or variation is important in the process of plant and is also basis of genetic fingerprinting (Sakata et al., 1991; Perez et al., 1998; Larisa, 2008). Assessment of the extent and distribution of genetic variation in a particular plant species along with its relatives is essential in understanding pattern of diversity and evolutionary relationships between accession that help to sample genetic resources in a more systematic fashion for conservation and improvement (Halldén et al., 1996; Patil et al., 2007).

Molecular markers are indispensable tools for understanding the genetic make-up of agricultural and horticultural crops. Molecular

markers allow researchers and molecular plant breeders to study and analyse genetic diversity or variation between two or more plant genomes. Molecular markers are similar to genetic markers and have received much attention these days (Haley et al., 1993; Haig et al., 1994; Larson et al., 1996). Genetic markers are seen as morphological differences. Since from the past three decades, phenotypic differences have been used to build genetic maps. Molecular markers differ from genetic markers in several ways: (1) molecular markers usually occur in greater numbers; (2) molecular markers can be distinguished without depending on complete growth of the plant that is tissue from a plantlet may be analysed rather than waiting for the plant to exhibit some morphological features; and (3) the environment does not alter the expression of molecular markers (Mullis et al., 1986; Hadrys et al., 1992; Prakash et al., 2006; Singh et al., 2006).

Generally, molecular markers are used for the genetic diversity, systematic and phylogenetic analysis. They are used in combination with other markers to construct genetic maps and gene factor genetic linkage studies (Bosland and Votava, 2000; Anderson, 2007; Bosland et al., 2012). Marker linked to a desired trait can be used by plant breeders in marker-assisted selection of qualitative and quantitative traits. When specific markers are identified with gene of interest and these microsatellite markers can be utilized as a selection criterion by molecular plant breeders and selection via marker-assisted selection speeds up the process of new variety development.

Cultivated plants have the market value and consumer preference (Sidhu et al., 2008). These plants perform in good sense against diseases, insects, nematodes and herbicides. These cultivated plants have good nutritive value when compared to the wild one. Present days many hybrid plants have more edible value and hence, every farmer concern about their crop, consumer need and marketing value. Now, consumer prefers hybrid varieties for their food because of different taste. The hybrids also have above mentioned good values when compared to its wild varieties (Nassar, 2000). Many of the breeders or farmers are growing hybrid varieties.

Brinjal eggplant (*Solanum melongena* L.) is an agronomically important crop of Solanaceae family, native to India, and widely cultivated across the globe. Its close relatives are *S. aethiopicum* and *S. macrocarpon* are of African origin. Karihaloo and Gottlieb (1995) proved genetic similarity between *S. melongena, S. incanum* (wild form) and *S. insanum* with allozyme studies and on the basis of the random amplified polymorphic DNA (RAPD) variation (Karihaloo et al., 1995). Much more studies have been done for disease resistance and abiotic stress by using RAPD markers (Doganlar et al., 2002; Frary et al., 2003; Sekara et al., 2007; Prabhu et al., 2009; Tümbilen et al., 2011; Sharmin et al., 2011; Laila et al., 2012; Manjusha et al., 2012; Singh and Malik, 2012; Mohd et al., 2013; Ramesh et al., 2013; Sifau et al., 2014). In this context, the paper presents an analysis of genetic diversity of eggplant cultivars using different RAPD markers.

MATERIALS AND METHODS

Plant Material

The present investigations were carried out at Department of Botany of Karnatak University in Dharwad, Karnataka. The experimental material consists of twelve hybrids of eggplant (Table 1).

Table 1. List of eggplant cultivars used in the present study

Hybrid seeds	Abbreviation
Mahya MEBH-11	MB
Kuduchi-601	KU
Syngenta EPH 718	SY
Sarpan SBH-65	SS65
Sarpan-25	S25
Ruchica	RU
Sarpan Green long	SGL
Sarpan Purple long	SPL
Simisi manjra	SM
Sarpan SBH-55	SS55
Green oval	GO
Ramya	RA

The hybrid seeds were sown in raised nursery bed and covered by the thin layer of sand and watered every day. Hybrid seeds were collected from Sarpan Hybrid Seeds Co. Pvt. Ltd., Dharwad and from farmers' fields in Kalagatagi, Jamakandi, and Belgaum. These are popularly eggplant growing areas of northern Karnataka.

Reagent for DNA isolation

200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS; the proper mixture of these solutions is called as Edwards Reagents.

Reagent for Agarose electrophoresis gel

Agarose; TAE buffer Stock (50X) - 1000ml (Tris base – 242 g, Glacial acetic acid – 57.1ml) - from this stock 0.5X is taken and made up to 2500 ml for running buffer; Blue loading dye; Ethidium Bromide solution - >10mg/ml.

DNA Isolation

For DNA isolation quick extraction method was used instead of CTAB method because of less period of time. This method includes Edward DNA isolation solution. For DNA extraction, Edwards solution was diluted to 10-fold to obtain extraction buffer. A 40 mg of leaf sample was weighed. The leaf sample was chopped to fine pieces with scissors and clean scalpel blade and fine leaf pieces were placed in an Eppendorf tube. About 400µl of DNA extraction buffer was added to the tube. The leaf tissue is grinded with a plastic rod against the tube wall. Once the solution turns to transparent green, the solution is centrifuged at 10000 rpm for 6min. The pellet was discarded and supernatant was stored at -20^{o}C. The collected supernatant is a source of DNA template for further experiments (Edwards, 1991; Lickfeldt et al., 2002; Amani et al., 2011). The quantification of DNA was done by the Agarose gel electrophoresis and Nanodrop method.

Agarose gel electrophoresis

Following the treatment of DNA samples, the electrophoresis of the samples were done according to the Sambrook procedure (Sambrook et al., 1989): 250mg of agarose was weighed and dissolved in 25 ml of TAE buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/1 liter) and boiled in microwave oven. The mixture was allowed to attain 55^{o}C, and then poured it into the gel cassette fitted with comb. Allow the gel to solidify and carefully comb was removed, later the gel was placed in the electrophoresis chamber flooded with TAE buffer. 10 µL of DNA sample (mixed with bromophenol blue dye @ 1:1 ratio) was loaded carefully into the wells, along with standard DNA marker and 50 V of electricity for around 45 min was passed. After the gel was eluted with ETBR solution (10 µL/ml) for 10-15 min and bands were observed under UV transilluminator and confirmed the DNA in sample.

DNA Quantification by Nanodrop technique

For PCR amplification, the concentration and purity of DNA is a primary requisite. Different DNA extraction procedures yield DNA of different quantity and quality, for higher resolution and better reproducibility it is necessary to optimize the concentration of DNA. RAPD amplification is no longer reproducible when certain critical concentration of genomic DNA is less than minimum quantity (Williams et al., 1990; Fraga et al., 2005). Hence, it is necessary to stay above this critical concentration. Moreover, excessive DNA concentration is likely to produce poor resolution or “smear” resulting in a lack of clearly defined bands in the gel. However It is the always best to carry out a series of RAPD reactions using a multiple set of primers and a set of serial dilutions of particular genomic DNA to verify the critical DNA concentrations for better reproducibility of RAPD pattern.

Amplification of RAPD markers by Polymerase Chain Reaction (PCR)

Twenty OPA decamer primers of random sequence were screened initially on a sub sample of four randomly chosen individuals to evaluate their suitability for DNA amplification, which could be accurately scored (Easmin et al., 2008). Primers suitability was evaluated based on the resolution of bands, repeatability of markers and compatibility and comparability within individuals and among varieties (polymorphism). The optimal template concentration was not identical for all primers and furthermore, not all primers performed equally well. Some, presumably due to lack of suitable priming sites in the genomic DNA,

gave poorly binding patterns, while other create polymorphic bands, even within a set of identical replicated DNA samples. Thus, oligo-nucleotides should be rigorously tested for priming ability and reproducibility before using as markers. For primer selection, PCR reactions were performed on each DNA sample in a 20 µl reaction mix containing the following reagents: Master Mix (10.0 µl), primer (1.0 µl), DNA sample (1.0 µl) and Nanopure water (8.0 µl).

Master Mix includes the composition as Ampli Taq polymerase buffer (l0X), dNTPs and Ampli Taq DNA polymerase. For electrophoresis, 2 µl loading dye was added in the reaction mix to make total volume 22 µL. To perform Polymerase Chain Reaction (PCR) Reaction mix preparation and conditions for RAPD amplification reactions were followed according to Williams et al. (1990) with slight modifications. PCR reactions were performed on each DNA sample using a 20 µl PCR reaction mix as mentioned elsewhere. During the experiment, Master Mix and primer solutions were thawed from frozen stocks, mixed by vortexing and placed on ice. DNA samples were allowed to attain room temperature and mixed gently. The primers were pipetted into PCR tubes (0.2 ml). For each DNA sample, a pre-mix was then prepared in the following order: Master Mix, DNA template and Nanopure water. The tubes were sealed and placed in thermocycler for amplification.

DNA amplification was performed accordingly as follows: preheating is 95°C for 5 min., 45 cycles of denaturation is 95°C for 1 min., annealing is 95°C for 2 min., elongation is 72°C for 2 min. then the final elongation is 72°C for 10 min. After completion of cycling program, reactions were held at 4°C and gel electrophoresis was performed.

Since RAPD markers are dominant in nature, each band represents the phenotype at a single allelic locus (Williams et al., 1990). One molecular weight marker, 1 kb DNA ladder was used to estimate the size of the amplification products by comparing the distance travelled by each fragment with that of the known fragments of molecular weight markers. All distinct bands (RAPD markers) were allotted with identical numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. Bands not identified by the readers were considered as non-scorable.

A single data matrix was created by summing scores of all primers used in RAPD analysis and the same was used to estimate polymorphic loci, gene diversity, population differentiation, gene flow, genetic distance and to construct UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using a computer program, POPGENE (Version 1.31) (Nei, 1973; Yeh et al., 1999; Ghose et al., 2014). The same program was also used to perform test of homogeneity in different loci between population pairs.

RESULTS AND DISCUSSIONS

RAPD can be considered to be essential tool in cultivar identification (DNA typing), assessment of genetic variability and relationship management of genetic resources and biodiversity, studies of phylogenetic relationships and in genome mapping (Hasibe, 2009). An attempt was made to analyse genetic variability among eggplant cultivars by Randomly Amplified Polymorphic DNA (RAPD) technique. We used 20 different RAPD primers (Table 2) for the present study. Among the 20 primers initially tested all primers with a single variety of DNA. It gives yield as comparatively maximum number of amplification products with high intensity, with minimal smearing, good resolution and also clear bands. The number of fragments amplified per primer varied. In case of 12 eggplant cultivars out of 20 OPA primers only four primers generate bands, which shows proliferation of DNA and these particular primers are binds with the DNA. OPA-04, OPA-06, OPA-07, OPA-08, OPA-14 and OPA-20 RAPD primers gave positive result with one variety of eggplant cultivar Syngenta but OPA-06 and OPA-08 primers shows very weak binding signals. After this result we selected four except OPA-06 and OPA-08 primers for further study, and perform four primers with the all eggplant cultivars and get maximum bands in OPA-07 (Figure 1) and OPA-14

(Figure 2). The generated bands show the highest numbers of polymorphic bands. Thus it showed a higher level of polymorphism. The four primers generated 5 to 10 scorable bands per primer and all bands shows polymorphic RAPD markers per primer but in OPA-14 one band shows monomorphic. Strong and weak bands were produced in the RAPD reactions. A weak band denotes low homology between the primer and the pairing site on the nucleotide sequence (Thormann et al., 1994).

Table 2. List of primers used for the RAPD analysis in the present study

Primers	Sequence	GC Content (%)
OPA-01	5'-CAGGCCCTTC-3'	70
OPA-02	5'-TGCCGAGCTG-3'	70
OPA-03	5'-AGTCAGCCAC-3'	60
OPA-04	5'-AATCGGGCTG-3'	60
OPA-05	5'-AGGGGTCTTG-3'	60
OPA-06	5'-GGTCCCTGAC-3'	70
OPA-07	5-GAAACGGGTG-3'	60
OPA-08	5'-GTGACGTAGG-3'	60
OPA-09	5'-GGGTAACGCC-3'	70
OPA-10	5'-GTGATCGCAG-3'	60
OPA-11	5'-CAATCGCCGT-3'	60
OPA-12	5'-TCGGCGATAG-3'	60
OPA-13	5'-CAGCACCCAC-3'	70
OPA-14	5'-TCTGTGCTGG-3'	60
OPA-15	5'-TTCCGAACCC-3'	60
OPA-16	5-AGCCAGCGAA-3'	60
OPA-17	5'-GACCAGCGAA-3'	60
OPA-18	5'-AGGTGACCGT-3'	60
OPA-19	5'-CAAACGTCGG-3'	60
OPA-20	5'-GTTGCGATCC-3'	60

Population genetic analysis in different eggplant cultivars was done using POPGENE software. The RAPD markers used in the study were able to differentiate the genetic diversity in the eggplant cultivars. The genetic diversity of eggplant was revealed by percentage of polymorphic loci. The genetic variations were analysed by the band presence *vs* band absence and this is due to incapability to prime a site in some strand difference or by deletions or insertions among priming sites on the DNA strand (Clark and Lanigan, 1993).

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated segregation of the 12 cultivars of eggplant into three main clusters: five entries were grouped into cluster 1, four entries were grouped into cluster 2 and remaining three entries were grouped into cluster 3. In cluster 1, Sarpan brinjal–65 alone makes sub cluster 1 and Kuduchi-601, Mahya MEBH-11, Sarpan brinjal green long, Sarpan brinjal purple long make all together to form cluster (Figure 3). Genetic distance higher between Sarpan brinjal- 65 *vs* other varieties. In cluster 2, Simisi manjra, Syngenta, Ruchica, Ramya cultivars of eggplant have same genetic distance between them. In cluster 3, Sarpan brinjal green oval alone make sub cluster 1 in cluster 3, Sarpan brinjal-25, Sarpan brinjal SPH-55 have same genetic distance but Sarpan brinjal green oval have some distance from the both varieties which are there along with it. Sarpan brinjal SPH-55 shows consistency of its resistance character to diseases has been proved over the years based on its field performance (Sarpan Seeds Pvt. Ltd., Dharwad).

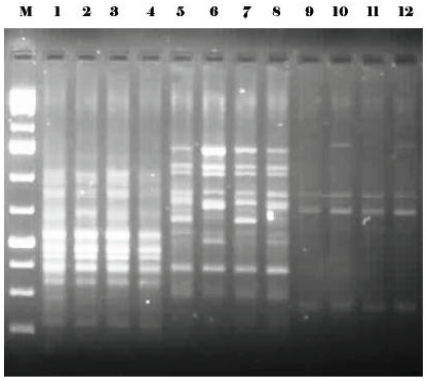

Figure 1. Different eggplant cultivar DNA Bands occur in gel electrophoresis with OPA-07

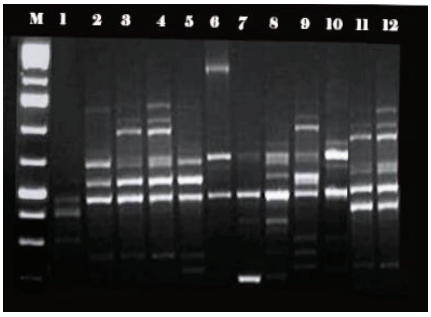

Figure 2. RAPD banding pattern obtained for twelve populations of eggplant with OPA-14

The RAPD analysis employed 20 random primers, four of which generated 20 polymorphic and 5 monomorphic markers with an average 2 to 4 bands per primer except OPA-07 it shows 7 to 9 DNA bands. Cluster

analysis of RAPD using POPGENE resulted into three clusters (Table 3). The present study reveals that among the PCR based fingerprinting techniques, RAPD is informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationship between different cultivars of eggplant with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. The information obtain from the present study could be of practical use for cultivar identification and validity as well as for classical and molecular breeding, as many of these cultivars are sources of vitamins and iron. The genetic similarity of among the cultivars is low as indicated by RAPD analysis. The informative primers identified in our studies will be useful in genetic and systematic analysis. The specific bands like OPA primers can be used as probes to certain eggplant cultivars they are in low or high copy numbers in the eggplant genome and such specific bands may be used for characterization and grouping eggplant cultivar. The study also provides a basis for crop breeders to make informed choices on selection of parental material based on genetic diversity to help overcome some of the problems usually associated with a cultivated species of eggplant. In a preliminary study, the genetic relationship between 12 populations, representative of the geographical distribution were investigated by the bulked genomic DNA-based methodology. This methodology has allowed the twelve populations for a detailed study of their genetic diversity. Most of these populations were indistinguishable from one another, but minimum genetic diversity among the 12 populations. The indistinguishable population was analysed further in this study to verify whether RAPD methodology using bulk DNA and individual samples give congruent results. Several primers were monomorphic with the bulk DNA procedure were revealed to be polymorphic with individual samples. Others, which were polymorphic with the bulk DNA were also polymorphic with individual populations, but were not retain in our study because the assignment of absence and presence of alleles was not clear for a number of sample. This can be explained by the fact that with bulk DNA, the alleles that are present in higher proportions are likely to be amplified with the random primers in such a way that rare alleles are not detected. Previous studies have shown that with the bulk DNA procedure, a polymorphic allele could be detected provided it represented at least 10% of the bulk (Paran et al., 1993).

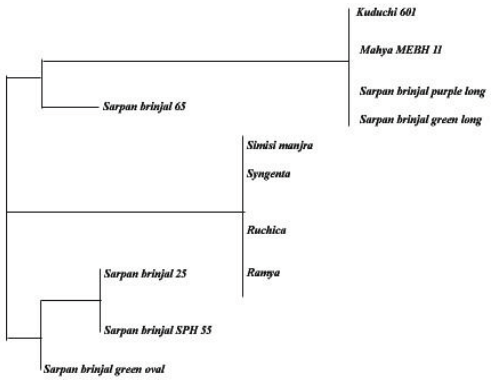

Figure 3. The differentiation between 12 eggplant genotypes based on RAPD analysis and Nei's (1972) genetic distance

Selection of polymorphic alleles was carried out very conservatively, and only clear and repeated polymorphic bands were selected. 14 out of 20 primers produced banding patterns that could be scored easily. The number of shared bands among individuals was evidence for the high degree of similarity among the populations (Table 4). The dendrogram constructed based on RAPD results obtained from four primers reflected the phenotypic variation observed in eggplant and related species during their collections. It is apparent from the dendrogram that collections originating from different places of the study area did not give well-defined distinctive clusters. They were interspersed with each other, indicating no association between RAPD pattern and the area of collection of accessions. This however, contrasted with the finding of Ge et al. (2013) who used SSR markers to get clusters among Chinese eggplant cultivers that resulted in clades subsequent to the geographic divisions.

Table 3. Scores of population genetic analysis in different eggplant cultivars; according to this DNA band data, dendrogram is drawn using POPGENE software

MB	1	1	1	1	1	0	0	0	0	0
KU	1	1	1	1	1	0	0	0	0	0
SY	1	1	1	1	1	0	0	0	0	0
SPL	1	1	1	1	1	0	0	0	0	0
SS55	0	1	0	0	0	1	1	1	1	1
SS65	0	1	0	0	1	0	1	1	1	1
S25	0	1	0	0	0	1	1	1	1	1
SGL	0	1	0	0	0	0	1	1	1	1
RU	0	0	0	0	0	0	1	1	0	0
GO	0	0	0	0	0	0	1	1	0	0
SM	0	0	0	0	0	0	1	1	0	0
RA	0	0	0	0	0	0	1	1	0	0

In Sarpan only many eggplant cultivars has showing wide spread genetic variability and Syngenta is close to the Mahya, Kuduchi and Sarpan purple long. Sarpan brinjal-65 have very wide genetic distance in cluster 1 based on the present RAPD analysis. Moreover, most of the eggplant cultivars shows low genetic variability when compared all other species, may be due to loss of alleles leads them to be low in number in the wild habitats needs further characterization and confirmation.

Morphological differences and similarities do not always reflect differences and similarity, because of genotype and environment interact-tion. As a result, the potential of making genetic progress is slow. In present study the Sarpan purple long and Sarpan green long are morphologically very similar and present in one cluster.

Therefore, in the future it is necessary to use DNA markers that will provide more rapid and precise information on the genetic diversity.

CONCLUSIONS

No molecular markers have been employed previously to quantify the genetic diversity within populations of eggplant cultivars in Northern Karnataka. This is the most comprehensive study to represent the genetic diversity available in populations of eggplant cultivars using molecular markers, which revealed a very narrow genetic base of the different collections. This study shows that if assay conditions are carefully controlled, the RAPD methodology may provide a cheap, rapid and effective means to evaluate the genetic diversity among a large number of populations and help devise sampling strategies to complement classical methodological agronomic descriptors. And the results also indicate that all the populations show more than 80% similarity irrespective of their flower colour and other morphological variability.

The results of RAPD showed that we need to take individuals from more different populations so as to preserve their diversity for the future. The study also confirms the suitability of RAPD as a rapid, cheaper, easy to handle and smart tool in molecular studies of different accessions in crop plants. Concurrently, it is also proved that the entries which were found to be similar in taxonomical classification based on morphological characters.

Table 4. RAPD bands generated by 4 primers in twelve cultivars of eggplant

Primer	Total number of bands	Total number of polymorphic bands	Number of polymorphic bands											
			A	B	C	D	E	F	G	H	I	J	K	L
OPA-04	3	2	2	0	2	0	2	0	1	1	2	2	2	2
OPA-07	10	10	9	8	8	9	8	9	8	8	1	2	1	2
OPA-14	8	6	2	3	4	6	3	2	1	4	6	2	4	6
OPA-20	4	2	2	2	2	2	2	2	2	2	2	2	2	2
Total	25	20	15	13	16	17	15	13	12	15	11	8	9	12
Polymorphism (%)			75	65	80	85	75	65	60	75	55	40	45	60

REFERENCES

Amani J., Kazemi R., Abbasi A.R., Salmanian A.H., 2011. A simple and rapid leaf genomic DNA extraction method for polymerase chain reaction analysis. Iranian Journal of Biotechnology, 9, 1.

Anderson N.O., 2007. Flower Breeding and Genetics: Issues, Challenges and Opportunities for the 21st Century. Springer, New York, 21, 586-587.

Bosland P.W., Votava E.J., 2000. Peppers: Vegetable and Spice Capsicums, CABI Publishing, Wallingford, UK, pp. 1–16.

Bosland P.W., Votava E.J., Votava E.M., 2012. Peppers: Vegetable and Spice Capsicums, CABI, Wallingford, UK, 76-77.

Clark A.G., Lanigan C.M.S., 1993. Prospects for estimating nucleotide divergence with RAPDs. Molec. Biol. Evol. 10:1096-1111.

Doganlar S., Frary A., Daunay M.C., Lester R.N., Tanksley S.D., 2002. A comparative genetic linkage map of eggplant (*Solanum melongena*) and its implications for genome evolution in the solanaceae. Genetics 161(4):1697–1711.

Easmin F., Rahman M.S., Islam M.S., Samad M.A., Alam M.S., 2008. Genetic variation and relatedness among high yielding rice varieties (*Oryza sativa* L.) revealed by RAPD markers. J. Genet Pl. Breed. Bangladesh. 21(1):7-14.

Edwards K., 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucl. Acids Res. 19(6):1349.

Fraga J., Rodriguez J., Fuentes O., Fernandez-Calienes A., Castex M., 2005. Optimization of random amplified polymorphic DNA techniques for use in genetic studies of *Cuban triatominae*. Rev. Inst. Med. trop. S. Paul. 47(5):295-300.

Frary A., Doganlar S., Daunay M.C., Tanksley S.D., 2003. QTL analysis of morphological traits in eggplant and implications for conservation of gene function during evolution of solanaceous species. Theor. Appl. Genet. 107:359-370.

Ge H., Liu Y., Jiang M., Zhang J., Han H., Cheng H., 2013. Analysis of genetic diversity and structure of eggplant populations (*Solanum melongena* L.) in China using simple sequence repeat markers. Sci. Hort. 162:71-75.

Ghose AK., Haque MS., Begum SN., Barua R., Hasan MK., Sagar MSI., 2014. Studies on the genetic diversity of lentil (*Lens culinaris* ssp. *culinaris*) using RAPD markers. Eco-friendly Agril. J. 7(9):85-92.

Hadrys H., Balick M., Schierwater B., 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol Ecol. 1(1):55-63.

Haig, S.M., Rhymer L.M., Heckel D.G., 1994. Population differentiation in randomly amplified polymorphic DNA of red-cockaded woodpeckers *Picoides borealis*. Mol. Ecol. 3:581-595.

Haley S.D., Miklas P.N., Stavely J.R., Byrum J., Kelly J.D., 1993. Identification of RAPD markers linked to a major rust resistance gene block in common bean. Theor. Appl. Genet. 86:505-512.

Halldén C., Hjerdin A., Rading I., Säll T., Fridlundh B., Johannisdottir G., Tuvesson S., Akesson C., Nilsson N., 1996. A high density RFLP linkage map of sugar beet. Genome 39:634–645.

Hasibe C.V., 2009. Genomic DNA isolation from aromatic and medicinal plants growing in Turkey. Sci. Res. Essay, 4(2):59-64.

Karihaloo J.L., Brauner S., Gottlieb L.D., 1995. Random amplified polymorphic DNA variation in the eggplant, *Solanum molengena* L. Theor. Appl. Genet 90:767-770.

Karihaloo J.L., Gottlieb L.D., 1995. Allozyme variation in the eggplant, *Solanum melongena* L. (Solanaceae). Theor. Appl. Genet. 90(3-4):578-583.

Laila R., Siddiqua M.K., Khalil I., Robin A.H.K., Meah M.B., 2012. Molecular characterization of *Solanum melongena* using RAPD marker for collar rot resistance. International Research Journal of Applied Sciences 1(4):38-65.

Larisa G., 2008. Genetic diversity in fruit and berry crops estimated with molecular markers. PhD Thesis, Swedish University of Agricultural Sciences, Alnarp, Sweden.

Larson S.E., Anderson P.L., Miller A.N., Cousin C.E., Richardss C.S., Lewis F.A., Knight M., 1996. Use of RAPD–PCR to differentiate genetically defined lines of intermediate host of *Schitosoma manasoni, Biomphalaria glabrata*. Journal of Parasitology 82:237-244.

Lickfeldt D.W., Hofmann N.E., Jones J.D., Hamblin A.M., Voigt T.B., 2002. Comparing three DNA extraction procedures for cost, efficiency, and DNA yield. HortScience 37(5), 822-825.

Manjusha V., Saurabh R., Munshi A.D., Arun K., Lalit A., Bhat K.V., Ravinder K., 2012. Genetic diversity of Indian brinjal revealed by RAPD and SSR markers. 517-522.

Mohd A.K., 2013. Genetic diversity analysis of brinjal (*Solanum melongena* L.) accessions using RAPD markers. International Journal of Current Research 5:1221-1223.

Mullis K., Faloona F., Scharf S., Saiki R., Horn G., Erlich H., 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symposium in Quantitative Biology 51:263-273.

Nassar N.M.A., 2000. Wild cassava, Manihot spp.: biology and potentialities for genetic improvement. Genetics and Molecular Biology 23(1):201-212.

Nei M., 1972. Genetic distance between populations. Am. Nat. 106:283-292.

Nei M., 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences 70:3321-3323.

Paran I., Mischelmore R.W., 1993. Development of reliable PCR based markers linked downey mildew resistance genes in lettuce. Theor. Appl. Genet. 85:985-993.

Patil M.D., Biradar P., Patil V.C., Janagoudar B.S., Nadaf H.L., 2007. Analysis of genetic diversity of cotton genotypes using rapd pcr technique. Karnataka J. Agric. Sci. 20(2):215-217.

Perez T., Albornoz J., Dominguez A., 1998. An evolution of RAPD fragment reproducibility and nature. Molec. Ecol. 7:1347-1357.

Prabhu M., Natarajan S., Pugalendhi L., 2009. Variability and heritability studies in F5 and F6 progenies of Brinjal (*Solanum melongena*). American-Eurasian Journal of Sustainable Agriculture 3(3):306-309.

Prakash S.P.J., Biji K.R., Gomez S.M., Murthy K.G., Babu R.C., 2006. Genetic diversity analysis of Sorghum (*Sorghum bicolor* L. Moench) accessions using RAPD Markers. Indian Journal of Crop Science 1(1-2):109-112.

Ramesh K.S., Arumugam T., Anandakumar C.R., Premalakshmi V., 2013. Genetic variability for

quantitative and qualitative characters in Brinjal (*Solanum melongena* L.). African Journal of Agricultural Research 8(39):4956-4959.

Sakata Y., Nishio T., Matthews P.J., 1991. Chloroplast DNA analysis of eggplant (*Solanum melongena*) and related species for their taxonomic affinity. Euphytica 55:21-26.

Sambrook J., Fritsch E.F., Maniatis T., 1989. Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sekara A., Cebula S., Kunicki E., 2007. Cultivated eggplants - origin, breeding, objectives and genetic resources, a review. Folia Horticulturae 19(1):97-114.

Sharmin D., Khalil M.S., Begum S.M., Meah M.B., 2011. Molecular characterization of eggplant crosses by using rapd analysis Int. J. Sustain. Crop Prod. 6(1):22-28.

Shiva V., 1994. Agriculture and food production. UNESCO/Environmental Education Dossiers 9:2–3.

Sidhu A.S., Bal S., Behera T.K., Mamta R., 2008. An outlook in hybrid eggplant breeding. In: Hybrid Vegetable Development, Singh P.K., Dasgupta S.K., Tripathi S.K. (Eds), Haworth Press, USA, pp. 15-30.

Sifau M.O., Ogunkanmi L.A., Adekoya, K.O., Oboh B.O., Ogundipe O.T., 2014. Partitioning and distribution of random amplified polymorphic DNA (RAPD) variation among eggplant *Solanum* L. in Southwest Nigeria. International Journal of Genetics and Molecular Biology 6(1):1-7.

Singh A.K., Singh M., Singh A.K., Singh R., Kumar S., Kalloo G., 2006. Genetic diversity within the genus *Solanum* (Solanaceae) as revealed by RAPD markers. Curr. Sci. 90(5):711-716.

Singh D., Malik C.P., 2012. Assesment of genetic diversity in *Verbesina encelioides* populations using randomly amplified polymorphic DNA (RAPD) markers. International Journal of Scientific and Technology Research 1(4):1-8.

Thormann C.E., Ferreira M.E., Camargo L.E.A., Tivanga J.G., Osborn T.C., 1994. Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. Theor. Appl. Genet. 88:973-980.

Tümbilen Y., Frary A., Mutlu S., Doğanlar S., 2011. Genetic diversity in Turkish eggplant (*Solanum melongena*) varieties as determined by morphological and molecular analyses. Inter Res J Biotech 2:16-25.

Williams J.G., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18(22):6531-6535.

Yeh F.C., Yang R.C., Boyle T., 1999. POPGENE 32 - version 1.31, Population Genetics Software, http.//www.ualberta.ca/~fyeh/fyeh/

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

STUDIES ON THE ANALYTICAL CHARACTERISTICS OF WINES OBTAINED FROM VINE VARIETY WITH BIOLOGICAL RESISTANT

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Abstract

Obtained grapes from vine varieties with biological resistance (the varieties of interspecific vines): Seyval, Admira, Radames, Valérien, Brumăriu and Purpuriu were vinified by traditional methods, and the obtained wines were analysed in terms of composition and as aromatic profile, compared with a control wine, of table wine, provided from an vinifera vines.
The chemical analysis of wines have referred to alcoholic strength, total acidity, volatile acidity, total dry extract, glycerol, phenolic compounds and anthocyanins for the Purpuriu variety etc. The aromatic profile of wines was determined by the gas chromatography method coupled with mass spectrometry.
The result of analysis showed that Admira, Seyval and Radames wines presents a high alcoholic strength (11.8 vol% alcohol, 12.1 vol% alcohol, respectively 11.0 vol% alcohol) as well as higher values of the total dry extract and glycerol, compared to the control variety.
Volatile acidity of wines was slightly elevated for most varieties with biological resistance, a higher value presenting at the Pupuriu wine. In the case of Purpuriu wine was analysed the content from the phenolic and anthocyanins compounds, their value being reduced.
In terms of the content in volatile compounds, the main analysis of compounds show a predominance of 3-methyl-1-butanol (Isoamyl alcohol), 2-methyl propanol, Isoamyl acetate, Ethyl propanoate, both in the case of varieties with biological resistance, even in the vinifera variety case. Isoamyl acetate showed high values in the case of varieties with biological resistance and very low values in the case of vinifera wine.
Overall, the wines from the two groups of grapes varieties showed different values of the main volatile compounds, standing out a clear difference between them.

Key words**:** *vine varieties with biological resistance, volatile compounds, gas chromatography/mass spectrometry methods.*

INTRODUCTION

The varieties with biological resistance are interspecific hybrids (American varieties x *vinifera* varieties) with a good and very good resistance to major diseases and pests of vine, including *phylloxera* (Grecu V., 2010). The limited number of treatments that they are required for the culture of these varieties of vines has made they to be called ecological varieties and the grape consumption, as the products resulting from their processing to show a increased interest for the consumer (Visan L. et al., 2007).
In terms of the vinification varieties with biological resistance, the current legislation in the field of the *Vine and of Wine* foresee their usage only as table wine, due to lower quality and a lower resistance in time, compared with the *vinifera* varieties.
However, some varieties with biological resistance for wine have the ability to accumulate a good concentration of sugars and respectively lead to the obtaining of wines with a high alcoholic strength, comparative with *vinifera* varieties.
Also, the composition parameters, such as the total content of dry extract, in glycerol etc., they can record higher values, wich can be compare with *vinifera* varieties.

MATERIALS AND METHODS

Grapes from the varieties with biological resistance, Admira, Seyval, Valerién, Radames, Brumăriu and Purpuriu and a variety of table *vinifera*, as a control variety, were vinified

after a classic scheme under similar conditions. The wines obtained were analyzed in terms of physico-chemical: alcoholic strength (vol% alcohol), total acidity (g/L sulfuric acid), volatile acidity (g/L acetic acid), total dry extract (g/L) and glycerol (g/L). Based analyzes were performed by standard methods (Tardea C., 1980): ebulliometer method for alcoholic strength; titrimetric method for total acidity; distillation method *Saunier-Cazenave* for acidity volatile; Tabarié method for total dry extract and volumetric method for glycerol.

The polyphenolic composition of Purpuriu wine was judged by the content in polyphenols and anthocyanins. Analyzes have been carried out in the wine by UV-VIS spectrometry techniques (Giusti M, 2001). Total content of polyphenols have been determined by IPT technique (g/L gallic acid) (Ribereau-Gayon J, 1978). The anthocyanins were determined by the discoloration technique with SO_2 (Dallas C., 1994).

Specific Extraction of Volatile Compounds.

200 mL of wine, placed in a conical flask, were successively extracted (3 x 20 min) at 0°C with 3 x 25 mL of freshly distilled dichloromethane and then centrifuged for 15 min. The three organic extracts were pooled, dried with anhydrous sodium sulfate and concentrated to 5 mL in a Danish concentrator (45°C), then to 1 mL under a stream of nitrogen (Baek H. et al., 1997; Serot Th. Et al., 2001).

Quantitative analysis of volatile compounds identified in wines by GC/MS.

1μL from each extract was injected into an HP 5-MS capillary column with dimensions: 30 m x 0.25 mm x 0.25 mm (film thickness). Column temperature: 30° C for 10 min., followed by temperature gradient 10° min^{-1} up to 80° C, then gradient of 25° C/min. up to 250°C where stationed 10 minutes. Detector and injector temperatures are: 280° C and 250°C resp. Carrier gas is He, flow-0.5 mL min^{-1}. MSD conditions are: temperature 180°C ion source, ionization energy 70 eV, mass limit of 20-400 amu, electronic multiplier voltage 1700V, scan rate 1.60 s^{-1}. Injection mode: split, opening after 60 sec. and the split flow: 20 mL min^{-1}.

Quantitative determination and identification of volatile compounds based on the comparison of retention indices (RI), mass spectra and the estate of odors. Identification is based on the standard MS library Wiley (Serot Th., 2001; Visan L., 2007).

RESULTS AND DISCUSSIONS

The results show that wine obtained from the control *vinifera* variety is slightly superior towards the studied varietals, regarding the quality of wine, which was expected.

However, among the varieties studied there are valuable varieties, that we can recommend for the production of table wines.

Of these it distinguish especially the varieties Admira, Seyval and Radames, which present superior values of main quality parameters. At the sensory analysis them were positive appreciated.

The analyzed varieties belong to the same ecopedological areas and were subject to the same cultural techniques.

Therefore, the examined parameter values reflects their own genetic potential of the variety. The obtained wine from the Seyval variety presents a *alcoholic strength* of 12.1 vol%, the variety being already known that it has a good ability to accumulate sugars, so with a high alcoholic potential. Also with a higher alcoholic strength, is presented the Admira wine (11.8 vol%) and Radames (11.2 vol%). The Brumariu variety accrues sugars in lower quantity and produce wine with a lower alcoholic strength (9,8 vol%). Below the average varieties lies the Purpuriu variety with 9,4 vol% alcohol (Figure 1).

Total dry extract represent an important element in the analytical characterization of a wine. Its value at the Romanian wines vary in larger limits between 13 and 35 g/L, higher values it show at the wines from quality varieties. From this point of view, the analyzed wines were within the permissible limits, even with high values. The Seyval variety (20.1 g/L), Admira (19 g/L) and Radames (17.4 g/L) shows superior values of the extract, values comparative with the control variety, *vinifera* (Figure 1).

The glycerol enters, together with other constituents in composing of dry extract of the wine; due the sweet taste, his presence prints a softness to the wine, harmony, suppleness; occurs, also in preserving the flavors.

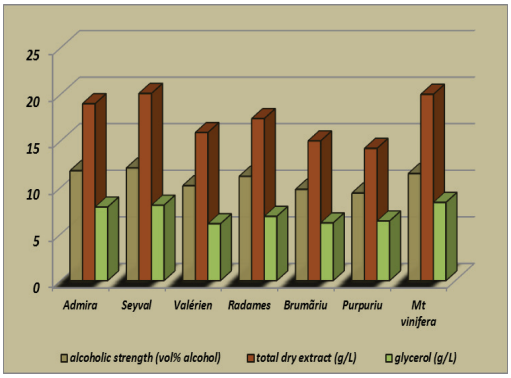

Figure 1. The Main Physico-Chemical Parameters Analyzed wines

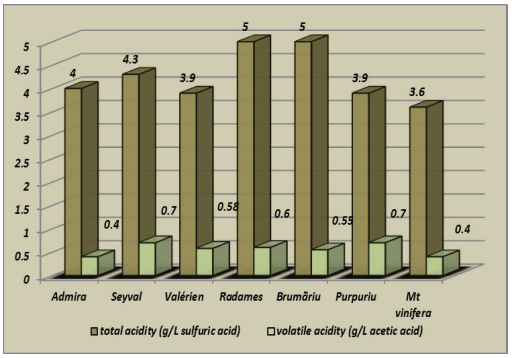

Figure 2. Total Acidity and Volatile Acidity of the Analyzed wines

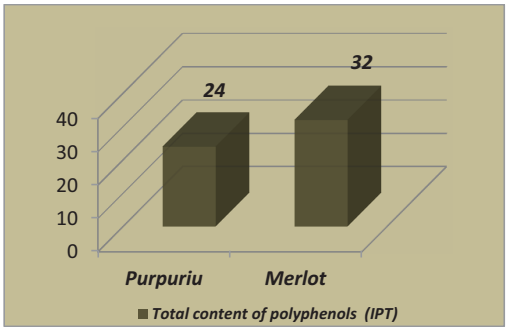

Figure 3 a. Polyphenol content in Purpuriu wine (IPT) compared to the control wine

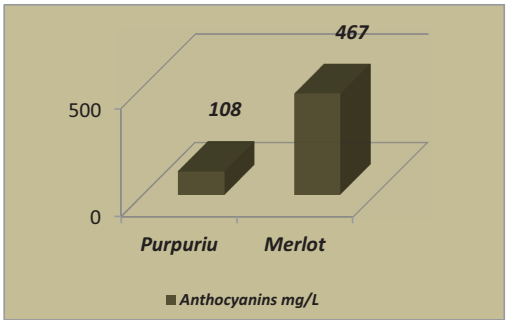

Figure 3 b. Anthocyanin content in Purpuriu wine (mg/L) compared to the control wine

The values content in glycerol are superior in the case of wines Seyval, Admira and Radames, representing approx. 1/11 from the alcoholic weight content of wines (Figure 1).

Total acidity has a great influence on the organoleptic characteristics of wines, concerning stability and maturation of wines.

It is noted from this point of view, that the total acidity of obtained wines from the biological resistance varieties is higher, compared with the *vinifera* variety, but without being affected the organoleptically balance of wine (Figure 2).

Volatile acidity is an extremely important component in the qualitative appreciation of wine. Volatile acids occur as by-products during alcoholic fermentation, as well as in other fermentations or processes, that occur during the wine evolution. It is known that the yeast influences the concentration in volatile acids from wine, which is why the wines were obtained by fermentation guided with yeasts selected from the same strain *Saccharomyces Cerevisiae.*

Thus, it appears that the most wines showed a high value of volatile acidity, except the Admira wine (0,40 g/L) with the same value of volatile acidity with the control wine (Figure 2).

In the case of Purpuriu wine, the value content in polyphenols and anthocyanin content (mg/L) have recorded low values (Figure 3, a, b).

Content analysis in wine flavors

Organic esters, originate from combining the organic acids with alcohols; many of esters have pleasant smell, some with a floral smell, fruity etc. In the analyzed wines, in higher concentration were identified many esters: *Isoamyl acetate*, pleasant smell, floral; Isoamyl acetate, the responsible ester for the bouquet the young wines, obtained from certain varieties; it is found in higher concentration in the studied wines from the varieties with biological resistance and in lower concentration in the *vinifera* wines (Table 1).

Table 1. Identified volatile compounds in the wines analyzed (µg/L)

Volatile compounds	Admira	Radames	Seyval	Valérien	Purpuriu	Mt *vinifera*
2-Methylpropan-1-ol	658	432	1587	6198	2500	78.2
3-Methyl-1-butanol (Isoamyl alcohol)	1020	9306	11245	13274	10121	4864
2-Methyl-1-butanol	842.9	789.1	823.4	989.5	1011.0	3854
1-Hexanol	43.9	28.7	17.9	121.8	40.1	18
2-Ethylhexanol	0.00	1.1	0.4	2.3	0.4	5.8
2-Phenylethanol	1.2	0.5	0.5	0.6	0.4	1.5
3-Methylbutanal	38.0	4.2	10.2	6.3	39.25	11.2
2-Methylbutanal	21.08	24.54	13.9	6.8	10.2	35.14
Heptanaldehyde	0.00	11.3	62.4	0.9	0.8	0.5
2-Ethylhexanal	0.00	0.2	4.1	0.00	1.9	2.8
Benzaldehyde	1.8	2.7	2.1	5.3	9.2	17.2
Methyl ethanoate	0.00	4.8	5.9	10.2	2.9	0
Ethyl propanoate	282.8	270.9	319.8	539.6	582.1	3775.2
Propyl acetate	0.00	0.00	0.00	133.1	124.5	87.9
(3-Methylthi opropylisothiocyanate	0.00	0.00	4.2	1.5	245	0
Methyl propanoate	984	541.2	632.5	501.2	412.0	42
2-Methylpropyl ethanoate	323.2	238.48	41.2	214.9	239.7	87.8
3-Methylbut-1-yl ethanoate	41	0.4	0.3	1.12	1.0	0
Ethyl butanoate	249.3	2.8	264.2	519.03	189.5	1915
(E)-2-Butenoic acid ethyl ester	0.00	4.9	5.2	1.4	1.0	0
2-Methylbutanoic acid, methyl ester	164.1	15.7	34.1	25.3	14.0	265
Isoamyl acetate	467.4	1152.4	730	1258.9	695.6	58.2
Ethyl pentanoate	0.00	0. 7	3.9	12.5	32.1	0
Methyl hexanoate	0.7	3	2.5	8.2	2.4	4
Ethyl octanoate	79.13	135.2	59.6	296.5	42.7	10.4
Ethyl hexanoate	512.3	640.1	390.1	998.9	378.4	15.3
Ethyl acetate	2.5	1.9	2.1	13.4	2.5	1.3
Methyl octanoate	0.7	1.2	0.9	4.3	1.1	6.4
2-Octanone	0.3	0.5	0.3	0.00	0.7	0
Linalol	0.2	4.5	0	2.1	0	15
Limonene	54.12	198.7	145.1	482	325.1	2.4
β-pinene	2.5	6.9	11.1	21.4	5.1	0.9

Other esters identified in higher concentration in the studied wines: *Ethyl propanoate* (pine-apple-like odor), with high concentration in *vinifera* wine; *Ethyl butanoate* (it has a fruity odor, similar to pineapple) with high concentration in *vinifera* wine;
Methyl propanoate (is a volatile ester with a sweet, fruity, rum-like odor); in the *vinifera* wine is found in low concentration; *2-Methylpropyl ethanoate* (like many esters it has a fruity or floral smell at low concentrations and occurs naturally in raspberries, pears and other plants.

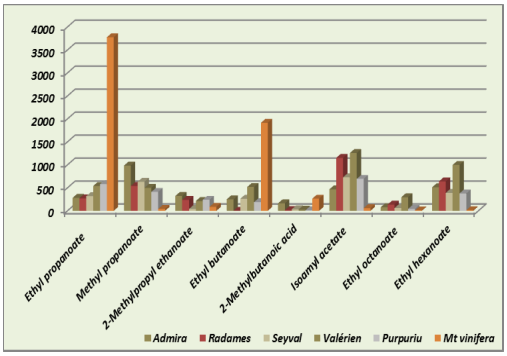

Figure 4. The main esters of a Analyzed wines (µg/L)

At higher concentrations the odor can be unpleasant and may cause symptoms of central nervous system depression such as nausea, dizziness and headache); *Ethyl hexanoate* ; is an apple-flavoured ester; in the *vinifera* wine is found in low concentration (Figure 4).

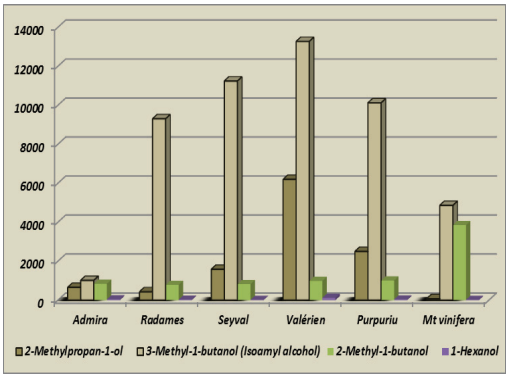

Figure 5. Concentration of higher alcohols

In terms of *higher alcohols*, were identified: *2-methylpropan-1-ol*, in high concentrations in wines of hybrids and in much lower concentration in *vinifera* wine. Isobutanol (2-methylpropan-1-ol) is produced naturally during the fermentation of carbohydrates and may also be a byproduct of the decay process of organic matter; 3-*Methyl-1-Butanol (Isoamyl alcohol);* it is one of the components of the aroma of *Tuber melanosporum*; *2-Methyl-1-butanol* (it is also one of the components of the aroma of *Tuber melanosporum*, the black truffle).
2-Methyl-1-butanol was found in higher concentration in all wines, but especially in the *vinifera* wine (Chisholm M. et al., 1994); *1-Hexanol* was found in higher concentrations in the hybrids wines and lower in the *vinifera* wine (Figure 5).
The aldehydes are very important compounds in wine bouquet formation (Figure 6); in the wines analyzed were identified: *3-Methylbutanal (isovaleraldehyde);2-Methylbutanal (Butyraldehyde);heptanaldehyde; 2-Ethylhexanal (2-Ethylhexaldehyde); Benzaldehyde etc.*

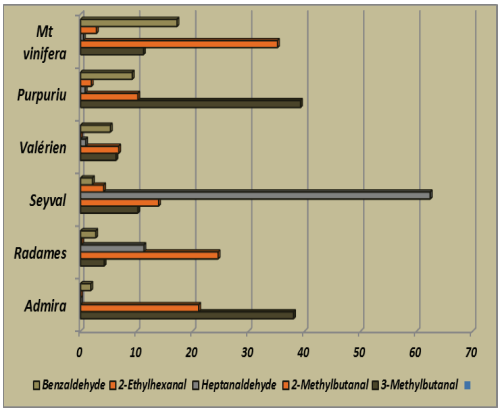

Figure 6. Aldehyde content of the Analyzed wines

Of the terpenes, were identified: *Linalool*, in low concentrations in the hybrid wines and in higher concentrations at the *vinifera* wine; *limonene*, characteristic to all the hybrid wines analyzed (Figure 7).

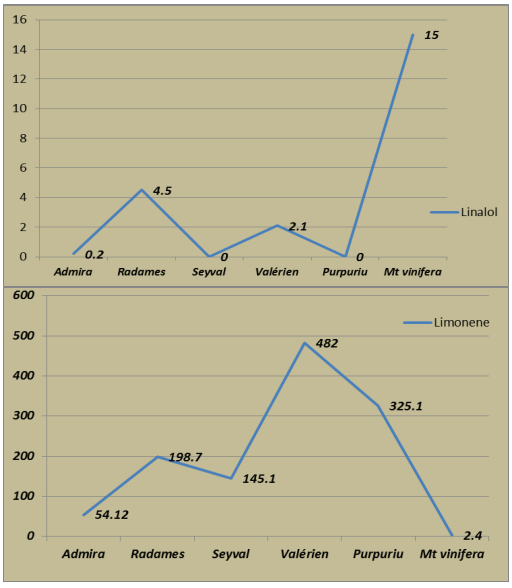

Figure 7. Concentration of terpenes

CONCLUSIONS

Of the analyzed wines, obtained from interspecific hybrids (wines with biological resistance) showed superior values of main quality parameters (alcoholic strength, extract, glycerol, total acidity and volatile acidity) the Admira, Seyval and Radames wines; the values of these parameters are comparable to the *vinifera* table wines.

At the GC/MS analysis, regarding the volatile compounds and the concentration in flavours of the wines were identified:

- *Ethyl butanoate, Ethyl propanoate*, with a floral smell or fruity, both in the hybrid and *vinifera* wines;
- *Isoamyl acetate*, the responsible ester for the bouquet the young wines; it is found in higher concentration in the studied wines from the varieties with biological resistance and in lower concentration in the *vinifera* wine;
- *Methyl propanoate, 2-Methylpropyl ethanoate*; *Ethyl hexanoate:* in higher concentration in the studied wines from the varieties with biological resistance and in lower concentration in the *vinifera* wine;
- Of the higher alcohols*, 2-Methylpropan-1-ol*, a was identified in higher concentrations in the wines of hybrids and in lower concentration in *vinifera* wine;
- Of terpenes, *linalool* is characteristic to the *vinifera* wine and *limonene* is characteristic to all wines of hybrids analyzed.

REFERENCES

Baek H., Cadwallader E., Marroquin E.., Silva J., 1997. Identification of predominant aroma compounds in *muscadine* grape juice. J. Food Sci., 62, 249-252;

Chisholm M. G., Guiher L. S., Vonah T. M. and Beaumont J. L., 1994. Comparison of some french-american hybrid wines with white *Riesling* using gas chromatography-olfactometry", Amer. J. Enol. Vitic., 45: 201-212;

Dallas C., 1994. Effect of SO_2 on the extraction of anthocyanins. Vitis 33, 42-51.

Grecu V., 2010. Soiurile rezistente de vita de vie si particularitatile lor de cultura. Ed. MAST;

Giusti M, Wrolstad R.E., 2001. Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy. Current Protocols in Food Analytical Chemistry.

Guth H., 1997. Identification of character impact odorants of different white wine varieties. J. Agric. Food Chem., 45, 3022-3026;

Serot Th., Proust C., Visan L., Burcea M., 2001. Identification of the Main Odor-active Compounds in Musts from French and Romanian Hybrids by Three Olfactometric Methods. J. Sci. Food Agric., 49, 1909-1914;

Tardea C., 1980. Metode de analiză şi control tehnologic al vinurilor", Ed. Ceres, Bucureşti,;

Visan L., Popa O., Babeanu N., Toma R., Serot T., 2007. Analytical Methods for Quantitative Identification of Aroma Compounds in Grape Juice of Resistant Varieties, Lucrari stiintifice-seria F-Biotehnologii ISSN 1221-7774, Vol XII.

BIOTECHNOLOGY IN VETERINARY MEDICINE

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MOLECULAR BIOLOGY TECHNIQUES USED IN ACTIVE SURVEILLANCE OF CRIMEAN–CONGO HAEMORRHAGIC FEVER IN RUMINANTS: A CRITICAL REVIEW

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Abstract

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne zoonotic disease with high risk of emergence in areas with uncontrolled high population of ixodid or argasid ticks. Disease is caused by a Nairovirus (family Bunyaviridae), one of the three known humans' pathogenic viruses of the genus, along with Dugbe virus and Nairobi sheep disease virus. Despite the important role played by livestock in the transmission and amplification of the CCHF virus, active surveillance of the disease is not part of the current actions of disease control in several countries. This situation may be due to subclinical evolution in animals and no economic effects on the livestock products. Nevertheless, CCHF virus is a major pathogen for humans, with haemorrhagic manifestations and mortality rate ranging from 5% to 80%. Consequently, implementation of public health measures in areas with high risk is decisive. This review summarized virus identification and immunological methods designed for CCHF and that may be used in active surveillance of disease in ruminants. Diagnostic tests for virus identification in animals can be used for surveillance (virus isolation in cell culture) or for determining the virus-free status (real-time reverse transcription polymerase chain reaction) In conclusion the front-line tool in diagnosis of CCHF seen to be reverse transcriptase polymerase chain reaction, and until the validation of serological methods seems to be the better for active surveillance of CCHF in ruminants.

Key words*: CCHF, tick-borne zoonotic disease, epidemiology, serological surveillance, RT-PCR.*

INTRODUCTION

Crimean-Congo haemorrhagic fever (CCHF) is a tick-borne zoonotic disease, endemic in Africa, Asia and Eastern Europe, including the Balkan Peninsula (Papa et al., 2002, 2004; Ahmeti, 2006), overlapping the distribution areal of *Hyalomma* spp. (Appannanavar and Mishra, 2011).

CCHF virus (*CCHFV*) is a *Nairovirus (family Bunyaviridae),* being one of the three viruses pathogenic for humans, belonging to this genus, along with Dugbe virus and Nairobi disease virus *(Swanepoel and Paweska, 2011).*

CCHFV has a spherical capsid (⌀100 nm) harbouring a genome single-stranded RNA, segmented in three linear fragments (large, medium and small) negatives, which encode: viral nucleocapsid (N), the precursor of the glycoprotein (GPC), and of the polymerase (L) (Schmaljohn and Hooper, 2001; Briese et al., 2013).

Life cycle of *CCHFV* can be hosted either by the arthropod-arthorpod (transovarial and transstadial) chain, either by the arthropod-vertebrate-arthropods chain. The ticks can suck blood on several wild and domestic animals, and, the last ones, in their turn, can infect other ticks. Also, the human can be exposed to the CCHF virus by direct contact with the blood or the tissues of the infected animal. (Kamboj and Pathak, 2013; OIE, 2014).

CCHFV has high risk of emergence in areas where the control policy of ixodid or argasid ticks is missing. (Bajpai and Nadkar, 2011).

Despite the important role played by livestock in the transmission and amplification of the *CCHFV*, most countries do not have sanitary veterinary programs for active surveillance of CCHF in animals. This situation may be due to the subclinical evolution in animals and also, to the weak impact on the economic performances in the livestock industry (OIE, 2014).

Despite the effects on the animals, *CCHFV* is a major pathogen for humans. Clinical signs in humans consist in fever, chills, severe headache, dizziness, photophobia, myalgia and arthralgia, ecchymosis, hematemesis, melena, epistaxis, haematuria, and haemoptysis (Kamboj and Pathak, 2013). Mortality rate is ranging from 5% to 80% (OIE, 2014).

In Balkans, CCHF is a zoonotic disease emerging, with high risk of exposure to this virus for animal workers, for animal handlers, for butchers, for the veterinarians, hunters, and foresters (Papa et al., 2002, 2004; Ahmeti and Raka, 2006; OIE, 2014).

PROBLEM FORMULATION

According of the above data, the implementation of public health measures in European states should be considered. This paper summarizes the available tools for the virus identification and immunological methods designed for CCHF and that may be used in active surveillance of disease in ruminants.

In order to propose the most appropriate molecular technique for the active surveillance of CCHF in ruminants, we compared the results reported by 19 teams of researchers in 23 scientific reports and reviews which used molecular biology techniques in detection of *CCHFV*, using different types of samples (Rodriguez et al., 1997; Burt et al., 1998; Drosten et al., 2002, 2003; Yashina et al., 2003; Nabeth et al., 2004; Yapar et al., 2005; Tonbak et al., 2006; Papa et al., 2004, 2007; Duh et al., 2007; Midilli et al., 2007; Wölfel et al., 2007, 2009; Deyde et al., 2009; Grard et al., 2011; Weidmann et al., 2011; Atkinson et al., 2012; Lindeborg et al., 2012; Al-Zadjali et al., 2013; Fajs et al., 2014; Kamboj et al., 2014; OIE, 2014).

PROBLEM SOLUTION

To set up preventive programs and therapeutic protocols for *CCHFV* infections, the first request to satisfy is the early diagnosis. (Al-Zadjali et al., 2013). An ideal preventive program should prevent human cases of disease, by keeping the infection only in animals or in vectors, at his lowest level and focusing the surveillance on the *CCHFV* screening in domestic or wild animals.

Until today, the disease surveillance is carried out by various virological methods, like *CCHFV* isolation (on cell cultures) or (reverse transcription PCR) (OIE, 2014).

The choice of one or other of method has different reasons: by serology can be counted the immune outcome to infection, meaning retrospective diagnosis, while the virus detection and the viral load by quantitative real-time PCR can be used in prediction of the clinical progress. (Duh et al., 2007; Papa et al., 2007; Wölfel et al., 2007).

Several serological methods (e.g. ELISA and immunofluorescence assay) are available to characterise the status of infection of a subject or of the population, or the prevalence of infection (a reliable indicator of virus circulation on the field) (OIE, 2014). In human infection, the tests used in quantification of IgM could not be used as a predictor of disease outcome because the levels of IgM were not correlated with the outcome of viral load, while levels of IgG were inversely correlated with viral loads (IgG might neutralize the virus) (Duh et al., 2007). All marketed kits, ELISA or IFA, are designed only for human samples, while for ruminants have been designed only in-house ELISA protocols. These tests have not undergone a formal validation process (OIE, 2014).

Real-time PCR for the small (S) *CCHFV* segment (amplifying a 127-bp product) (Wölfel et al., 2007) was used in monitoring the influx of migratory birds carrying *CCHFV* infected ticks; this study supports that the real-time PCR can be a tool for predicting the risk of emergence of new *CCHFV* foci (Lindeborg et al., 2012).

In India, Appannanavar and Mishra (2011) emphasizes the need of active surveillance not only for existing pathogens in any geographic

location (e.g. Ganjam virus of Nairobi sheep disease) but also for those that pose future threat. They recommend real-time PCR in *CCHFV* surveillance instead of Serological tests (Appannanavar and Mishra, 2011).

Drosten et al. (2002) evaluated several viral hemorrhagic fevers by real-Time reverse Transcription-PCR and identified some problems that can impair the sensitivity and specificity of molecular biology diagnostic. For instance, in design of a multiplex PCR is difficult to achieve optimal reaction conditions for each individual pair of primers used for each virus. Therefore, Drosten et al. (2002) recommend additional tests (e.g. virus isolation, ELISA, singleplex PCRs, IgM/IgG detection by ELISA or IFA) in confirmation of a humane case of CCHF (Drosten et al., 2002).

Reliable, specific, sensitive and simple RT-PCR assays were described by Rodriguez et al. (1997), Burt et al. (1998), Drosten et al. (2002), Yashina et al. (2003), Yapar et al. (2005), Tonbak et al. (2006), Midilli et al. (2007), Deyde et al. (2009), and Grard et al. (2011), some of them assessed and certified in complex molecular investigations and genetic analysis.

Low-Density Microarray for the Rapid Detection and Identification of the *CCHFV* was already described, and the results seem to exceed those of real-time PCR protocols (Wölfel et al., 2009), but CCHF virus strains cannot be completely characterised by this method. Unfortunately, this technology is running only in few diagnostic laboratories and its implementation in surveillance or diagnostic is still expected.

CONCLUSIONS

According to data above presented, the front-line tool in CCHF diagnosis is the reverse transcription polymerase chain reaction and, as well as in humans, can be used successfully in ruminants. Also, absence of validated serological methods for active surveillance of CCHF in ruminants, support the introduction of reverse transcription polymerase chain reaction in surveillance programs.

ACKNOWLEDGEMENTS

This work was funded by MEN-UEFISCDI PN II "Partnerships in priority areas" program, Grant No. 295/2014.

REFERENCES

Ahmeti S., Raka L., 2006. Crimean-Congo haemorrhagic fever in Kosova: a fatal case report, Virol J, 3:85.

Al-Zadjali M., Al-Hashim H., Al-Ghailani M., Balkhair A., 2013. A Case of Crimean-Congo Hemorrhagic Fever in Oman. Oman Medical Journal, 28(3), 210-212.

Appannanavar S.A., Mishra B., 2011. An Update on Crimean Congo Hemorrhagic Fever, J Glob Infect Dis., 3(3): 285–292.

Atkinson B., Chamberlain J., Logue C.H., Cook N., Bruce C., Dowall S.D., Hewson R., 2012. Development of a real-time RT-PCR assay for the detection of Crimean-Congo hemorrhagic fever virus, Vector Borne Zoonotic Dis.,12(9):786-93

Bajpai S., Nadkar M.Y., (2011). Crimean Congo hemorrhagic fever: requires vigilance and not panic. J Assoc Physicians India, 59:164-167.

Briese T., Calisher C.H., Higgs S., 2013. Viruses of the family Bunyaviridae: Are all available isolates reassortants? Virology, 446(1-2), 207-216.

Burt F.J., Leman P.A., Smith J.F., Swanepoel R., 1998. The use of a reverse transcription-polymerase chain reaction for the detection of viral nucleic acid in the diagnosis of Crimean-Congo haemorrhagic fever, J Virol Methods., 70(2):129-137.

Deyde V.M., Khristova M.L., Rollin P.E., Ksiazek T.G., Nichol S.T., 2009. Crimean-Congo hemorrhagic fever virus genomics and global diversity. J Virol., 80(17):8834-8842.

Drosten C., Göttig S., Schilling S., Asper M., Panning M., Schmitz H., Günther S., 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR, J Clin Microbiol, 40(7):2323-2330.

Drosten C., Kümmerer B.M., Schmitz H., Günther S., 2003. Molecular diagnostics of viral hemorrhagic fevers, Antiviral Res, 57(1-2):61-87

Duh D., Saksida A., Petrovec M., Ahmeti S., Dedushaj I., Panning M., Drosten C., Avšič-Županc T., 2007. Viral Load as Predictor of Crimean-Congo Hemorrhagic Fever Outcome. Emerging Infectious Diseases, 13(11): 1769-1772.

Fajs L., Jakupi X., Ahmeti S., Humolli I., Dedushaj I., Avšič-Županc T., 2014. Molecular Epidemiology of Crimean-Congo Hemorrhagic Fever Virus in Kosovo, PLoS Negl Trop Dis. 8(1): e2647

Grard G., Drexler J.F., Fair J., Muyembe J.-J., Wolfe N. D., Drosten C., Leroy E.M., 2011. Re-Emergence of Crimean-Congo Hemorrhagic Fever Virus in Central Africa. PLoS Neglected Tropical Diseases, 5(10), e1350. doi:10.1371/journal.pntd.0001350

Kamboj A., Pateriya A.K., Mishra A., Ranaware P., Kulkarni D.D., Raut A.A., 2014. Novel Molecular Beacon Probe-Based Real-Time RT-PCR Assay for Diagnosis of Crimean-Congo Hemorrhagic Fever Encountered in India, BioMed Research International, vol. 2014, Article ID 496219, 4 pages

Kamboj A., Pathak H., 2013. Crimean-Congo hemorrhagic fever: a comprehensive review, Veterinary World, 6(10): 812-817.

Lindeborg M., Barboutis C., Ehrenborg C., Fransson T., Jaenson T.G.T., Lindgren P.E., Lundkvist A., Nyström F., Salaneck E., Waldenström J., Olsen B., 2012. Migratory Birds, Ticks, and Crimean-Congo Hemorrhagic Fever Virus, Emerging Infectious Diseases, 18(12):2095-2097.

Midilli K., Gargılı A., Ergonul O., Şengöz G., Ozturk R., Bakar M., Jongejan F., 2007. Imported Crimean-Congo hemorrhagic fever cases in Istanbul, BMC Infectious Diseases, 7:54

Nabeth P., Cheik, D.O., Lo B., Faye O., Vall I.O., Niang M., Wague B., Diop D., Diallo M. Diallo B., Diop O.M., Simon F., 2004. Crimean–Congo hemorrhagic fever, Mauritania, Emerg Infect Dis, 10 (12):2143–2149.

OIE, 2014. Terrestrial Manual, Chapter 2.1.3b. Crimean-Congo haemorrhagic fever. Version adopted by the World Assembly of Delegates of the OIE in May 2014. http://www.oie.int/fileadmin/Home/eng/Health_standards /tahm/2.01.03b_CCHF.pdf

Papa A., Bino S., Llagami A., Brahimaj B., Papadimitriou E., Pavlidou V., Velo E., Cahani G., Hajdini M., Pilaca A., Harxhi A., Antoniadis A., 2002. Crimean-Congo hemorrhagic fever in Albania, 2001, Eur J Clin Microbiol Infect Dis., 21:603-606.

Papa A., Christova I., Papadimitriou E., Antoniadis A., 2004. Crimean-Congo hemorrhagic fever in Bulgaria, Emerg Infect Dis., 10:1465-1467.

Papa A., Drosten C., Bino S., Papadimitriou E., Panning M., Velo E., Kota M., Harxhi A., Antoniadis A., 2007. Viral Load and Crimean-Congo Hemorrhagic Fever, Emerging Infectious Diseases, 13(5):805-806.

Rodriguez L.L., Maupin G.O., Ksiazek T.G., Rollin P.E., Khan A.S., Schwarz T.F., Lofts R.S., Smith J.F., Noor A.M., Peters C.J, Nichol S.T., 1997. Molecular investigation of a multisource outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates, Am J Trop Med Hyg, 57(5):512-8.

Schmaljohn C.S., Hooper J. W., 2001. Bunyaviridae: the viruses and their replication, In D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott-Raven Publishers, Philadelphia, Pa. pp. 1581-1602.

Swanepoel R., Paweska J.T., 2011. Crimean-Congo hemorrhagic fever. In: Oxford Textbook of Zoonosis: Biology, Clinical Practise and Public Health Control, Second Ed. Palmer S.R., Soulsby L., Torgerson & Brown eds, Oxford University Press, UK, pp. 287–293.

Tonbak S., Aktas M., Altay K., Azkur A.K., Kalkan A., Bolat Y., Dumanli N., Ozdarendeli A., 2006. Crimean-Congo hemorrhagic fever virus: genetic analysis and tick survey in Turkey, J Clin Microbiol., 44(11):4120-4124.

Weidmann M., Sall A.A., Manuguerra J.C., Koivogui L., Adjami A., Traoré F.F., Hedlund K.O., Lindegren G., Mirazimi A., 2011. Quantitative analysis of particles, genomes and infectious particles in supernatants of haemorrhagic fever virus cell cultures, Virol J., 8:81.

Wölfel R., Paweska J.T., Petersen N., Grobbelaar A.A., Leman P.A., Hewson R., Georges-Courbot M.C., Papa A., Heiser V., Panning M., Günther S., Drosten C., 2009. Low-Density Macroarray for Rapid Detection and Identification of Crimean-Congo Hemorrhagic Fever Virus. J Clin Microbiol., 47(4):1025-1030.

Wölfel R., Paweska J.T., Petersen N., Grobbelaar A.A., Leman P.A., Hewson R., Georges-Courbot M.C., Papa A., Günther S., Drosten C., 2007. Virus detection and monitoring of viral load in Crimean-Congo hemorrhagic fever virus patients, Emerg Infect Dis, 13(7):1097-100.

Yapar M., Aydogan H., Pahsa A., Besirbellioglu B.A., Bodur H., Basustaoglu A.C., Guney C., Avci I.Y., Sener K., Setteh M.H., Kubar A., 2005. Rapid and quantitative detection of Crimean-Congo hemorrhagic fever virus by one-step real-time reverse transcriptase-PCR, Jpn J Infect Dis., 58(6):358-62.

Yashina L., Vyshemirskii O., Seregin S., Petrova I., Samokhvalov E., Lvov D., Gutorov V., Kuzina I., Tyunnikov G., Tang Y.W., Netesov S., Petrov V., 2003. Genetic analysis of Crimean-Congo hemorrhagic fever virus in Russia, J Clin Microbiol., 41(2):860-2.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

IN VITRO ASSESSMENT OF EMD BASED BIOMATERIAL BIOCOMPATIBILITY

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Abstract

Tissue engineering is a rapidly developing area that might restore, maintain or improve tissue functions. The major elements of tissue engineering are: integrate cells, scaffolds and biologically active molecules. These components act synergistically to regulate stem cell propagation and differentiation thus ensuring tissue regeneration. For evaluation of biocompatibility of enamel matrix derivative (EMD) based biomaterial, palatal mesenchymal stem (PMSCs) cells at a density of $1x10^5$ cells were co cultured. The level of adherence was daily assessed. The viability of cells was evaluated after 24h, 72h and 7 day using FDA (fluorescein diacetate) assay. Our study revealed that the selected biomaterial are biocompatible and can be used as scaffolds for mesenchymal stem cells delivery especially for periodontal regeneration.

Key words*: biomaterials, biocompatibility, stem cells, regenerative therapy.*

INTRODUCTION

Stem cells are definied as cells with clonogenic ability, self-renewing capacity that can differentiate in one or more specialized cell lineage (Rodriguez-Lozano et al., 2011). Biomaterial-based scaffolds are the most important tool in providing a 3D environment for cells, both in culture and inside the body. The main properties of biocompatible scaffolds (synthetic or natural) consist in optimal fluid transport, delivery of bioactive molecules, material degradation, cell-recognizable surface chemistries, mechanical integrity and the ability to induce signal transduction (Shin et al., 2003, Drury et al., 2003)

Natural biomaterials used for stem cells cultivation can consist of components found in the ECM such as alginate, cellulose, chitosan, collagen, fibrinogen, hyaluronic acid, silk fibroin, glycosaminoglycans (GAGs), hydroxyapatite (HA) etc., and therefore have the advantage of being bioactive, biocompatible, and with of similar mechanical properties as native tissue (Chung et al., 2008). Ideally the scaffold must provide certain properties (1) directed and controlled degradation; (2) promote cell viability, differentiation, and ECM production; (3) allow for the diffusion of nutrients and waste products; (4) adhere and integrate with the surrounding native cartilage; (5) span and assume the size of the defect, and (6) mechanical integrity depending on the defect location (Pati et al., 2012). Regenerative therapy requires such biomaterials and biocompatibility assessement of these cells is very important. Enamel matrix derivative, EMD (Emdogain®) (Straumann) is a purified acidic extract from the tooth germs of 6-month old piglets. The major component of Emdogain is a hydrophobic protein ameiogenin, is a widely used biologic agent

capable to support periodontal tissue regeneration (Koop et al., 2012, Gruber et al., 2013) formation of new cementum, periodontal ligament and alveolar bone (Miron et al., 2013).
The aim of the present study was to investigate the effects of EMD on palatal mesenchymal stem cells proliferation.

MATERIALS AND METHODS

Characterized palatal mesenchymal stem cells passages 7 (Roman et al., 2013) were used for our experiment. The cells were cultured in a 96-well plate in DMEM/F12 (Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham) (Sigma-Aldrich, St.Louise, MO, USA) supplemented with 10% FCS (fetal calf serum) (EuroClone, MI, Italy), 2 mM glutamine, 1% Non Essential Amino-Acids (NEAA) (Sigma-Aldrich, St.Louise, MO, USA). After 5 h of initial cultivation the culture medium were changed with serum free medium supplemented with EMD in different concentration (10, 20, 50, 100 µg/ml) for 24, 72h and 7 day. The level of adherence was daily assessed. The viability of cells was evaluated after 24h, 72h and 7 day using fluorescein diacetate (FDA) staining and the cells proliferation were evaluated using MTT assay. Experiments were performed in triplicate. All the results are expressed as mean ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSIONS

Palatal mesenchymal stem cells used in our study were previously characterized for their stemness and trilineage differentiation capacity, performed according to the criteria of the International Society for Cellular Therapy (unpublished data). For assessments of EMD based biomaterials biocompatibility the cells were stimulated with four different concentration of Emdogain® (Straumann). Cells viability, proliferation capacity and the potential citotoxicity level were evaluated after 24h, 48h and 7 days. After EMD treatment the level of adherence were assessed daily.
After 24h of treatment the palatal mesenchymal stem cells were showed elongated phenotype a small part of the cells were in suspension, especially in culture treated with 20 µg/ml of EMD (fig.1, fig. 2).
The viability was assessed in three different periods, using MTT assay. The formazan were dissolved with dimethyl sulfoxide (DMSO), and the absorbance was measured at 550nm. The results were compared with control plates.

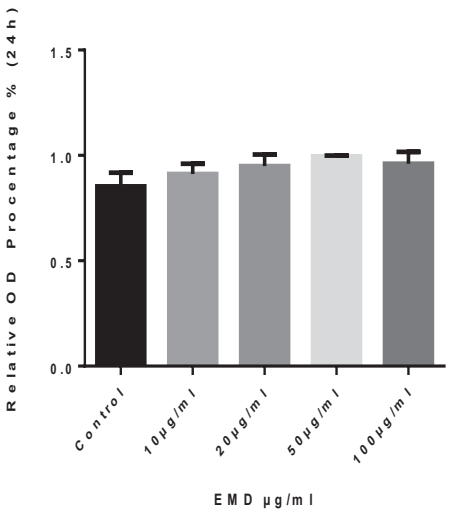
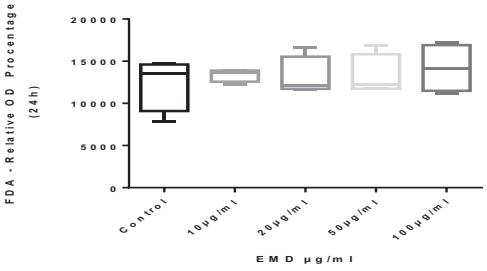


Figure 1. Palatal mesenchymal stem cells proliferation after suplimentation of propagation medium with EMD for 24h

FDA assay results reveal no significant difference compared with the control group.



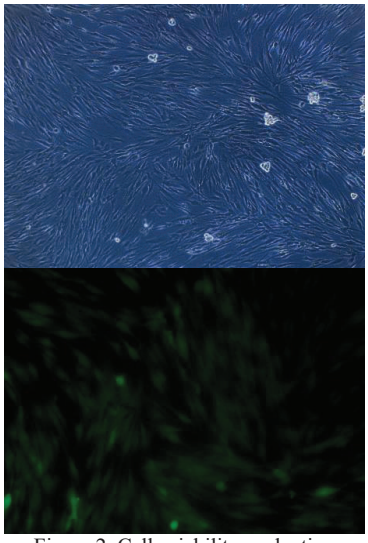


Figure 2. Cells viability evaluation using FDA assay

After 72 h of cultivation were observed an intense proliferation with a significant reduction of the non adherent cells (fig. 3, fig.4, fig. 5).

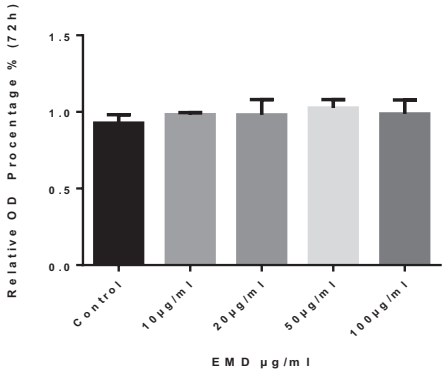


Figure 3. Palatal mesenchymal stem cells proliferation after suplimentation of propagation medium with EMD for 72 h

Also were observed organization of small cells clusters, in special in cultures treated with 50 and 100 µg/ml EMD. No citotoxicity were observed compared with control. Instead cell morphology shows some changes.

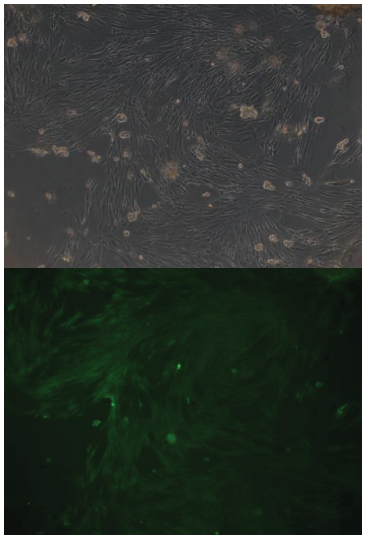


Figure 4. Viability evaluation using FDA assay

Compared with untreated cultures the degree of cell proliferation was significantly higher in cultures stimulated with EMD.

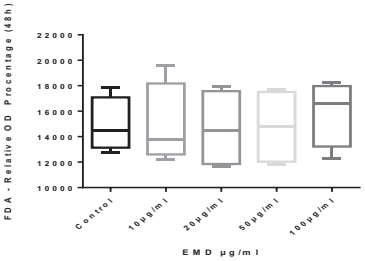


Figure 5. Cells viability after 48h of stimulation with EMD

There were no significant differences between the four concentrations (10, 20, 50, 100 µg/ml) of EMD in terms of toxicity.

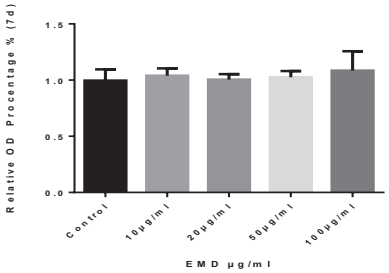
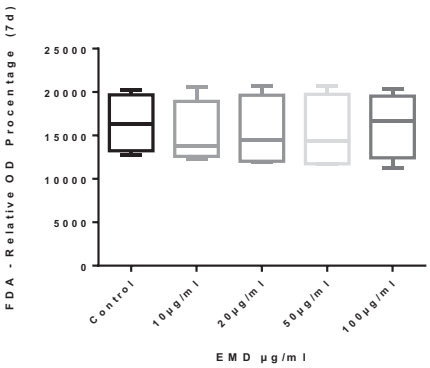


Figure 6. Palatal mesenchymal stem cells proliferation after suplimentation of propagation medium with EMD for 7 day

In 7 day in culture treated with 50 and 100µg/ml EMD the clusters increased in number and sizes (fig.6, fig.7).



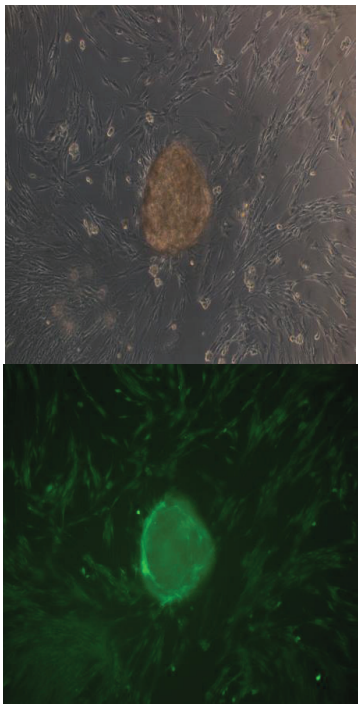


Figure 7. Cell cluster after 7 day of stimulation with EMD

In vitro studies indicated that EMD support cells proliferation and osteogenic differentiation (Gestrelius et al., 1997, Carinci et al., 2006, Wu et al., 2014). EMD also stimulate the signal transduction of bone morphogenic protein and transforming growth factor-ß and promote osteoclastogenesis (Hatakeyamaet al., 2006, Sculean et al., 2007, Fujishiro et al., 2008, Wu et al., 2014).

Mesenchymal stem cells (MSCs) which can be found in almost all postnatal organs and tissues (Patel et al., 2013) are stromal unspecialized cells that have the ability to self-renewal through cell division and also exhibit multilineage differentiation and immune-suppressive functions (Kim et al., 2012, Zhang et al., 2012, Patel et al., 2013).

Oral cavity such as dental pulp, dental follicle, dental papilla and periodontal ligament (PDL), coronal pulp, apical papilla, subepithelial layers of oral mucosa, the gingival tissues, exfoliated deciduous teeth have been identified as easily accessible sources of multipotent stem cells that could be cryopreserved and used for autogenic or allogenic cell therapy (Patel et al., 2013, Bojic et al., 2014).

An important subject for the development of differentiated cells is the practical aspects of producing optimal culture conditions (substrates, cultures medium, growth factors, etc) of these cells (Baharvand et al., 2005) for prolonged expansion (Ng et al., 2014) One of the major interests of regenerative therapy requires the cultivation of stem cells on different specific substrates namely degradable scaffolds to obtain all kinds of tissues through the control and guidance of their differentiation (Battista et al., 2005).

CONCLUSIONS

Our study revealed that the selected biomaterial are biocompatible and can be used as scaffolds for mesenchymal stem cells delivery especially for periodontal regeneration.

New biomaterials are being continuously developed, and their interaction with inserted cells and growth factors has a decisive role for regenerative medicine. Understanding the complex mechanisms involved in stem cells adhesion and division allows us to obtain useful biodegradable biomaterials combinations for cell therapy. Successful combination of these scaffolds lead to mimicking cellular microenvironment and maintaining pluripotent ability of these cells.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources

Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/138776.

REFERENCES

Baharvand H., Mahnaz A., Kazem P., Saeid K.A., 2005. The effect of extracellular matrix on embryonic stem cell-derived cardiomyocytes. Journal of Molecular and Cellular Cardiology 38: 495–503.

Battista S., Guarnieri D., Borselli C., Zeppetelli S., Borzacchiello A., Mayol L., Gerbasio D., Keene D.R., Ambrosio L., Netti P.A., 2005.The effect of matrix composition of 3D constructs on embryonic stem cell differentiation. Biomaterials 26:6194–6207.

Bojic S., Volarevic V., Ljujic B., Stojkovic M., 2014. Dental stem cells - characteristics and potential. Histol Histopathol 29: 699-706.

Carinci F., Piattelli A., Guida L., Perrotti V., Laino G., Oliva A., Annunziata M., Palmieri A., Pezzetti F., 2006. Effects of Emdogain on osteoblast gene expression.OralDis 12:329–342.

Chung C., Burdick J.A., 2008.Engineering Cartilage Tissue. Adv. Drug.Deliv.Rev14:243-262.

Drury J.L., Mooney D.J., 2003. Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials 24:4337–4351.

Fujishiro N., Anan H., Hamachi T., Maeda K., 2008. The role of macrophages in the periodontal regeneration using Emdogain gel. J Periodontal Res 43:143–155.

Gestrelius S., Andersson C., Lidstrom D., Hammarstrom L., Somerman M., 1997. In vitro studies on periodontal ligament cells and enamel matrix derivative. J Clin Periodontol 24:685–692.

Gruber R., Bosshardt DD., Miron RJ, Gemperli AC., Buser D., Sculean A., 2013Enamel Matrix Derivative Inhibits Adipocyte Differentiation of 3T3-L1 Cells via Activation of TGF-βRI Kinase Activity, PLoS One. 8(8): e71046.

Hatakeyama J., Philp D., Hatakeyama Y., Haruyama N., Shum L., Aragon M.A., Yuan Z., Gibson C.W., Sreenath T., Kleinman H.K., Kulkarni A.B., 2006. Amelogenin-mediatedregulation of osteoclastogenesis, and periodontal cell proliferation and migration. JDentRes, 85:144–149.

Influence of enamel matrix derivative on cells at different maturation stages of differentiation. PLoS One. Aug 12;8(8):e71008.

Kim R.H., Mehrazarin S., Kang M.K., 2012. Therapeutic Potential of Mesenchymal Stem Cells for Oral and Systemic Diseases. Dent Clin North Am 56:651–675.

Koop R., Merheb J., Quirynen M., 2012, Periodontal Regeneration with Enamel Matrix Derivative in Reconstructive Periodontal Therapy: A Systematic Review, Journal of Periodontology. 83, No. 6, Pages 707-720.

Miron RJ, Caluseru OM, Guillemette V, Zhang Y, Gemperli AC, Chandad F, Sculean A., 2013.

Ng C.P., Mohamed Sharif A.R., Heath D.E., Chow J.W., Zhang C.B., Chan-Park M.B., Hammond P.T., Chan J.K.Y., Griffith L.G., 2014. Enhanced ex vivo expansion of adult mesenchymal stem cells by fetal mesenchymal stem cell ECM. Biomaterials 35:4046-4057.

Patel D.M., Shah J., Srivastava A.S., 2013. Therapeutic Potential of Mesenchymal Stem Cells in Regenerative Medicine. Stem Cells Int 1-15.

Pati F., Datta P., Adhikari B., Dhara S., Ghosh K., Mohapatra P.K.D, 2012. Collagen scaffolds derived from fresh water fish origin and their biocompatibility. Journal of Biomedical Materials Research Part A 100:1068–1079. Porada C.D., Zanjani E.D., Almeida-Porad G, 2006. Adult mesenchymal stem cells: a pluripotent population with multiple applications. Current stem cell research & therapy 1:365–369.

Rodriguez-Lozano F.J., Bueno C., Insauti C.L., Meseguer L., Ramirez M.C., Blanquer M., Marin N., Martinez S., Moraleda J.M., 2011. Mesenchymal stem cells derived from dental tissues. International Endodontic Journal 44:800-806.

Sculean A., Windisch P., Dori F., Keglevich T., Molnar B., Gera I., 2007. Emdogain in regenerative periodontal therapy. A review of the literature. Fogorv Sz. 100:220–232.

Shin H., Jo S., Mikos A.G., 2003. Biomimetic materials for tissue engineering. Biomaterials 24:4353–4364.

Wu S-M., Chiu H-C., Chin Y-T., Lin H-Y., Chiang C-Y., Tu H-P., Fu M.M., Fu E., 2014, Effects of enamel matrix derivative on the proliferation and osteogenic differentiation of human gingival mesenchymal stem cells, Stem cells research and therapy, 5:52

Zhang Q.Z., Nguyen A.L., Yu W.H., Le A.D., 2012. Human Oral Mucosa and Gingiva A Unique Reservoir for Mesenchymal Stem Cells. J Dent Res 91:1011–1018.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

AN APPLICATION OF ANALYSIS OF VARIANCE TO A PROBLEM OF BIOLOGY

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Abstract

In this paper, we discuss about an application of Analysis of Variances from biology. We are interested to determine the effect of adding the NaCN (sodium cyanide) on the uptake in vitro of a particular amino acid by intestinal preparations from certain species of fish. First, we introduce some theoretical notions about this statistical method; we present the most important calculation formulas and the way they are used for comparing the treatment means in case of an experiment with k treatments, b blocks and n replicates per treatment per block. Afterwards, we consider a two-treatment experiment (with and without NaCN) and the results obtained for three replicates from each of the 4 species of fish are expressed as μ *mol* g^{-1} *dry weight per 20 min period. After we calculate the sums of observational data per sub-class, per treatment and grand totals, we determine the elements from the table of analysis of variances (sums of squares, degrees of freedom, the error mean square) and we use the Fisher test for establishing the conclusions. Then we find that the difference between treatment means is 0,49, thus with 95 % confidence probability, we may state that the addition of the NaCN to the medium reduces uptake by 0,68* μ *mol* g^{-1} *dry weight per 20 min period.*

Key words*: sums of squares, degrees of freedom, the error mean square, randomized block.*

INTRODUCTION

With certain and many nutritional qualities, fish has an important role in human nutrition. Content rich in amino acids, fats and minerals provide both basic nutritious properties and increasing energy needs.

Numerous studies have proven that all living aquatic shows accumulation and concentration capabilities even in natural environmental conditions, therefore no direct sources of pollution, which continues to justify concern for maintaining a habitat as unpolluted.

With wide application in technological processes, NaCN is a dangerous pollutant of the environment. Discharge of wastewater containing this substance must be achieved through strict observance of the rules, otherwise it may cause, real environmental disasters. Studies show that cyanide reacts with other elements and is broken down into hundreds of compounds containing various concentrations of cyanide in accumulating in fish tissues.

In this context, the work shows the effect of adding the NaCN (sodium cyanide) on the uptake in vitro of a particular amino acid by intestinal preparations from certain species of fish, this topic constituting the subject of a previous article (Varga, 2010), the conclusions being established by applying analysis of variance.

MATERIALS AND METHODS

It is considered an experiment in which are preserved constant all of the factors that influence the character of a statistical population *Y*, except one, which will be variable *X*. The homogeneous population is grouped in *m* homogeneous subpopulations associated with variations $X_i, i=\overline{1,m}$ of the factor *X* (Parker, 1973).

In the case of a heterogeneous population group, it can be done in *l* sub-populations, homogeneous in relation to the acquisition of *Y* under study. Experimental material will also be heterogeneous consisting of the *l* polls of the subpopulations, each sample consisting of *n* repetitions corresponding to the *m* variants, denoted $x_{ij}, i=\overline{1,m}, \quad j=\overline{1,n}$

If the number of repetitions is different from the variants of the factor *X* $(m \neq n)$ is used the

model (drawing) of the randomized blocks $B_1, B_2, ...B_l$. Each repetition is designated by a box which is denoted applied variant factor *X*, ie $X_i, i=\overline{1,m}$ and the response repetition x_{ij}^b , $i=\overline{1,m}, j=\overline{1,n}$, $b=\overline{1,l}$

The results can be organized in the following table:

Table 1.The experimental data

B_1	X_1 $x_{11}^1, x_{12}^1, ...x_{1n}^1$	X_2 $x_{21}^1, x_{22}^1, ...x_{2n}^1$	…	X_m $x_{m1}^1, x_{m2}^1, ...x_{mn}^1$
B_2	X_1 $x_{11}^2, x_{12}^2, ...x_{1n}^2$	X_2 $x_{21}^2, x_{22}^2, ...x_{2n}^2$		X_m $x_{m1}^2, x_{m2}^2, ...x_{mn}^2$
…	……..	……..	… …	……
B_l	X_1 $x_{11}^l, x_{12}^l, ...x_{1n}^l$	X_2 $x_{21}^l, x_{22}^l, ...x_{2n}^l$	… …	X_m $x_{m1}^l, x_{m2}^l, ...x_{mn}^l$

The responses are placed in a new table to be processed by bi-factorial analysis of variance with *n* repetitions for each cell.

Table 2. The totals/block

Block	Totals/ block
B_1	$s_1 = \sum_{j=1}^{n} x_{1j}^1 + \sum_{j=1}^{n} x_{2j}^1 + ... + \sum_{j=1}^{n} x_{mj}^1$
B_2	$s_2 = \sum_{j=1}^{n} x_{1j}^2 + \sum_{j=1}^{n} x_{2j}^2 + ... + \sum_{j=1}^{n} x_{mj}^2$
.....	……
B_l	$s_l = \sum_{j=1}^{n} x_{1j}^l + \sum_{j=1}^{n} x_{2j}^l + ... + \sum_{j=1}^{n} x_{mj}^l$

If $GT^2 = s_1^2 + s_2^2 + ...s_n^2$ and $C = \frac{GT^2}{lmn}$, it is determined the sums of squares, the degrees of freedom and the mean squares to calculate the experimental value of the Fisher test in order to complete the analysis of variance table.(Varga, 2014)

Table 3.The analysis of variance table

Sources of variation	Sums of-squares	Degrees of freedom *GL*	Mean squares S^2	*F*
X	*SSX*	*m*-1	S_X^2	$F_X = S_X^2 / S_E^2$
B	*SSB*	*l*-1	S_B^2	$F_B = S_B^2 / S_E^2$
Q	*SSQ*	*n*-1	S_Q^2	$F_Q = S_Q^2 / S_E^2$
E	*SSE*	(m-1)(n-1)	S_E^2	
T	*SS*	*mnl*-1		

Where,

$$SSX = \sum_{i=1}^{m} (T_i)^2 / nl - C, \quad T_i = \sum_{b=1}^{l} x_{ij}^b, \quad i = \overline{1,m}$$

$$SSB = \sum_{b=1}^{l} (s_b)^2 / mn - C \quad SS = \sum_{i,j,b} (x_{ij}^b)^2 - C$$

$$SSE = SS - (SSX + SSB) \quad SSQ = (\sum_{b=1}^{l} s_b^2) / n - C$$

$$S_X^2 = SSX / GL_X \ , \ S_B^2 = SSB / GL_B \ , \ S_E^2 = SSE / GL_E$$

The values F_X and F_B are compared with the critical values $F_{0,05}$, $F_{0,01}$, $F_{0,001}$ extracted from the Fisher distribution tables for pairs of the corresponding degrees of freedom (GL_X, GL_E) respectively (GL_B, GL_E). If the Fisher test experimental values (last column in Table 3) are lower than the values tabulated $F_{0,05}$, $F_{0,01}$, $F_{0,001}$, then different versions of the *X* factor significantly influences the character *Y*.

It is considered an experiment conducted on four species of fish, to which they apply two types of treatments (with and without NaCN in their environment); for the investigation on the effect of NaCN on the uptake in vitro of a particular amino acid by intestinal preparatio The results (expressed in $\mu mol\, g^{-1}$ dry weight per 20 min period) are grouped in the following table (Table 4).

Table 4.The experimental data

Fish	$X_1 =$ Treatment without NaCN	$X_2 =$ Treatment with NaCN
Fish 1	1,54; 1,92; 2,26	1,10; 1,42; 1,04;
Fish 2	1,52; 2,02; 1,91	1,31; 1,15; 1,51
Fish 3	1,00; 1,12; 1,13	0,79; 0,84; 0,86
Fish 4	1,58; 1,78; 1,52	1,24; 0,81; 1,32

RESULTS AND DISCUSSIONS

First, it is calculated the amounts of observational data sub-classes, treatments, and total (Varga, 2014).

1. Sums of squares:

$$C = \frac{GT^2}{lmn} = \frac{32,69^2}{24} = 44,52$$

$$SS = 1,54^2 + 1,92^2 + ...1,32^2 - 44,52 = 3,81$$

$$SSX = \sum_{i=1}^{m} (T_i)^2 / nl - C = \frac{T_1^2 + T_2^2}{12} - C = 1,45$$

$$SSB = \frac{9,28^2 + 9,42^2 + 5,74^2 + 8,25^2}{6} - C = 1,45$$

$$SSE = SS - (SSX + SSB) = 0,91$$

2. Degrees of freedom:
$GL_X = 1$; $GL_B = 3$; $GL_T = 23$; $GL_E = 19$.

3. Mean-squares:
$S_X^2 = 1,45$, $S_B^2 = 0,48$, $S_E^2 = 0,04$

4. The experimental values of Fisher test are:
$F_X = S_X^2 / S_E^2 = 30,38$; $F_B = S_B^2 / S_E^2 = 10,1$

Next, it is completed the table of variances analysis (Table 5)

Table 5.The analysis of variance table for the problem studied

Sources of variation	Sums of-squares	Degrees of freedom	Mean squares	F
X	1,45	1	1,45	$F_X = 30,38$
B	1,45	3	0,48	$F_B = 10,1$
E	0,91	19	0,04	-
T	3,81	23		-

The population is heterogeneous with respect to *Y*, but can be divided into homogeneous blocks (l = 4): B_1(Fish 1), B_2(Fish 2), B_3(Fish 3) B_4(Fish 4).
There are m = 2 variants of *X* factor (NaCN Without Treatment, Treatment with NaCN) and n = 3 repetitions / treatment / block.

5. From the tables of Fisher distribution for (1; 19) GL, the critical values are obtained: $F_{0.05}$ = 4,38, $F_{0.01}$ = 8,18 and $F_{0.001}$ = 15,08; for (3,19) GL, $F_{0.05}$ = 3,13, $F_{0.01}$ = 5,74 şi $F_{0.001}$ = 8,28.

6. By comparing the values $F_X = 30,38 > F_{0,001}$ $F_B = 10,1 > F_{0,001}$ we obtain the conclusion that the treatments with and without Na CN and block variation leads to very significant differences.
The contributions of the variation of *X*-factor and *B* to the variation of *Y* are:

$$A_X = \frac{SSX}{SS} = \frac{1,45}{3,81} = 38\%,$$

$$A_B = \frac{SSB}{SS} = \frac{1,45}{3,81} = 38\%,$$

$$A_X = 100\% - A_X - A_B = 24\%$$

Because the experiment was performed considering the many replicas/block, the variation of the averages is studied assuming that blocks the effects of treatments gathered. Again is applied Fisher test, first there are been calculated the sizes of variance analysis table, necessary to determine the experimental value of the test (Samboan and Bad, 1986).

$$SSQ = 5,72^2 + 5,45^2 + ...3,37^2 / 3 - 44,52 = 3,06$$

$$GL_Q = n - 1 = 3, \quad S_Q^2 = SSQ / GL_Q = 0,05,$$

$$SSE = SS - (SSQ + SSX + SSB) = 0,74$$

$$GL_E = GL_T - (GL_Q + GL_X + GL_B) = 16$$

$$S_E^2 = SSE / GL_E = 0,04 \quad F_Q = S_Q^2 / S_E^2 = 1,25$$

For (3,16) GL and the confidence probability of 95%, the tabular value is 3.24.
Such, it follows that the interaction is not significant, so it can make a quantitative expression of the difference between the

treatments, which is applicable to all blocks (Feller, 1996).

The difference between treatments averages is

$$D = {(19,30 - 13,39)}/{12} = 0,49$$

If considered

$$S_E^2 = 0,04, \quad GL_E = 16, \quad \alpha = 5\%, \quad t_{\alpha/2} = 2,12,$$

The confidence interval for D

$$(0,49 - 0,1; 0,49 + 0,1)$$

CONCLUSIONS

In conclusion, we may state that the addition of the NaCN to the medium of fish, reduced uptake by $D = 0,49 \pm 0,1\ \mu mol\, g^{-1}$ dry weight per 20 min period.

REFERENCES

Feller W. 1996. An Introduction to Probability Theory and its Applications, John Wiley & Sons, Inc., New York, London, 40-55

Parker R.E. 1973. Introductory Statistics for Biology, London, 67-75.

G. Samboan G., Bad M.D., 1986. Elements of Mathematics for biologists, Bucharest. Univ. Ed., Bucharest, 289-313.

Varga M., 2014. Elements of Mathematics and Statistics with applications in biotechnology, Printech Ed., Bucharest, 199-204.

Varga M., 2010. Some results on effect of NaCN on the uptake in vitro of a particular amino acid by intestinal preparations from a certain species of fish,Proceedings of the International Symposium on New Researches in Biotechnology, Bucharest, 128-133.

FOOD BIOTECHNOLOGY

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

RESISTANCE PROFILE OF PLANT-DERIVED LACTIC ACID BACTERIA AGAINST HERB EXTRACTS

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Abstract

It was examined the possibility for growth of representatives from genera Enterococcus and Streptococcus, isolated from different parts of the herbs Salvia and Geranium in the presence of relevant crude plant extracts. There have been established relationships between the type and concentration of the herbal extracts and tested microorganisms. It was determined a resistance profile of examined lactic acid bacteria against the effect of natural plant extracts. It was established that Streptococcus thermophilus and Enterococcus faecium (isolated from Geranium) grow in the presence of extract of Geranium sanguineum L. in concentrations up to 30 mg/ml whereas some strains S. thermophilus and Ent. faecium (isolated from Salvia) show higher resistance - in range of 30 mg/ml to 100 mg/ml extract of Salvia officinalis L. This is an evidence of selective inhibitory activity of plant extracts to examined lactic acid bacteria and it is a prerequisite for their incorporation in food fermentation that have a lot of economic value, as well as in helping improve human health.

Key words*: lactic acid bacteria, plant extract, resistance profile, Salvia, Geranium.*

INTRODUCTION

In recent years there is a trend of increased interest in lactic acid bacteria (LAB), isolated from non-dairy products due to their diverse metabolic profile and unique flavor-forming activities. Plant – derived lactobacteria, have shown tolerance to high values of pH and salt concentration, high level of stress resistance and an ability to ferment more types of carbohydrates, compared to those of dairy origin. Furthermore, no significant differences were noted in fermentation characteristics and profiles of enzymes (lipases, peptidases and phosphatases) required for obtaining various fermented dairy products with plant and commercial strains lactobacteria (Nomura et al., 2006; Michaylova et al., 2007; Siezen et al., 2008; Venugopalan et al., 2010). Medicinal plants are an important ecosystem for isolation of LAB (Siezen et al., 2008; Venugopalan et al., 2010; Cakir, 2010; Baradaran et al., 2012). Each specified plant species provides a unique environment in terms of competing microorganisms, natural plant antagonists, as well as accessibility, type and concentration of the substrate in the various physical factors. These conditions allow growth of typical epiphytic flora, from which derives a population and a chain of fermentation processes, when the plant material is collected and prepared for fermentation.

Recently, it has been formed a new direction in scientific research, related to: obtaining bioactive or biogenic substances, extracted from different plants or synthesized, during food fermentation; subsequent creation of novel foods (defined as healthy and functional) by additional introduction into their technological schemes of such exogenous functional components or use of microorganisms, producers of biogenic substances, as well as microorganisms, which have probiotic characteristics (Gobbetti et al., 2010).

The genus *Salvia* is a broad genus belonging to the family Lamiaceae. *Salvia* comprises one of the largest genera of flowering plants in the world with 900 to 950 species occurring worldwide except in Australia (Ali and Aboud, 2010). Different species of *Salvia* genus are grown and used as a spice and in traditional and folk medicine, because of their antibacterial,

antioxidant, anti-inflammatory and analgesic properties (Ibrahim, 2012). Phytochemical studies conducted on plants of this genus have led to the isolation of diterpenoids (Habibi et al., 2000; Nieto et al., 2000), triterpenes, sterols (Rauter et al, 2007), anthocyanins, coumarins, polysaccharides, flavonoids and phenolic acids (Lu and Foo, 2002). *Salvia officinalis* (common sage) is known as one of the herbs that has antimicrobial activity, it is a useful resource in combating many illnesses. (Eidi et al., 2006; Ali and Aboud, 2010). It is cultivated in several countries mainly to be used in medicine, perfumery and food industry (Santos et al, 2002).

Geranium sanguineum L., commonly called Bloody Cranesbill, is an herbaceous plant species in the Geraniaceae family. It is native from Europe and temperate Asia (Hammami et al., 2011). It is wide-spread in Bulgaria and known with the popular name bloody geranium. Extracts from various parts of *G. sanguineum* L., have significant antiviral, antibacterial, anti-inflammatory and antioxidant activities (Serkedjieva and Manolova, 1992; Hammami et al., 2011). The *Geranium* genus phytochemistry is well known in present with the most studied classes of active compounds (tannins, volatile oils, flavonoids and polyphenols) (Fodorea, et al., 2004; Kobakhidze and Alaniya, 2004).

The aim of this study was to evaluate resistant profile of LAB isolated from medicinal plants *G. sanguineum* and *Salvia* species against extracts from plants from which they are isolated and to compare them to LAB isolated from dairy products; to select resistant LAB suitable for their potential cultivation in the presence of plant extract during *in situ* cultivation in milk.

MATERIALS AND METHODS

Microorganisms

Thirty one strains *Streptococcus thermophilus* and *Enterococcus faecium* used in this study were isolated from medicinal plants *Geranium sanguineum* L. (Teneva et al., 2014) and various *Salvia* species (*S. scabiosifolia* Lam., *S. ringens* Sibth. & Sm., *S. officinalis* L. and *S. blepharophylla* Brandegee ex Epling), collected from their natural habitats in Bulgaria as well as the Botanical garden of the Technical University in Dresden, Germany. LAB were grown in M17 broth (pH 6.6) (Merck, Germany, Darmstadt) at 37 ºC for 48 h.

Plant Materials and Preparation of crude extract

Plant materials of *Salvia officinalis* L. (young leaf) and *Geranium sanguineum* L. (root) were collected in June 2013 from their natural habitat - field near by town of Kroumovgrad and Vitosha region (Iskar Dam), respectively. The plant materials were washed 2-3 times with running tap water and once with sterile distilled water, air-dried, powdered and used for extraction.

100 g powder of each plant materials were extracted (in triplicate) with 70 % ethanol (1:10 w/v) for 24 h at room temperature. The crude extracts were pooled, filtrated by filter paper and concentrated in a vacuum evaporator and then they were lyophilized. Lyophilized extracts were dissolved in 30% ethanol and in water and subsequently filtrated through a 0.22 μm syringe filter.

HPLC analyses

The phenolic acid, flavonoids and quercetin glycosides were analyzed by HPLC system consisting of Waters 1525 Binary Pump (Waters, Milford, MA, USA), Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA), controlled by Breeze 3.30 software. Supelco Discovery HS C18 column (5 μm, 25 cm × 4.6 mm) operated at 26 °C was used for separation. The following mobile phases were used for separation: of the phenolic acids - 2% (v/v) acetic acid (solvent A) and 0.5 % (v/v) acetic acid : acetonitrile (1:1, v/v) (solvent B); of the flavonoids - 2.0 % (v/v) acetic acid (solvent A) and methanol (solvent B), of the quercetin glycosides - 2.0 % (v/v) acetic acid (solvent A) and acetonitrile (solvent B). The gradient programs used for the phenolic acids and the flavonoids was described by Marchev et al. (2011) and for the quercetin glycosides by Ivanov et al. (2014). Eluting compounds were detected by monitoring the eluate at 280, 380 and 370 nm, respectively.

Rosmarinic acid was analyzed by the same Waters HPLC system and column. It was used

an isocritic elution with the following composition of the mobile phase and conditions: methanol : k. H_3PO_4 (85 %) :H_2O = 50 : 0.3 : 49.7 (solvent A) and temperature 26 °C. The detection was carried out at 327 nm.
Data represent the mean values of three independent experiments and standard deviation.

Analysis for resistance profile of LAB against *Geranium* and *Salvia* extracts

The agar well diffusion method was used to detect resistance profile of plant - derived LAB against *G. sanguineum* L. and *S.officinalis* L. extracts. The sterile M17 medium (20ml) was inoculated with test bacteria ($1x10^6$ cfu/ml) and was poured into Petri plates. A sterile cork borer of diameter (8 mm) was used to cut uniform wells in the agar. A 50µl volume of each concentration of the extracts (5 – 150 mg/ml) was added in the wells into M17 plates. The 30% ethanol was used as control. The plant extracts and 30 % ethanol were sterilized by filtration using 0.22 µm syringe filters. All test plates were incubated at 37°C for 48 h. The diameter (mm) of inhibition zones of the extracts was measured. Data represent the mean values of three independent experiments and standard deviation.
The inhibitory activity of *S. officinalis* L. and *G. sanguineum* L. extracts on the growth of the LAB, belonging to the collection of the laboratory and isolated from various dairy products, as well as used in our previous studies (*Streptococcus thermophilus* ST3, *Enterococcus faecium* EF4) was examined.

RESULTS AND DISCUSSIONS

G. sanguineum L. and *Salvia officinalis* L. are plants containing compounds from the group of polyphenols. (Pantev et al., 2006; Martins et al., 2015) Data for content of flavonoids and phenolic acids in *G. sanguineum* L. and *S. officinalis* L. extracts showed significant variety of these biologically active components (Table 1). It was observed a wider range of phenolic compounds in the extracts of *S. officinalis* L., compared to those of *G. sanguineum* L. Furthermore, it was observed also strict specificity of presence of individual phenolic acids in the studied extracts (gallic acid, 3,4-dihydroxy-benzoic acid, 2-hydroxy-benzoic acid – *G. sanguineum* L.; vanillic acid, syringic acid, p-coumaric acid, ferulic acid, rosmarinic acid - *Salvia officinalis* L. A similar trend was established for the content of flavonoids in the studied extracts. Specific flavonoids (myricetin, luteolin, rutin, hyperozide) contained in the extracts of *S. officinalis* L. were not detected in the extracts of *G. sanguineum* L., except for rutin and hyperozide, found in insignificant concentrations (Table 1).

Table 1. Concentration of polyphenolic compounds in the extracts of *G.sanguineum* L. and *S.officinalis* L.

	Compound, mg/g extract	*G.sanguineum* L.		*S.officinalis* L.	
		Hydroethanolic extract	Aqueous extract	Hydroethanolic extract	Aqueous extract
Phenolic acids					
	Gallic acid	2.72 ± 0.07	1.92± 0.05	nd	nd
	3,4-dihydroxy-benzoic acid	0.34 ± 0.01	0.29 ± 0.03	nd	nd
	2-hydroxy-benzoic acid	2.06 ± 0.08	2.50 ± 0.07	nd	nd
	Vanillic acid	nd	nd	0.25 ± 0.01	0.25 ± 0.03
	Syringic acid	nd	nd	0.70 ± 0.05	0.46 ± 0.04
	Caffeic acid	nd	nd	nd	nd
	Chlorogenic acid	nd	nd	nd	nd
	p-Coumaric acid	nd	nd	3.65 ± 0.07	3.01 ± 0.07
	Sinapic acid	nd	nd	nd	nd
	Ferulic acid	nd	nd	0.51 ± 0.05	0.24 ± 0.03
	Cinnamic acid	nd	nd	nd	nd
	Rosmarinic acid	nd	nd	48.69±0.23	22.18±0.17
Flavonoids					
Flavonols	Myricetin	nd	nd	0.13 ± 0.02	0.14 ± 0.01
	Kaempferol	nd	nd	nd	nd
	Quercetine	nd	nd	nd	nd
Flavanone glycoside	Hesperidin	nd	nd	nd	nd
Flavone	Apigenin	nd	nd	nd	nd
	Luteolin	nd	nd	0.04 ± 0.01	0.03 ± 0.01
Quercetin glycoside	Rutin	0.04 ± 0.01	nd	9.96± 0.12	nd
	Hyperozide	nd	0.04 ± 0.01	17.24± 0.13	15.75± 0.14

nd - not detected

The species *S. thermophilus* and *Ent. faecium*, isolated from representatives of the genus *Salvia* (*S. blepharophylla* Brandegee ex Epling, *S. scabiosifolia* Lam., *S. ringens* Sibth. & Sm., *S. officinalis* L.), showed a wide variety concerning their resistance to the extract of *S. officinalis* L. (Table 2). A similar trend was established also in testing the extract of *G. sanguineum* L. on the development of these types of lactobacteria, but isolated from *G. sanguineum* (Table 3). Interspecies as well as interstrain differences in the extent of growth inhibition, depending on the concentration of the tested extracts were found (Table 2, 3).

S. thermophilus strains (Sbf352, Sbf373, Sbf401) isolated from *Salvia,* were inhibited by the ethanol extracts, at low studied concentrations (25-30 mg/ml), while to aqueous extracts the strains demonstrated resistance to all the tested concentrations (20-150 mg/ml) (Table 2).

54 % of *Ent. faecium* strains (isolated from *Salvia*) showed resistance to the ethanol extracts up to a concentration of 100 mg/ml, while insignificant part of the strains (Sof271, Sof277 and Sof279), showed a high sensitivity to the ethanol extracts (40-50 mg/ml). *Ent. faecium* strains showed resistance to the aqueous extracts, throughout the whole studied range. The only exception is *Ent. faecium* Sof279, whose growth was inhibited at the concentration of 40 mg/ml (Table 2).

The controls didn't inhibit the growth of studied strains.

Table 2. Effect of different concentrations of *S. officinalis* L. extracts on the growth of *S. thermophilus* and *Ent. faecium*

Strains	Inhibition Zone (diameter, mm)															
	Hydroetanolic extract, mg/ml								Aqueous extract, mg/ml							
	150	**100**	**75**	**50**	**40**	**30**	**25**	**20**	**150**	**100**	**75**	**50**	**40**	**30**	**25**	**20**
***S. thermophilus*[1]**																
Sbf352	15,0 ±0,5	14,4 ±1,0	12,2 ±0,8	11,0 ±0,6	10,0 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-
Sbf373	19,0 ±1,0	18,0 ±1,1	15,4 ±0,8	13,0 ±0,6	12,4 ±0,6	11,1 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-
Sbf401	18,2 ±1,0	17,3 ±1,0	15,3 ±1,0	12,2 ±0,6	11,0 ±0,6	9,3 ±0,6	-	-	-	-	-	-	-	-	-	-
***Ent. faecium*[2]**																
Ssf21	10,4 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf22	12,3 ±0,5	11,4 ±0,5	10,3 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf32	10,4 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf33	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf34	10,5 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Srs161	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sof271	12,0 ±0,5	11,4 ±0,7	10,3 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-
Sof277	12,0 ±0,9	11,0 ±0,5	10,0 ±0,4	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-
Sof279	14,0 ±1,1	13,0 ±0,5	12,4 ±0,6	10,3 ±0,5	9,3 ±0,6	-	-	-	14,5 ±1,0	13,5 ±0,8	12,0 ±0,5	10,0 ±0,5	9,3 ±0,6	-	-	-
Sol284	9,3 ±0,6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sos301	9,5 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sos312	11,5 ±1,2	10,3 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sos328	10,5 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] *S. thermophilus* strains isolated from *Salvia* species (*S. blepharophylla* Brandegee ex Epling); [2] *Ent. faecium* strains isolated from Salvia species (*S. scabiosifolia* Lam., *S. ringens* Sibth. & Sm., *S. officinalis* L.) (-) – resistant, without zone of inhibition;

Table 3. Effect of different concentrations of *G.sanguineum* L. extracts on the growth of *S. thermophilus* and *Ent. faecium*

Strains	Inhibition Zone (diameter, mm)																	
	Hydroetanolic extract, mg/ml									Aqueous extract, mg/ml								
	75	50	30	25	20	15	10	8	5	75	50	30	25	20	15	10	8	5
***S. thermophilus*[1]**																		
Gsf60	15,5 ±0,5	13,0 ±0,8	10,8 ±0,5	10,0 ±0,5	9,2 ±0,3	-	-	-	-	15,0 ±1,2	11,2 ±0,6	10,5 ±0,5	10,1 ±0,7	9,2 ±0,3	-	-	-	-
Gsf65	14,3 ±0,6	12,0 ±0,9	10,2 ±0,6	9,3 ±0,6	9,2 ±0,3	-	-	-	-	14,1 ±0,7	13,5 ±0,5	10,0 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-
Gsf69	14,1 ±0,7	13,5 ±1,0	12,2 ±0,7	11,0 ±0,6	10,5 ±0,5	9,2 ±0,3	-	-	-	13,1 ±0,5	12,4 ±1,2	11,0 ±0,5	-	-	-	-	-	-
Gsf76	13,1 ±0,5	11,0 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-	11,2 ±0,6	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-	-
***Ent. faecium*[2]**																		
Gsf123	12,0 ±1,0	10,5 ±0,5	9,3 ±0,6	-	-	-	-	-	-	12,0 ±1,0	11,0 ±0,5	9,3 ±0,6	-	-	-	-	-	-
Gsf124	12,0 ±1,0	10,2 ±0,5	9,3 ±0,6	-	-	-	-	-	-	11,0 ±0,5	10,0 ±0,3	9,2 ±0,3	-	-	-	-	-	-
Gsl123	12,4 ±0,9	11,2 ±0,5	10,4 ±0,4	10,0 ±0,5	-	-	-	-	-	10,4 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-
Gsl124	13,2 ±1,2	11,2 ±0,5	10,3 ±0,5	9,3 ±0,6	-	-	-	-	-	12,5 ±1,0	11,3 ±0,9	-	-	-	-	-	-	-
Gsl213	16,3 ±0,9	14,3 ±1,1	13,0 ±0,5	12,5 ±1,0	11,2 ±0,5	10,4 ±0,4	10,0 ±0,5	9,2 ±0,3	-	15,0 ±0,5	13,5 ±1,0	13,1 ±0,5	11,2 ±0,6	10,3 ±0,5	9,5 ±0,5	9,2 ±0,3	-	-
Gsf313	15,3 ±1,2	13,5 ±1,0	11,5 ±1,2	11,0 ±0,4	11,0 ±0,4	10,5 ±0,5	10,0 ±0,5	-	-	15,0 ±0,5	13,0 ±0,5	12,0 ±1,0	11,4 ±0,8	10,0 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-
Gsl312	16,1 ±0,9	14,5 ±0,5	12,0 ±1,0	11,0 ±0,5	10,5 ±0,2	10,3 ±0,5	9,2 ±0,3	-	-	15,2 ±0,6	14,3 ±0,7	12,0 ±1,0	11,0 ±0,5	10,0 ±0,3	9,2 ±0,3	9,2 ±0,3	-	-
Gsf2115	12,5 ±1,0	11,0 ±0,4	10,4 ±0,4	10,0 ±0,5	9,3 ±0,6	-	-	-	-	12,1 ±0,5	11,2 ±0,6	10,0 ±0,5	9,5 ±0,5	9,2 ±0,3	-	-	-	-
Gsf2101	12,0 ±0,5	11,2 ±0,5	10,2 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-	12,5 ±1,0	11,1 ±0,6	10,3 ±0,5	10,0 ±0,5	9,3 ±0,6	-	-	-	-
Gsl2227	11,0 ±0,5	10,5 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-	11,4 ±0,8	10,0 ±0,3	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-
Gsl2212	12,0 ±1,0	11,5 ±0,5	10,0 ±0,5	9,2 ±0,3	-	-	-	-	-	11,5 ±0,5	10,5 ±0,5	10,4 ±0,4	9,2 ±0,3	-	-	-	-	-

[1] *S. thermophilus* strains isolated from *G. sanguineum* L.; [2] *Ent. faecium* strains isolated from *G. sanguineum* L.; (-) – resistant, without zone of inhibition;

The ethanol and the aqueous extracts of bloody geranium showed a similar inhibitory influence on *S. thermophilus* and *Ent. faecium* strains, isolated from *Geranium*. Inhibition of *S. thermophilus* strains was observed at a concentration of ethanolic extract between 15-25 mg/ml and of aqueous extract between 20-30 mg/ml (Table 3). *Ent. faecium* strains showed resistance to lower concentrations – 5 - 25 mg/ml and 8- 30 mg/ml, of ethanol and aqueous extract, respectively (Table 3). The controls didn't inhibit growth of studied strains. The available information concerning studies of resistance profile of plant - derived LAB to relevant plant extracts is scarce (Saguibo et al, 2012).

Most collectives study the effect of various plants, spice, vegetable extracts on LAB species, but not from plant origin (Sagdic et al., 2003; Sagdic et al, 2005; Michael et al., 2010; Saguibo and Elegado, 2012; Ekren et al., 2013). It was also examined the influence of different concentrations of the extracts on the growth of LAB isolated from dairy products (*Streptococcus thermophilus* ST3, *Enterococcus faecium* EF4). It was observed that LAB of diary origin demonstrate similar resistance to the tested plant extracts regarding plant - derived LAB.

CONCLUSIONS

Most of the LAB strains (*S. thermophilus*, *Ent. faecium*) isolated from *Geranium* showed a similar resistance profile to those of dairy origin, both to the ethanol and to aqueous extracts of *Geranium*. Exceptions are 2 strains of *Ent. faecium* (Gsl123, Gsl124), which demonstrated a higher level of resistance to the aqueous extract.

S. thermophilus and *Ent. faecium*, isolated both from *Salvia* and from dairy products, showed resistance to the aqueous plant extract. Regarding the ethanol extract, *S. thermophilus* of plant and dairy origin also showed similar behavior - high level of inhibition.

The trend for total similarity of resistant profiles is not valid for *Ent. faecium*, isolated from *Salvia* species and dairy products - about 60% of *Ent. faecium*, showed higher level of resistance compared to *Ent. faecium* from diary origin.

Probably some strains of plant – derived LAB are more resistant to extracts of the plant, from which they are isolated, in comparison to those isolated from dairy products. Out of all isolated plant-derived LAB, highest resistance was reported for the strains of *Ent. faecium* isolated from *Salvia*.

REFERENCES

Ali M.R., Aboud A.S., 2010. Antimicrobial activities of aqueous and methanolic extracts from *Salvia officinalis* and *Salix acmophylla* used in the treatment of wound infection isolates. Ibn Al-Haitham Journal For Pure And Applied Science, 23(3):25-38.

Baradaran A., Foo H.L., Sieo C.C., Rahim R.A., 2012. Isolation, identification and characterization of lactic acid bacteria from *Polygonum minus.* Romanian Biotechnological Letters, 17(3):7245-7252.

Cakir I., 2010. Antibacterial and antifungal activities of some lactic acid bacteria isolated from naturally fermented herbs. Journal of Food, Agriculture & Environment, 8(2):223-226.

Eidi A., Eidi A., Baharc, M.(2006). Effects of Salvia officinalis L. (sage) leaves on memory retention and its interaction with the cholinergic system in rats. Ap plied and Basic Nutritional Science, 22(3): 321-326.

Ekren S., Yerlikaya O., Tokul H., Akpınar A., M. Açu, 2013. Chemical composition, antimicrobial activity and antioxidant capacity of some medicinal and aromatic plant extracts. African Journal of Microbiology Research, 7(5):383-388.

Fodorea C.S., Vlase L., Suciu S., Tamas M., Leucuta S.E., Bersan L., HPLC study on some polyphenols of *Geranium macrorrhizum* L. (Geraniaceae). Ovidius University Annals of Medical Science – Pharmacy**, 2(**2):70.

Gobbetti M., Di Cagno R., De Angelis M., 2010. Functional microorganisms for functional food quality. Critical Reviews in Food Science and Nutrition 50(8):716-727.

Habibi Z., Eftekhar F., Samiee K., Rustaiyan A., 2000. Structure and antibacterial activity of a new labdane diterpenoid from *Salvia leriaefolia*. Journal of Natural Products, 63(2):270–271.

Hammami I., Triki M.A., Rebai A., 2011. Chemical compositions, antibacterial and antioxidant activities of essential oil and various extracts of *Geranium sanguineum* L. flowers. Archives of Applied Science Research, 3(3):135–144.

Ibrahim T.A., 2012. Chemical composition and biological activity of extracts from *Salvia bicolor* Desf. growing in Egypt. Molecules, 17(10):11315-11334.

Ivanov I., Vrancheva R., Marchev A., Petkova N., Aneva I., Denev P., Georgiev V., Pavlov A., 2014. Antioxidant activities and phenolic compounds in Bulgarian *Fumaria* species. International Journal of Current Microbiology and Applied Sciences, 3(2):296-306.

Kobakhidze K.B., Alaniya M.D., 2004. Flavonoids from Geranium robertianum. Chemistry of Natural Compounds, 40(1):89-90.

Lu, Y., Foo, L.Y., 2002. Polyphenolics of *Salvia*. A review. Phytochemistry, 59(2):114–140.

Marchev A., Georgiev V., Ivanov I., Badjakov I., Pavlov A, 2011. Twophase temporary immersion system for *Agrobacterium rhizogenes* genetic transformation of sage (*Salvia tomentosa* Mill.). Biotechnology Letter, 33 (9):1873-1878.

Martins N., Barros L., Santos-Buelga C., Henriques M., Silva S., Ferreira I.C.F.R., 2015. Evaluation of bioactive properties and phenolic compounds in different extracts prepared from *Salvia officinalis* L. Food Chemistry, 170:378–385.

Michael M., Phebus R.K., Schmidt K.A., 2010. Impact of a plant extract on the viability of Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus in nonfat yogurt. International Dairy Journal, 20(10):665-672.

Michaylova M., Minkova S., Kimura K., Sasaki T., Isawa K., 2007. Isolation and characterization of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* from plants in Bulgaria. Federation of European Microbiological Societies Microbiology Letters, 269(1):160-169.

Nieto M., García E.E., Giordano O.S., Tonn C.E., 2000. Icetexane and abietane diterpenoids from *Salvia gilliessi*. Phytochemistry, 53(8):911–915.

Nomura M., Kobayashi M., Narita T., Kimoto-Nira H., Okamoto T., 2006. Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. Journal of Applied Microbiology, 101(2):396-405.

Pantev A., Ivancheva S., Staneva L., Serkedjieva J., 2006. Biologically Active Constituents of a Polyphenol Extract from *Geranium sanguineum* L.

with Anti-Influenza Activity. Zeitschrift für Naturforschung c, 61(7-8):508-516.

Rauter A.P., Branco I., Lopes R.G, Justino J., Silva V.M.F., Noronha J.P., Cabrita E.J., Brouard I., Bermejo J. A., 2007. New lupenetriterpenetriol and anticholinesterase activity of *Salvia sclareoides*. Fitoterapia, 78(7-8):474–481.

Sagdic O., Kaharan A.G., Ozkan M., Ozkan G., 2003. Effect of some spice extracts on bacterial inhibition. Food science and technology international, 9(5):353-356.

Sagdic O., Yasar S., Kisioglu A.N., 2005. Antibacterial effects of single or combined plant extract. Annals of Microbiology, 55(1):67-71.

Saguibo J.D., Elegado F.B., 2012. Resistance Profile of Probiotic Lactic Acid Bacteria Against Inhibitory Effects of Selected Plant Extracts. The Philippine Agricultural Scientist, 95(1):22-32.

Saguibo J.D., Jimeno B.T., Calapardo M.R., Perez MT. M., Ramirez G.A., Elegado F.B., 2012. Isolation and screening of resistant lactic acid bacteria against Guava leaf extract and the hypoglycemic effect of its fermentation on mice. Journal of Engineering Technology and Education,The 2012 International Conference on Green Technology and Sustainable Development, 59-65.

Santos,P.C., Seabra, R.M., Andrade, P.B., Fernandes,F.M., 2002. Phenolic antioxidant compounds p roduced by *in vitro* shoots of sage (*Salvia officinalis L.*). Plant Sci*ence,* 162(6):981 – 987.

Serkedjieva J., Manolova N., 1992. A plant polyphenolic complex inhibits the reproduction of influenza and herpes simplex viruses. In: Hemingway R.W. and Laks P.E. (Eds), Plant polyphenols. Basic Life Sci. 59, 705-715.

Siezen, R.J., Starrenburg, M.J.C., Boekhorst, J., Renckens B., Molenaar, D., van Hylckama Vlieg, J.E.T., 2008. Genome-scale genotype-phenotype matching of two *Lactococcus lactis* isolates from plants identifies mechanisms of adaptation to the plant niche. Applied and Environmental Microbiology, 74(2):424-436.

Teneva Ts., Beshkova D., Marchev A., Nikolova M., Frengova G., Pavlov A., 2014. *Geranium sanguineum* L. - an alternative source for isolation of lactic acid bacteria. Ecological engineering and environment protection, 1:4-11.

Venugopalan V., Dinesh M.S., Geetha K.S., 2010. Enhancement of antimicrobial potential of phyllanthus niruri by fermentation. Journal of Herbal Medicine and Toxicology, 4(2):167-175.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

DEVELOPMENT OF SOME INNOVATIVE BAKERY PRODUCTS BASED ON WHEAT FLOUR ENRICHED IN BIOACTIVE COMPOUNDS WITH FUNCTIONAL ROLE

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Abstract

In this work an extensive analysis of the composition of Cannabis sativa L., has been done in order to further exploit the functionality of it in bakery products based on wheat flour.
*Cannabis sativa L., is already known, (*J. C. Callaway, 2002*,) as an excellent source of unsaturated fatty acids (omega 3 and omega 6 - in an optimum ratio), fiber, amino acids (lysine, alanine, arginine), and high content of mineral which should recommend it as an important functional ingredient in food.*
In this study, partially Cannabis sativa L. skimmed flour has been used in different proportions for enrichment of wheat flour with functional ingredients such as: bioactive carbohydrates (dietary fiber), bioactive protein, essential fatty acids and minerals.
Fatty acid composition was determined by ^{1}H-NMR and minerals evaluation was done by using ICP analysis.
The results demonstrated that Cannabis sativa L, partially skimmed, should be used in food industry, especially in bakery processing, as additional valuable source of bioactive compounds.

Key words*: hemp, bread, fatty acids, dietary fiber.*

INTRODUCTION

Hemp *(Cannabis sativa* L.) is one of the first plants cultivated by humans for use in the manufacture of various fabrics, rope and paper. Later it was used widely in food and medicinal purposes (Fitzpatric, 2007).
Currently, industrial hemp and marijuana are both classified as *Cannabis sativa*, a species with hundreds of different varieties. Industrial hemp is bred to maximize fiber, seed and/or oil, while marijuana varieties seek to high content of THC (9-tetrahydro-cannabinol; the primary psychoactive component). In the European Union, only cultivars containing less than 0.3% THC in their flower portions are permitted as commercial crops (Ivan Svec, Marie Hruskova, 2015). Hemp seed oil has a high content of polyunsaturated fatty acids and the ratio of omega-3 and omega-6 (1:2 and 1:3) is optimal for human health (S. Rezapour-Firouzi et al., 2013).
With a valuable nutritional content, hemp seed is an excellent source of protein, dietary fiber, vitamins (A, C and E) and minerals (JC Callaway, 2002, 2004).
The hemp seed proteins have a good potential to be used as a valuable source of protein in nutrition. (X.-S. Wang et al., 2008)
Currently there are few works that reveal valuable potential of hemp seed flour partially defatted. X.-S. Wang et al., have obtained and studied a protein isolate from defatted flour hemp seed. The findings of this study were that the proportion of essential amino acids to the total amino acids for hemp seed protein isolate and their in vitro pepsin plus trypsin digestibility was significantly higher, as

compared to that of soy protein isolate. "Thus, hemp proteins can be suitable for human consumption as a more superior source of protein nutrition, relative to widely recognized soy proteins" (X.-S. Wang et al., 2008).
Therefore, the main objective of this study was to evaluate the content of valuable compounds from wheat flour enriched with different percentages of hemp seed flour, partially defatted, for use in bakery
Adding partially defatted hemp flour in bakery products improves the dietary intake of most micronutrients and fiber. Daily consumption of these products is recommended to help prevent major non-communicable diseases such as cardiovascular diseases and certain cancers (OMS, 2003). Therefore, evaluation of the minerals and other chemicals elements contained of these mixtures these two flours are important.

MATERIALS AND METHODS

2.1. Materials

Partially defatted hemp seed flour, a byproduct during manufacture of the hemp seed oil, was kindly supplied by SC Hofigal Export Import SA, (Bucharest, Romania). This meal has been obtained from hemp (*C. sativa* L.) seeds on a large scale through dehulling, grinding and degreasing at low temperatures of less than 45^0C. The degree of damage to the components of this material may be considered to be low because all steps were performed at low temperature.
550 type wheat flour used in the study was provided by Titan S.A. (Bucharest, Romania).

2.2. Preparation of wheat flour types enriched in bioactive compounds

We have manufactured 6 samples of wheat flour type 550, obtained by addition of different percentages of partially defatted hemp seed flour. The types of mixtures of flours used in this study is: P1 - 100 % wheat flour type 550; P2 - 95% wheat flour + 5% defatted hemp seed flour; P3 - 90% wheat flour + 10% defatted hemp seed flour; P4 - 85% wheat flour + 15% defatted hemp seed flour; P5 - 80% wheat flour + 20 defatted hemp seed flour; - P6 - 100% defatted hemp seed flour.

2.3. Chemical analysis

Moisture was determined at 103 ^{0}C (±2 ^{0}C) until constant weight (ICC Standard No. 110/1). The ash content was determined by incineration at 525 ± 25^0C (ICC No 104/1). Total fat was determined by extracting 10 g of sample with petroleum ether 40-65^0C, using a semi-automatic Soxhlet Foss Extraction System 2055 (Foss, Sweden); Total nitrogen (N) and crude protein content (N · 6.50, conversion factor) was estimated by the Macro Kjeldahl Method (Kjeltec System, FOSS, Sweden). Total fiber was measured using the enzymatic gravimetric method, Mes-Tris buffer, AOAC (1995) method 991.43. The determination was performed using Fibertec 1023 system (FOSS Sweden). Carbohydrate contents were calculated as the difference of 100 - (ash + protein + fat + moisture). Using ^{1}H-NMR spectral technique was determined the fatty acids composition, especially the concentrations of short-chain saturated fatty acids (C_4-C_8), di-unsaturated fatty acids, mono-unsaturated fatty acids and long-chain saturated fatty acids (>C8).
The mineral contents were determined with inductively coupled plasma-mass spectrometer equipment (ICP-MS; Perkin Elmer NexION 300Q). Total ash was determined by incineration at 550 °C in a muffle. Quantitation was performed using external standards (Merck, multi element standard solution) and all the standard curves were obtained at 6 different concentrations. Total mineral content was measured using their most abundant isotopes. The dried samples were digested in a mixture of concentrated HCl.
All experiments were performed in triplicate.

2.4. Statistical analysis

All the measurements were performed at least in triplicate. The values of different parameters were expressed as the mean ± standard deviation (s_r), to a confidence interval of 95%.

RESULTS AND DISCUSSIONS

The composition of wheat flour, partially defatted hemp seed flour, and mixtures of the two flours is shown in Table 1 from where it can be observed that P2 sample (95% wheat

flour+5% partially defatted hemp seed) contains more than 3 grams of dietary fiber per 100 g total, which allow the provision of nutritional term "source of fiber".

Table 1. Physicochemical characterization of samples

Composition % d.m.	Sample					
	P1	P2	P3	P4	P5	P6
Protein	12.9±0.24	13.8±0.25	14.7±0.25	15.61±0.21	16.56±0.20	31.26±0.21
Ash	0.55±0.01	0.89±0.02	1.27±0.02	1.63±0.03	1.987±0.04	7.84±0.06
Total Fat	1.03±0.07	1.51±0.06	2.03±0.07	2.59±0.07	3.11± 0.08	11.63±0.08
Carbohydrate	85.52±0.12	83.8±0.12	82±0.11	80.17±0.11	78.34±0.11	49.27±0.12
Dietary Fiber	1.9±0.12	4.01±0.35	6.2±0.38	8.39±0.56	10.59±0.78	45.87±0.98

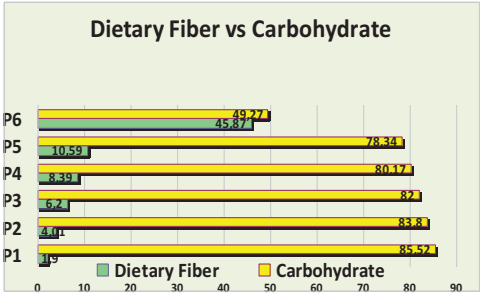

Fig. 1. Dietary fiber and carbohydrate content in mixtures flours

These data confirm that partially defatted hemp seed flour is a good source of bio-compounds, especially total fibers (45.87%, d.m). Partially defatted hemp seed should be considered a source of interesting added value carbohydrate compounds with potential known prebiotic properties, useful to formulate functional foods as well as nutraceuticals.

In the present study, the contents of four biologically essential mineral elements were analysed: calcium (Ca), potassium (K), magnesium (Mg) and sodium (Na), and two essential trace elements: copper (Cu) and zinc (Zn).

Table 2. Minerals contents of wheat flour and thereof mixtures

Sample	Minerals (mg/100g)					
	Ca	Mg	Na	K	Cu	Zn
P1	43.8±0.59	47.9±0.95	30.6±0.26	187.8±0.35	0.77±0.01	5.43±0.02
P2	76.13±0.96	76.13±0.98	31.31±0.26	243.41±0.35	0.83±0.01	5.5±0.02
P3	68.06±0.98	104.37±1.04	32.01±0.28	299.02±0.45	0.89±0.02	5.58±0.03
P4	80.19±1.01	122.52±1.09	32.72±0.29	354.63±0.45	0.94±0.01	5.65±0.03
P5	92.32±1.02	132.6±1.10	33.42±0.30	410.24±0.43	1±0.01	5.72±0.02
P6	286.42±1.19	612.58±1.14	44.7±0.32	1300±0.44	1.93±0.01	6.9±0.03

From performed analyses regarding minerals content it can be observed that partially defatted hemp seed is a material having important minerals content.

It is noticed that, compared to the low mineral content of wheat flour sample, (P1), mixtures of wheat flour and partially defatted hemp seed have a higher content of minerals as percentage increases flour mixtures.

In Fig. 2 is presented the fatty acids profile of samples. The addition of partially defatted hemp seed in mixtures flours modifies the lipid profile of samples compared to the control sample P1.

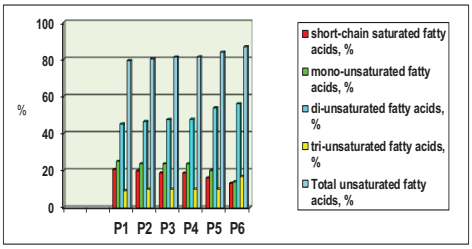

Fig. 2. *Fatty acids content in fresh sample*

In short, study of the food potential of hemp seed are not yet available in the scientific literature. Taking into accout that consumers are more and more aware about the food quality, especially from the nutritive point of view, the new food resources rich in bioactive compounds are necessary to be found. In this respect, hemp seeds meet the expectations of such consumers.

CONCLUSIONS

The aim of this study was to evaluate the functional potential of partially defatted hemp seed flour in order to be used for getting bakery products rich in valuable biocompounds.

Chemical composition of some four mixtures flours using both classical and spectral methods was analyzed.

The increasing the content of partially defatted hemp flour lead to the increasing of dietary fiber, minerals, protein, ash, and total fat content.

The chemical characterization performed in this study proved that the partially defatted hemp flour is a valuable source of nutritional components as well as a "source of fiber", according the Nutritional Claims approved by EFSA.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

REFERENCES

AOAC, 1995, *Official methods of analysis (14th ed.)*, Washington DC: Association of Official Analytical Chemists.

Callaway, J. C., 2002, Hemp as Food at High Latitudes, Journal of Industrial Hemp, 7(1).

Callaway, J. C., 2004, *Hempseed as a nutritional resource*, an overview. Euphytica, 140, 65–72.

Fitzpatric, K., 2007, Innovation in Western Canadian functional food ingredients", Cereal Food World, 52, 288-289.

Ivan. Svec, M. Hruskov, 2015, *The Mixolab parameters of composite wheat/hemp flour and their relation to quality features",* LWT - Food Science and Technology, 60, 623-629.

Soheila Rezapour-Firouzi, 2013, *Immunomodula-tory and therapeutic effects of Hot-nature diet and co-supplemented hemp seed, evening primrose oils intervention in multiple sclerosis patients*, Complementary Therapies in Medicine, 21, 473—480.

X.-S. Wang et al., 2008, *Characterization, amino acid composition and in vitro digestibility of hemp (Cannabis sativa L.) proteins*, Food Chemistry, 107, 11–18.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BACTERIA PRESENCE WITHIN THE DAMAGED CANNED MEAT PACKAGING AND SURFACE SAMPLES OBTAINED FROM BATH, UNITED KINGDOM MARKET

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Abstract

The study was carried to survey a total of 60 samples of canned meat (20 samples of canned pork, 20 samples of canned meat with sauce and 20 canned fish).The samples were obtained from local markets in Bath city and Wiltshire County within the United Kingdom, during three months January – March 2015. The samples were relocated to laboratory and kept at room condition (temperature and humidity). Thus, the scope of the present study was to conduct bacteriological measurements for the quality control of canned meat stored at 20°C for up to 15 days. In this paper, variations of the cans integrity are analysed to reveal the correlation between cans structure and the evolution of the spoilage organisms developing in food cans, semi-finished goods meat product and on fish packed meat. The study was focused on analysing the growth behaviour of the spoilage flora in the processed meat product, and on liquid medium rather than in fresh meat. Based on these analyses it was observed that all cans that are damaged deeply, more than cans that are superficial scratched, had a growth of microbiological spoilage. The bacteriological examinations of external surface showed negative results, so there no pathogenic microorganisms attached to the surface, but some presence of moulds was highlighted in this study.

Key words*: bacteriological measurement, spoilage organisms, canned meat, shelf life.*

INTRODUCTION

Canned meat and derivate products are considered as an alternative way to keep food away from microbiological and other contaminations, but there are some specific situations when it fails to be a good shelf preserver and the food matrix can be a good media to develop microorganisms.

Not all microorganisms are dangerous for human health, but most of them are developing toxins that are poisonous for ingestion.

The pasteurization and sterilization of cans transform meat, vegetables and fruits into safe food products, hermetically closed by the metallic tins. Though, some external forces can shift them into unsafe products. Even when the tins are perfectly closed, contamination may appear due to poor hygiene of the storage spaces and of the handling tools (Fraser, O. P., Sumar, S., 1998).

The meat acidity of the properly processed products can be altered by the low and very low modification of its metal tin structure and in consequence spoilage can appear if incubated at different temperatures. Such an example is the occurrence of *Escherichia*, which is one of the main food safety indicators.

For many people, the canned meat represents an important source of nutrients within the daily nourishment. Meat is an important food element, which keeps suffering chemical and biochemical alterations even after its complex processing and transformation into secondary food products (Le Loir, Y. et al., 2003). As it has a high content of water, meat represents a good environment for bacteria development (Krieg, N. R., 1984).

MATERIALS AND METHODS

The study was conducted to evaluate a total of 60 samples of canned meat (20 samples of canned pork, 20 samples of canned meat with sauce and 20 canned fish). The samples were collected from local market in Bath, Somerset, UK.

Diluted canned meat samples in standard saline was practiced onto these micro well substrates and incubated at 35 - 37°C for 18 - 24 hours, except detection of fungi, which were incubated at 25°C for 5 days. Staphylococcus segregates were exposed by microscopic,

cultural and habitual biochemical tests. Progressing dilutions up to sixty were prepared for the microbiological analysis.

One of the materials used was Microgen Bacillus-ID (MID-66), a miniaturised biochemical identification system designed to identify those Mesophilic *Bacillus* spp. and related genera associated with food and beverage spoilage and food poisoning. The method uses classical biochemical substrates modified in such a way that they can be employed for the identification of these organisms. The Microgen *Bacillus*-ID identification system consists of 2 microwell test strips (labelled BAC 1 and BAC 2), each containing 12 dehydrated substrates for the performance of either carbohydrate fermentation tests or other biochemical based tests. The last well in the second test strip is a carbohydrate fermentation control well for use as a reference well in the interpretation of these tests. The selection of the substrates included in the test panel has been determined using computer based analysis of all available substrates for the identification or differentiation of this group of organisms.

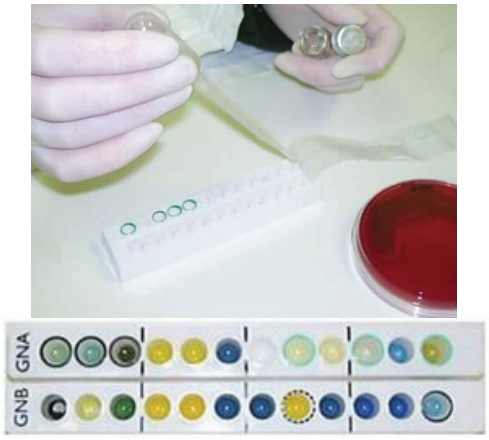

Figure 1. Microgen Bacillus-ID identification system

The second type of assessment was obtained using Microgen GNA Identification system for Gram Negative Bacilli consists of traditional or conventional biochemical substrates presented in a miniaturised format. When these substrates are inoculated with a suspension of test organism and the organism is able to metabolise the active components, the end products are detected either through a change in colour of a pH indicator or the development of a coloured end product after the addition of a supplementary reagent. The colours are scored as positive or negative and then translated into a digital Reaction Code which when input into the associated software produces an identification.

The Microgen GNA Identification system offers a number of features which are able to set it apart from other systems. The Microgen GNA-ID system is suitable for use in the identification of isolates from food or medical samples including urinary pathogens, faecal pathogens and common wound isolates. For canned meat applications and better results of current research due the multiple species of Enterobacteriaceae and an extensive range of oxidase-positive Gram Negative Bacilli that might be identified it was used the combination of the Microgen GNA + GNB identification panels i.e. 24 substrates. The microwell test strips are stable in the unopened foil pouches at 2 - 8°C.

It was selected a single colony of the isolate by the time to be identified. The colony was emulsified in 3ml saline without using of PBS and Distilled Water. The optimum density to MacFarland was 0.5. Final suspension reached the visibly turbid or cloudy appearance. The adhesive tape was peeled back without removing completely. The next run was adding 3 - 4 drops (100µL) of the suspension to each well of the strips. The quantity went be approximately 30 - 40% full. The next phase was overlaying appropriate of wells with mineral oil, the wells indicated by black highlight. After that 3 drops of mineral oil were used. Re-sealing of the inoculated strip with the adhesive tape and incubating 18 - 24 hours at 35 - 37°C.

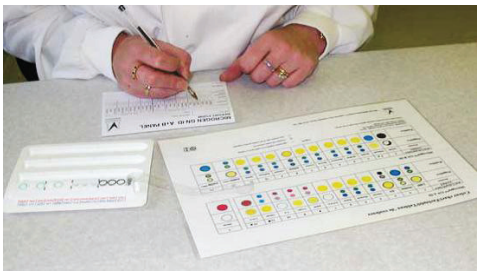

Figure 2. Microgen Bacillus-ID identification system – results monitoring sheets

The next phase was adding reagents Indole, read within a few seconds and VP1, VP 11 reagents were added in order to colour development of positive reactions that started within five minutes and the intensity increased over the next 15 – 20 minutes.

RESULTS AND DISCUSSIONS

Considerable types of microorganisms, whose action may have a relevant influence on the quality of canned meat, were monitored in samples of meat with sauce and fish tins during microbiological analysis. The surface of cans becomes contaminated during storing, in relation with the hygiene conditions.

A diminished presence of microorganism was found in pork canned meat and partially damaged tins for which their inner integrity was not affected. Storing tins in a temperature controlled environment influences the numbers of microorganisms.

The numbers of coliform bacteria were not present for the tins that were not damaged. The numbers of coliform bacteria in damaged cans, especially fish and meat with sauce, duplicated much more than safe undamaged tins.

The first values were low in both cases and their numbers increased only intensely slowly, particularly during long periods of storage.

No visible growth of mould was discovered on the samples of meat stored. Any spores present were in an inactive state.

Low culture amounts of moulds were discovered following cultivation during the storage period. The amplification of moulds was inhibited by competing microflora and for the hermetically cans an insufficiency of oxygen.

The existence of yeasts were also low at the beginning of storage, though their appearance moderately increased by storage time and conditions.

Coliform results

Coliform presence was confirmed in canned sauce meat and fish samples for 20 samples that were damaged (cracks and pin holes). It was found that fish tin contained significantly higher numbers of coliform compared to other tins samples.

However, no significant difference was found between the hermetically closed cans and the canned meat tins with insufficient damage. These results are also due to the aseptic techniques implied by the correct food processing.

Staphylococcus aureus

A total of 18 out of 60 canned meat samples were found positive in total *Staphylococcus aureus*. The mean staphylococcal counts of sauce meat sample and fish tins were significantly high ($P<0.05$) than other samples. The results of present research showed that *Staphylococcus aureus* were isolated from sauce meat and fish tins samples.

Salmonella and Shigella

The lack of Salmonella in the meat product samples indicate the quality of raw meat and that the process conditions and food hygiene include the quality of the raw materials, water and tools used in process.

Fungal

No fungus was detected in the sample of undamaged meat except sample 2 samples with leakage presence on the outside surface. Within this study, it was observed that in the initial condition of normal and undamaged cans, the fungal flora was not present in a high percentage which could harm human health after consumption. Moreover, for the damaged cans, other contaminants were detected in a small percentage.

Table 1.Test results for the main categories – Undamaged Cans

Undamaged Food Cans	Pork cans	Sauce meat cans	Fish cans
Coliform presence	-	-	-
Staphylococcus aureus	-	-	-
Salmonella Shigella	-	-	-
Fungal presence	-	+	-

The study revealed that the count of detailed bacteria in canned sauce meat was higher than acceptable values, making the product a potential public health hazard.

Table 2.Test results for the main categories – Damaged Cans

Damaged Food Cans	Pork cans	Sauce meat cans	Fish cans
Coliform presence	+	+	+
Staphylococcus aureus	+	+	+
Salmonella Shigella	+	+	+
Fungal presence	+	+	+

Commercially canned food is considered safe as it is processed under carefully controlled conditions (Waliullah, S., Ahsan C.R., 2011). If canned food shows signs of spoilage, like bulging can ends, leakage of spurting liquid, odd odour or mould, it should not be consumed by any way. Canned meat may contain toxin if it is not properly processed.

This study's purpose was to indicated the mean count of mould and yeast of canned beef and fish. It revealed that canned meat contains a lower presence of mould than un-canned meat. However, the processing environment and product handling and packaging may introduce microorganism, including pathogens, into packaged product (Smoot, L. M., Pierson, M. D., 1997).

CONCLUSIONS

Particularly more rapid development of contaminating microflora was found in canned meat with sauce and fish rather than pork stored under aerobic conditions. In the final phase of monitoring the numbers of microorganisms, i.e. on day 8 for canned fish meat, meat with sauce and on day 16 for pork tins, extremely similar numbers of all groups of microorganisms were found in all tins.

Restricting the supply of air and the protective action of the packaging material led to numbers of microorganisms growing considerably more slowly in hermetically closed meat tins – the meat continued to be of acceptable sensory quality even after 14 days of storage.

To meet the food safety requirements of canned meat is important to ensure contamination suppression within the manufacturing process and supply chain, rather than end-product testing. As canned meat was not massively contaminated with microorganisms, just the damages and alteration of hermetically issues are possible drives of food borne illnesses, hence conditions of hygiene on the market shelves and employees respect all conditions and regulation for food market.

REFERENCES

Arumugaswamy, R. K., et al. 1995, Prevalence of Salmonella in raw and cooked foods in Malaysia, Food Microbiology 12, 3-8

Ayulo, A. M. R. et al., 1994, Enterotoxigenic Escherichia coli and Staphylococcus aureus in fish and seafood from the southern region of Brazil, International Journal of Food Microbiology 24, 171-178

Fraser, O. P., Sumar, S., 1998, Compositional changes and spoilage in fish (part II) – microbiological induced deterioration, Nutrition & Food Science 98, 325–329.

Jarvis, B., 1989, Statistical aspects of the microbiological analysis of foods, Prog. Industrial Microbiol, 21-36.

King, R. D., 1980, The Determination of Food Colours, Developments in food analysis techniques, Applied Science Publishing, Essex UK, , 79-106

Krieg, N. R., 1984. Bergey's manual of systematic bacteriology, vol. 1, The Williams & Wilkins Co., Baltimore, 498.

Le Loir, Y. et al., 2003, Staphylococcus aureus and food poisoning, Genetics and Molecular Research 2, 63-76

Skinner, G. E., 1991, Status summary: Moffett food safety index, Food and Drug Administration, Summit-Argo, Ill.

Smoot, L. M., Pierson, M. D., 1997, Indicator microorganisms and microbiological criteria, Food microbiology fundamentals and frontiers, ASM Press, Washington DC, 66-75

Waliullah, S., C. R. Ahsan, 2011, Assessment of microbiological quality of some meat-based fast foods collected from street vendors., Journal of Innovation, 44-46

Internet, The Genus Streptococcus, An Overview, Microlab newsletter, accessed at 03.01.2015, Online at www.microgenbioproducts.com/pdf/Microlab%20Newsletters/MLAB_25.pdf

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

OBTAINING AND NUTRITIONAL CHARACTERISATION OF FUNCTIONAL BISCUITS WITH CEREAL GERMS AND *MOMORDICA CHARANTIA* EXTRACT

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Abstract

Because of the high content of active principles and better bioavailability of nutritional compounds, sprouted grain flours are used in functional and dietary foods composition. Using white wheat flour, germinated wheat flour in different percent and Momordica charantia extract (with hypoglycemic effect), we obtained a dietary product, the MOMGERMBISC biscuits, which was characterized in terms of nutritive and energetic contribution. Proximate composition (humidity, protein content, fat content, ash, alkalinity) was determined according SR ISO methods. Also, it was determined the carbohydrate content and energy value of biscuits. The product is distinguished by a protein and lipid content, comparable with simple biscuits, but with an increased intake of minerals. The energy value of the biscuits with the addition of germinated wheat flour falls within the range between 449.1 and 453.44 kcal. The carbohydrate content of the biscuits with the addition of M.charantia and sprouted wheat is between 67.1 and 70.61%. The content of carbohydrates and energetic value is reduced, the product being recommended in hypoglycemic diet.

Key words: *germinated cereals, Momordica charantia extract, biscuits.*

INTRODUCTION

Current trends in nutrition are oriented to functional foods consumption with an important biological role in all metabolic processes of the body. In health insurance, functional foods promotes body growth and development, optimize the metabolic processes, physiological activity of organs, immune system, cognitive performance and the defense against oxidative stress (Hurgoiu, 2004). Also called protection food, functional food can be used for a long time without causing secondary negative reactions, having cytoprotective and fortifying effects, increasing the body's natural immunity (Dawidziak et al., 2014). Cereals represent the first vegetable matrices used as functional foods. It is known the role of dietary fiber from cereals in ensuring a healthy diet (Alexa, 2009). Germination as a process of grain processing, leading to a significant increase in the content of bioactive compounds (vitamins, bioelements, enzymes) of the product, transforming it from food in functional product (Alexa et al., 2009). Germinated cereal flours have a higher bioavailability of nutritive compounds and in combination with medicinal herbs extracts permit the production of functional foods. Germinated wheat is the ideal food for a perfectly healthy diet. Given the large number of nutrients that are found in wheat germ, they bring a multitude of benefits to our health, being an important source of protein, unsaturated fats, fiber, vitamins A, B_1, B_2, B_6, D, E and K, iron, phosphorus, magnesium and zinc. Germinated wheat are recommended in treating anemia, but also helps to strengthen the immune system, protects the muscles, circulatory system, lungs and improves visual acuity. Being very rich in antioxidants, they protect our cells against free radical damage. Treatment with wheat germ is indicated for strengthening immunity being recommended for older people and children. The chemical composition of germinated cereals and their positive effect on organism have been reported in previous studies. (Donkor et al., 2012; Hidalgo et al., 2013; Bennett et al., 2013; Marton, 2010; Pandhre, 2011). Also, the positive effect of plant extracts

in alimentation was previously investigated. The seeds obtained from the fruit *Momordica charantia,* called bitter cucumber or vegetable insulin, have many health benefits. Juice of *Momordica charantia* is indicated for the relief of type 2 diabetes, metabolic syndrome and dyslipidemia (Sarandan et al., 2010). It also strengthens the immune system, especially in adolescents, people with advanced age, smoking, or who suffer from alcoholism. The aim of the research was to obtain a dietary product from white wheat flour, wheat flour germinated in different percentage and *Momordica charantia* extract (with hypoglycemic effect), nutritional and energetic characterization of the product for patients with type 2 diabetes.

MATERIALS AND METHODS

Fabrication of biscuits. The raw material used to obtain MOMGERMBISC is represented by white wheat flour, sprouted wheat flour, *Momordica charantia* extract, butter and sugar. The producing recipe is presented in Table 1. Technological stages of the product are: dosage of auxiliary raw materials according to the producing recipe, kneading of the dough, modeling of the dough into characteristic forms, baking at 180^0C, for 20 minutes, cooling and storage. (Alexa, 2004; Alexa, 2010).

Table 1. Production recipes

Biscuits with added	MOMGERMBISC I	MOMGERMBISC II	MOMGERMBISC III
Wheat flour	500 g	500 g	500 g
Butter	250 g	250 g	250 g
Germinated wheat flour	40 g	65 g	90 g
Momordica extract	10 g	10g	10 g
Egg yolk	16 g	16 g	16 g
Honey	50 g	50 g	50 g
Baking powder	1 g	1 g	1 g

Chemical and nutritional characterization. Proximate composition was determined according SR ISO methods: humidity SR ISO 6496/2001, protein content SR EN ISO 5983-1/2006/AC: 2009, fat content SR ISO 6492/2001, ash SR ISO 5984. Determination of biscuits alkalinity was done according SR ISO 1227/3-90. All analyses are performed in triplicate and the reported value represents the average. The carbohydrate content was determined by difference.

Energy value, respectively caloric intake was calculated by summing of caloric intake given by fats, carbohydrates and proteins, considering the following: 1 g fat = 9 kcal, 1 g protein = 4 kcal, 1 g carbohydrate = 4 kcal.

Statistical data analysis. Data processing was performed by analysis of variance and *t*-test (Ciulca, 2006).

RESULTS AND DISCUSSIONS

Figure 1 presents images with obtained biscuits. Figure 2 shows the proximate composition (moisture, fat, protein, ash) and in figure 3 carbohydrates content and energetic value of MOMORGERM biscuits are presented.

Figure 1. *Biscuits with wheat germ and Momordica charantia extract*

By comparing the obtained humidity quality with standards of quality for biscuits is observed that they fall within the limits of admissibility for all samples, so humidity is less than 9% (Figure 2).

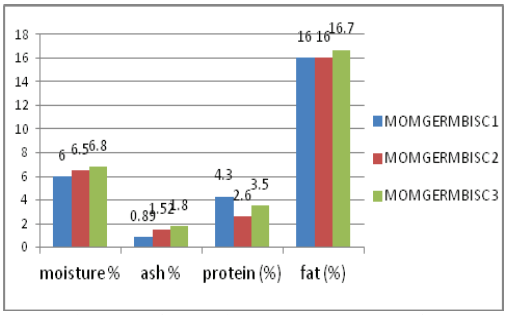


Figure 2. The proximate composition of the MOMGERMBISC biscuits

The ash content of the biscuits with the addition of *M.charantia* and germinated wheat is superior to ordinary gluten biscuits (0.22%) or sugar biscuits (0.42%) reported in literature, contributing to the high nutritional value of the

finished product. Mineral food supplement is due to the addition of bitter cucumber (*M.charantia*) representing a matrix rich in micro- and macroelements. Maximum mineral content is at the addition of 20% mixture. Proteic content of biscuits with germinated wheat and *M.charantia* added is low, but it is within the limits of admissibility reported for gluten and sugar biscuits (Figure 2). The fat content of simple biscuits varies in restricted limits (16 to 16.7%). Lipid contribution is given by animal fat (butter) introduced in the formulation which, used vegetable matrices having a low fat (Figure 2). The product obtained has high lipid content and its use is not recommended in the diet of the population with metabolic disorders, cardiovascular or indications for a hypolipidic diet.

The carbohydrate content of the biscuits with the addition of *M.charantia* and sprouted wheat (Figure 3) is between 67.1 and 70.61%. The addition of *M. charantia* leads to a reduction in carbohydrate content, which recommends these products for use in hypoglucidic diet.

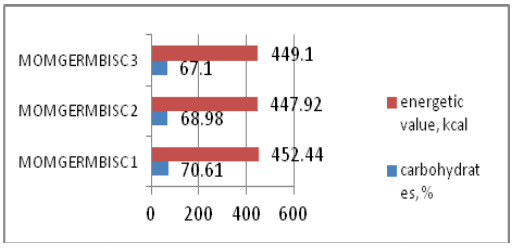


Figure 3. The carbohydrates and energetic value content of MOMGERMBISC biscuits

From the results of the Table 1 it is observed that MOMGERMBISC 1 biscuits associate highest values for protein and carbohydrate content and MOMGERMBISC 3 associates moisture, minerals and fat content. MOMGERMBISC 2 shows the mean values except protein content which is the lowest of all three types of biscuits analyzed. Also, the fat content has the same value as biscuits MOMGERMBISC 1.

The energy value of the biscuits with the addition of germinated wheat flour falls within the range between 449.1 and 453.44 kcal and is comparable with values reported in the literature (Alexa, 2010).

The biscuit assortments alkalinity value obtained is less than 3%, falling within the admissibility standards.

The content of mineral substances and lipids had the highest values at the MOMGERMBISC 3 biscuits type, thus demonstrating the supplementary mineral contribution by the addition of wheat germ flour to wheat flour.

High fat content due to the addition of wheat germ flour, make a significant contribution of unsaturated fatty acids, increasing the nutritional value of the product. Protein content presented maximum values at the MOMGERMBISC 1 biscuits.

Statistical analysis of the values obtained by measurements shows that the humidity, minerals and fat content of MOMGERMBISC 3 product were significantly superior to other types of biscuits analyzed. Product MOMGERMBISC 1 showed a significantly higher value compared to other varieties in terms of protein content (Table 2).

The assortment of biscuits MOMGERMBISC 3 presented superior to average about significant values on humidity, minerals and fats and MOMGERMBISC 1 recorded significantly lower than the mean values of these attributes, but the value was significantly superior to average about protein content. MOMGERMBISC 2 shows a significant value, lower than the average in protein content. With turning to the energy, alkalinity and carbohydrate content of varieties of biscuits (Table 3), the highest values for all these qualities stand out from the assortment of biscuits MOMGERMBISC 1. It is found that the values obtained for these three qualities vary inversely with content of germinated wheat flour added to wheat flour. The lowest values were obtained from cookies MOMGERMBISC 3. For a hypoglucidic diet is recommended MOMGERMBISC 3.

The low energy value (449.10 kcal) of MOMGERMBISC 3 also determines ownership of this product diet and increased acidity in comparison with the other two types of biscuits, due to higher amounts of enzymes, which gives superior nutritional value.

Table 2. Humidity and contents of minerals, proteins and lipids of the biscuits varieties

Assortment biscuits	Humidity (%)	Minerals (%)	Proteins (%)	Lipids (%)
MOMGERMBISC 1	6,00b	0,89b	**4,30a**	16,00b
MOMGERMBISC 2	6,50ab	1,52ab	2,50b	16,00b
MOMGERMBISC 3	**6,80a**	**1,80a**	3,50ab	**16,70a**
Average	**6,43±0,23**	**1,40±0,27**	**3,43±0,52**	**16,23±0,23**
DL5%	0,64	0,73	1,42	0,64
DL1%	1,00	1,15	2,23	1,00
DL0,1%	1,70	1,96	3,79	1,70

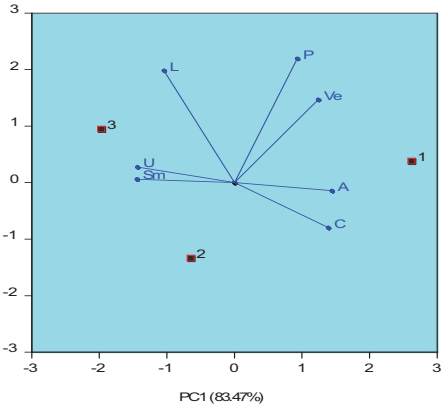
There are considered significant differences between genotypes denoted by different letters

Table 3. The energy value, alkalinity and carbohydrate content for varieties of biscuits

Assortment biscuits	Energy value (kcal)	Alkalinity	Carbohydrates (%)
MOMGERMBISC 1	**452,44a**	**2,70a**	**70,61a**
MOMGERMBISC 2	447,92a	2,50ab	68,98a
MOMGERMBISC 3	449,10a	2,40b	67,10a
Average	449,82±1,35	2,53±0,09	68,90±1,01
DL5%	4,92	7,72	13,15
DL1%	0,24	0,38	0,64
DL0,1%	3,69	5,78	9,85

There are considered significant differences between genotypes denoted by different letters

Following statistical analysis it was found that only in terms of alkalinity was no significant difference. The biscuits were worth one MOMGERMBISC significantly superior to those found in other varieties and the average. MOMGERMBISC 3 showed a significantly lower value compared to other varieties and the average.



U-Humidity; Sm-Minerals; P-Proteins; L-Lipids; Ve-Energetic value; A-Alkalinity; C-Carbohydrates.

Figure 4. Biplot for biochemical features of different biscuits types

Biplot representing the biochemical characterristics of varieties of biscuits (Figure 8) shows that MOMGERMBISC 1 associates the values significantly higher of carbohydrate content, acidity, energy and protein compared to the average. MOMGERMBISC 3 relates signifycantly superior values of fat content, minerals and moisture.

CONCLUSIONS

Biscuits with added germinated wheat flour and *Momordica charantia* extract represents a functional product with hypoglycemic value and additional contribution of macro- and microelements, which contributes to improving the health of the population segment that uses this type of product.

Momordica charantia addition ensure faster and easier metabolizing of carbohydrates (due to hypoglycemic active principles), which recommends the product in the diet of patients with type 2 diabetes (noninsulinodependent).

The protein content is low, but the lipid content is high, so that the product is not recommended in the diet of patients with metabolic diseases, cardiovascular or indications for hypolipidic diet.

REFERENCES

Alexa, E. (2004). *Tehnologia alimentelor făinoase*, Ed. Eurobit;

Alexa, E. (2009). Dietetic flouring supplies for children nourishment, *Journal of Agroalimentary Processes and Technologies*, Timişoara, XIII(1), 163-168;

Alexa, E. (2010). *Alimente făinoase dietetice. Materii prime, caracterizare, tehnologii de obţinere*, Ed. Eurobit, Timişoara;

Alexa, E., Voica, V.D., Stoin, D., Pop, G., Rosu, I., Negrea, M. (2009). Reserches concerning the physical-chemical and rheological properties of ecological flours, *Analele Universitatii din Oradea - Fascicula Chimie XVI*, ISSN 1224-7626, 19-24;

Bennett, R., De Vasconcelos, M.C.B.M., Castro, C., Cardoso, P., Saavedra, M.J., Rosa, E.A. (2013). Study of composition, stabilization and processing of wheat germ and maize industrial by-products, *Industrial Crops and Products,* 42, 292–298;

Ciulca, S., (2006). Metodologii de experimentare în agricultură şi biologie, Timişoara, România: Agroprint;

Dawidziak, M., Piasecka, Kwiatkowska, D., Warchalewski, J.R., Makowska, A., Gawlak, M., Nawrot, J. (2014). Sprouted wheat grain with ferritin overexpression as a potential source of iron for cereal product fortification, *Eur. Food Res. Technol.,* 238, 829-835;

Donkor O.N., L. Stojanovska, P. Ginn, J. Ashton, T. Vasiljevic, (2012). Germinated grains – Sources of bioactive compounds, Food Chemistry, Volume 135, Issue 3, 1 December, pp. 950-959;

Hidalgo, A., Brusco, M., Plizzari, L., Brandolini, A. (2013). Polyphenol oxidase, alpha-amylase and beta-amylase activities of Triticum monococcum, Triticum turgidum and Triticum aestivum: A two-year study, *Journal of Cereal Science*, 58(1), 51–58;

Hurgoiu, V. (2004). *Alimente funcţionale*, Casa Cărţii de ştiinţă, Cluj –Napoca;

Marton, M., Mandoki, Z., Csapo, K.Z. (2010). The role of sprouts in human nutrition. *Alimentaria*, 3, 81-117;

Pandhre, G.R., Satwase, A.N., Syed Imran, H. (2011). Studies on drying characteristics and nutritional composition of sprouted wheat and finger millets, *International Journal of Current Research,* 3(7), 218-221;

Sarandan, H., Botau, D., Ianculov, I., Radu, F., Rada, O., Morar, D., Sărăndan, M., Serb, M., Anghel, A. (2010). The hypoglicemic effect of *Momordica charantia* Linn in normal and alloxan-induced diabetic rabbits, *Anim. Sci. Biotechnol.*, 43, 516–518.

*** SR ISO 6496/2001 Determination of humidity from cereals.

*** SR EN ISO 5983-1/2006/AC: 2009 Determination of protein content from cereals.

*** SR ISO 6492/2001 Determination of fat content.

*** SR ISO 5984/2001 Determination of ash.

*** SR ISO 1227/3-90 Determination of biscuits alkalinity.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PHENOLIC PROFILE, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF *PELARGONIUM GRAVEOLENS* LEAVES' EXTRACTS

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Abstract

Pelargonium graveolens, commonly known as rose geranium, is an aromatic and medicinal plant belonging to the Geraniacea family. The aim of the present study was to evaluate the in vitro antioxidant and antibacterial activity of Pelargonium graveolens, as well as to determine the total phenolic content of the studied extracts. Four spectrophotometric assays were used for the radical scavenging ability analysis, namely ABTS, DPPH, CUPRAC, and FRAP. The inhibitory activity of the leaves' extracts was tested against S. aureus, L. monocytogenes, E. coli and Salmonella.
The total phenolics ranged from 1.65 to 8.23 mg GAE/g FW. Antibacterial activity, whose zone of inhibition varied from 15 mm to 19 mm depending on the extract quantity, was shown only against L. monocytogenes.

Key words*: Pelargonium graveolens, antioxidants, antimicrobial activity, phenolics*.

INTRODUCTION

Antioxidants are compounds that can delay or prevent the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu et al., 1998). There are two basic categories of antioxidants, namely, synthetic and natural. Natural antioxidants are more readily acceptable than synthetic ones. They inhibit the oxidative damage of food products and may prevent inflammatory conditions and neurodegenerative disease (Mahdieh et al., 2013). The antioxidant activity is a fundamental property important for life. Many of the biological functions, such as antimutagenicity, anticarcinogenicity, and antiaging, among others, originate from this property (Cook and Samman, 1996; Huang et al., 1992).
Plants are potential sources of natural antioxidants, and certain species are particularly significant because they may be used for the production of raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (Exarchou et al., 2002). The antioxidative effect is mainly due to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (Shahidi et al., 1992; Chung et al., 1998; Pietta, 2000). Flavonoids and other polyphenolic compounds are a much investigated group of antioxidants in plants exerting bio-protective effects and having strongly positive influence on human health. Many studies have reported that phenolic compounds in spices and herbs significantly contributed to their antioxidant and pharmaceutical properties (Cai et al., 2004; Shan et al., 2005; Wu et al., 2006). Their effect in reducing many chronic, cardiovascular and carcinogenic diseases is remarkable (Slezák et al., 2007). Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al., 2004).
The demand of producing high-quality, safe (pathogen-free) food relies increasingly on natural sources of antimicrobials to inhibit food-spoilage organisms and food-borne pathogens and toxins. The discovery and development of new antimicrobials from natural sources for a wide range of applications requires that knowledge of traditional sources

for food antimicrobials is combined with the latest technologies in identification, characterization and application.

Some plants have been added to food since ancient times, not only as flavoring agents, but also as folk medicine and food preservatives (Beuchat, 1994; Nakatani, 1994; Cutler, 1995). Spices and herbs are well known for their antimicrobial and antioxidant properties and have the ability to produce multidimensional flavors in food (Uhl, 2000).

Rose geranium is a species which belongs to the *Pelargonium genus, Geraniaceae* family. It has a woody, straight stem with branches; its leaves are usually alternate, palmately lobed or pinnate, often on long stalks, and sometimes with light or dark pattern; covered with short, rough hairs, which give the plant a strong, pleasant rose-like scent (Balchin et al., 1995).

It had been brought in Europe from South Africa in the beginning of the eighteenth century (Miller, 2002). At present days, it grows in different parts of the world and is cultivated, mostly for its repellent activity against mosquitoes (Pohlit et al., 2011). It is also widely used in cosmetic industry and as flavoring for foods (Lis-balchin, 2006). Rose-scented geranium (*P. graveolens* L'Hér.) is also widely known as one of the medicinal herbs with the highest antioxidant activity (Newman et al., 2007). In herbal medicine its leaves are used for the treatment of gastrointestinal diseases, throat infections, and bleeding (Saraswathi et al., 2011).

According to the data on chemical composition the dominant volatiles of the *P. graveolens* essential oil were citronellol, geraniol and citronellyl formate (Verma et al., 2010; Ghannadi et al., 2012).

Through several studies it was shown that extracts of *Pelargonium graveolens* possess antibacterial and antifungal activity (Baratta et al., 1998; Dorman and Deans, 2000). The antimicrobial and antimalarial activity of *P. graveolens* extracts was also studied by Lalli (2006). In addition to that, antioxidant and antitermitic activity of *P. graveolens* has also been reported (Zheng and Wang, 2001; Fayed, 2009; Seo et al., 2009; Čavar and Maksimović, 2012).

The objective of the present work is to provide much needed information concerning the antioxidant and antimicrobial activity of extracts of Bulgarian *Pelargonium graveolens* which have not yet been extensively studied and evaluated.

MATERIALS AND METHODS

Extract preparation

Fresh plant material of *Pelargonium graveolens* was subjected of three different types of extractions:

- *decoction* – extraction by boiling of the plant material for 30min with distilled water;
- *infusion* - extraction by boiling water and then pouring it over the herb, which is then allowed to steep in the liquid for 30 min
- *heat reflux extraction* - alcoholic extraction (70 % ethanol as solvent) for 30min;

The resulting extracts solutions were filtered before analyzed.

Determination of total phenolics

A modified Kujala et al. (2010) method with Folin - Ciocalteu's reagent was used for the determination of the total polyphenolic content (TPC). Gallic acid was employed as a calibretion standard and the results were expressed as mg gallic acid equivalents (mg GAE) per gram of plant fresh weight.

Determination of antioxidant activity

DPPH radical scavenging activity

The ability of the extracts to donate an electron and scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams, Cuvelier, and Berset (1995). Freshly prepared $4x10^{-4}$ M methanolic solution of DPPH was mixed with the samples and a standard solution in a ratio of 2:0.5 (v/v). The light absorption was measured at 515 nm and the percentage of inhibition of $DPPH^{\bullet}$ by the obtained extracts was calculated for each sample using the following formula:

$$\% \text{ Inhibition} = [(A_B - A_E)/A_B] \times 100$$

Where: A_B = absorbance of the control without sample; A_E = absorbance of the test sample with $DPPH^{\bullet}$

The DPPH radical scavenging activity was presented as a function of the concentration of Trolox. The unit of Trolox equivalent antioxidant capacity (TEAC) was defined by the concentration of Trolox having equivalent antioxidant activity expressed as μM TE/g FW.

ABTS radical scavenging assay

The radicals scavenging activity of the extracts against radical caption ($ABTS^{\bullet+}$) was estimated according to Re et al. (1999) with some modifications. $ABTS^{\bullet+}$ was produced by reacting 7 mM of ABTS solution with 2.45 mM potassium persulphate, and the mixture was kept in the dark at room temperature (20 - 22°C) for 12-16 h. At the moment of use, the ABTS solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30°C. Each sample (0.01 ml) was added to 1 ml of ABTS diluted (working) solution and mixed vigorously. After reaction at 30°C for 6 min, the absorbance at 734 nm was measured. The percentage of inhibition of $ABTS^{\bullet+}$ by the obtained extracts was calculated for each sample using the following formula:

$$\% \text{ Inhibition} = [(AB-AE)/AB] \times 100,$$

Where: A_B = absorbance of the control without sample; A_E = absorbance of the test sample with $ABTS^{\bullet+}$.

The TEAC value was defined as the concentration of Trolox having equivalent antioxidant activity expressed as µM TE per gram fresh weight (µM TE/g FW).

CUPRAC assay

The CUPRAC assay was carried out according to the procedure of Apak et al., 2008. To a test tube were added 1 mL of $CuCl_2$ solution (1.0×10^{-2}M), 1 mL of neocuproine methanolic solution (7.5×10^{-3}M), and 1 mL NH_4Ac buffer solution (pH 7.0), and mixed; 0.1 mL of herbal extract (sample) followed by 1 mL of water were added (total volume = 4.1 mL), and mixed well. Absorbance against a blank reagent was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of extracts was expressed as µM TE/g FW.

FRAP assay

The FRAP assay was carried out according to the procedure of Benzie & Strain (1996) with slight modification. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe (II)-tripyridyltriazine compound from colourless oxidized Fe (III) form by the action of electron donating antioxidants. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37°C in a water bath prior to use. One hundred and fifty microliters of plant extracts were allowed to react with 2850 µl of the FRAP reagent solution for 4 min at 37°C. The absorbance of the reaction mixture was recorded at 593 nm. The results were expressed as µM TE/g FW.

Antimicrobial analysis

Antibacterial activity was tested against Gram-positive bacteria - *Listeria monocytogenes* NCTC 11994 and *Staphylococcus aureus* ATCC 25093, and Gram-negative bacteria – *Escherichia coli* ATCC 8739 and *Salmonella enterica subsp. Enterica serovar Abony* NCTC 6017. The selective growth media, used in the analyses respecticely were: Listeria Oxford Agar Base with cicloheximide supplement /Biolife/; ENDO agar /Merck/; LEIFSON Agar /Merck/; Baird Parker Agar Base with Egg Yolk Tellurite emulsion supplement /Biolife/.

The media were inoculated with 24-hour suspension of the corresponding bacterial species.

For the microbial analyses a crude extract of rose geranium leaves was obtained under aseptic surroundings by mashing fresh leaves after preliminary washing under tap water, sterilized distilled water and soaking for 5 min with ethanol and exposing under the influence of ultraviolet illumination for 20 min.

Antimicrobial assay by agar diffusion method

The agar diffusion test was used to determine the antibacterial activity of crude extract of *P. graveolens*. Melted and cooled to the temperature at about 45°C selective media were inoculated with the tested microorganisms and after setting of media, small amount of crude extract, respectively 0.05; 0.10 and 0.15 cm^3, was placed into sterile metal rings (Ø 6 mm). Plates were incubated at 37°C for required incubation periods (24h or 48h) according to the strain type and then the distinct zone of growth inhibition around the rings was measured.

Statistical analysis

All measurements were carried out triplicates. The results were expressed as mean ± SD and statistically analysed using MS-Excel software.

RESULTS AND DISCUSSIONS

Since there have been numerous studies suggesting a direct correlation between phenolic substances and antioxidant activity, the estimation of the total phenolics is very important. The phenolic content could be an indicator of the antioxidant capacity of the studied extracts. The total content of phenolics was determined using the FC reagent, which is sensitive to many classes of phenolic compounds. The results are given on Fig. 1.

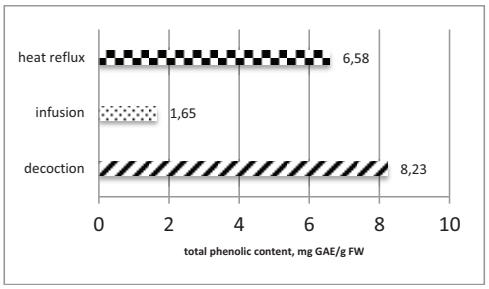

Figure 1.Total phenolic content of extracts of fresh *R. geranium* leaves, mg GAE/g FW

The decoction extract contained 1.2 times more phenolic substances than the heat reflux extract, while the infusion led to the lowest phenolic values. Water was also proven to be more suitable in other herb extracts (Alexieva et al., 2014). Bichra et al. (2013) reported significantly lower TPC values (0.12 mg/g EGA) from an aqueous extract.

Measuring the antioxidant activity of food products such as natural compounds began to present a great interest in recent years. There are several methods to determine the antioxidant capacity of plant extracts. However, the chemical complexity of extracts could lead to scattered results obtained from different techniques, depending on the test employed. Therefore, an approach with multiple assays in the screening work is highly advisable.

Antioxidant activity was measured by four different assays DPPH, ABTS, FRAP and CUPRAC using Trolox equivalents to express the results. The systems DPPH and ABTS are excellent tools for determining the antioxidant activity of hydrogen donating and chain breaking antioxidants (Thaipong et al., 2006). The FRAP and CUPRAC methods are based on the measurement of the ferric and cupric reducing ability. Both methods are based on electron transfer and are considered to be a good indicator for total antioxidant power because total reducing power is the sum of the reducing powers of individual compounds presented in a sample (Tezcan et al.,2011).

The antioxidant activity of the tested samples is given in Table 1.

Table 1. *In vitro* antioxidant activity (μM TE/g FW) of *P. graveolens* extracts

Method/ Plant sample	Decoction	Infusion	Heat reflux
$TEAC_{ABTS}$	198.91 ± 10.71	23.15 ± 0.59	223.76 ± 1.26
$TEAC_{DPPH}$	120.26 ± 1.37	36.81 ± 0.46	121.26 ± 1.10
$TEAC_{FRAP}$	218.16 ± 0.71	55.77 ± 0.46	231.64 ± 3.57
$TEAC_{CUPRAC}$	122.96 ± 7.67	166.74 ± 7.82	176.98 ± 0.01

The ABTS scavenging capacity ranged from 23.15 (infusion) to 223.76 (heat reflux) μM TE/g FW. The highest DPPH values were found to be 121.26 μM TE/g FW. Čavar and Maksimović (2012) published the radical scavenging activity of extracts and essential oils of *P. graveolens*. They measured the DPPH antioxidant capacity and reported values of 63.70 mg/ml for the leaves. In another study the antiradical activity of the geraniol oil was found to be ranging from 14.49 mg/ml to 66.45 μg/ml EC_{50} value (Fayed, 2009). Džamić et al. (2014) reported that the oil exhibited antioxidant activity and reduced DPPH to 50% at EC_{50} value of 0.802 mg/ml of oil solution.

Significant FRAP activity was evident in the heat reflux extract of *P. graveolens* leaves. In accordance with the FRAP, ABTS and DPPH assays, the highest values in the CUPRAC assay were also found in the heat reflux extract. Ethanol appeared to be a better extractant as far as antioxidant activity is being measured. This is in disagreement with the TPC values, where water was the most suitable medium, and is probably due to the different mechanism of contribution of each individual component to the total radical scavenging activity of the studied samples.

Many herbs and plants are known to have therapeutic and antimicrobial properties, and their biological activity is currently the subject of renewed interest (Okigbo et al., 2008).

However, only few of them have been characterized for their antibacterial activities (Halcon and Milkus, 2004). The antimicrobial activity of the crude extract of *Pelargonium graveolens* leaves were evaluated against four bacteria species. The results of the antibacterial activity are presented in Table 2.

Table 2. Zones of growth inhibition (mm) of tested pathogenic bacteria by crude extract of *Pelargonium graveolens* leaves

Plant sample / Bacteria	*P. graveolens*		
	0.05cm3	0.10cm3	0.15cm3
St. aureus	0,6	0,6	0,6
L. monocytogenes	1,5	1,7	1,9
E. coli	0,6	0,6	0,6
S. enterica	0,6	0,6	0,6

The tested extract possessed antibacterial activity against Gram (+) bacteria - *Listeria monocytogenes* NCTC 11994. On the basis of inhibition zone diameters, *Listeria monocytogenes* NCTC 11994 was more sensitive to the extract than the other bacterial species. Recent research on *P. graveolens* oil revealed that it manifests a strong inhibitory effect on Gram-positive bacterial the extract than the other bacterial species. Recent research on *P. graveolens* oil revealed that it manifests a strong inhibitory effect on Gram-positive bacterial strains such as *Staphylococcus aureus* (Silva and Fernandes, 2010).

CONCLUSIONS

The outcomes of the current investigation prompt the necessity for further studies of the *P. graveolens*, focusing on the isolation and structure elucidation of its antioxidant compounds, since they have potential use as therapeutic agents in managing diseases associated with free radicals and also have the potential to be employed as additives in the food industry.

REFERENCES

Alexieva J., Mihaylova D, Popova A., 2013, Evaluation of the antioxidant capacity of aqueous extracts of fresh Chrysanthemum balsamita L. leaves growing in Bulgaria, University of Ruse Procedeeings, 52 (10.2), 89-91

Apak R., Guclu K., Ozyurek M., Karademir S.E., 2004, Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method, JAgricFoodChem, 52, 7970–7981

Balchin-Lis M., Hart S.L., Deans S.G., Eaglesham E., 1995, Potential agrochemical and medicinal usage of essential oils of Pelargonium species, J Herbs Spices Med Plants, 3:11-22

Baratta M.T., Dorman H.J., Deans S.G., Figueiredo A.C., Barroso J.G., Ruberto G., 1998, Antimicrobial and antioxidant properties of some commercial essential oils, FlavFragJ, 13:235-244

Benzie I.F.F., Strain J.J., 1996, The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. Analytical Biochemistry, 239, p. 70-76, PMid: 8660627.Biol. Med., 23(2): 302-313.

Beuchat, L.R., 1994, Antimicrobial properties of spices and their essential oils, Dillon, Y.M., Board, R.G. (Eds.), Natural Antimicrobial Systems and Food Preservation, CAB International, Oxon, 167–179

Bichra M., Cherkaoui M., Abdelilah A., Hafida B., Fatiha B., 2013, Antioxidant activities and phenolic profile of six Moroccan selected herbs, JMBFS, 2(4)2320-2338

Brand-Williams W., Cuvelier M.E., Berset C., 1995, Use of a free radical method to evaluate antioxidant activity, FoodSciTechnol, 28, 25-30

Cai Y.Z., Luo Q., Sun M., Corke H., 2004, Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer, LifeSci., 74, 2157–2184

Čavar S., Maksimović M., 2012, Antioxidant activity of essential oil and aqueous extract of Pelargonium graveolens L'Her., Food Control, 23:263-267

Chung K.T., Wong T.Y., Huang Y.W., Lin Y., 1998, Tannins and human health: A review, Crit. ReV.Food Sci.Nutr., 38, 421-464

Cook N.C., Samman S., 1996, Flavonoids-Chemistry, metabolism, cardioprotective effects, and dietary sources, Nutr. Biochem., 7, 66-76

Cutler H.G., 1995, Natural product flavor compounds as potential antimicrobials, insecticides, and medicinals, Agro-Food-Industry Hi-Tech, 6, 19–23

Dorman H.J.D., Deans S.G., 2000, Antimicrobial agents from plants, antibacterial activity of plants volatile oils, JApMicrobiol., 88: 308-316

Džamić A.M., Soković M.D., Ristić M.S., Grujić S.M., Mileski K.S., Marin P.D., 2014, Chemical composition, antifungal and antioxidant activity of Pelargonium graveolens essential oil, J App Pharm Sci, 4(03):001-005

Exarchou V., Nenadis N., Tsimidou M., Gerothanassis I.P., Troganis A., Boskou D., 2002, Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory, J. Agric. Food Chem., 50, 5294-5299

Fayed S.A., 2009, Antioxidant and anticancer activities of Citrus reticulatae (Petitgrain mandarin) and Pelargonium graveolens (Geranium) essential oil, ResJ.Agric.Biol. Sci., 5:740-747

Ghannadi A., Bagherinejad M.R., Abedi D., Jalali M., Absalan B., Sadeghi N., 2012, Antibacterial activity and composition of oils from Pelargonium graveolens

L'Her and Vitex agnus-castus L., IranianJMicrobiol, 4:171–176
Halcon L., Milkus K., 2004, Staphylococcus aureus and wounds: a review of tea tree oil as a promising antimicrobial, Am.J.Infect.Control, 32:402-408
Hara-Kudo Y., Kobayashi A., Sugita-Konishi Y., Kondo K., 2004, Antibacterial activity of plants used in cooking for aroma and taste, JFoodProtection, 67, 2820–2824
Huang M.-T., Ho C-T., Lee C.Y., 1992, Phenolic compounds in food and their effects on health. II. Antioxidants and cancer prevention, ACS 507, American Chemical Society: Washington, DC
Kujala T.S., Loponen J.M., Klika K.D., Pihlaja K., 2000, Phenolics and betacyanins in red beetroot (Beta vulgaris) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds, J.Agric.FoodChem., 48, 5338-5342
Lalli J.Y.Y., 2006, In vitro pharmacological properties and composition of leaf essential oils and extracts of selected indigenous Pelargonium (Geraniaceae) species, MPharm Thesis, University of the Witwatersrand, Johannesburg, South Africa
Lis-balchin M., 2006, Aromatherapy science: A guide for healthcare professionals, London: Pharmaceutical Press
Mahdieh M., Yazdani M., Mahdieh S., 2003, The high potential of Pelargonium roseum plant for phytoremediation of heavy metals, Environ Monit Assess, 185(9):7877-81
Miller D.M., 2002, The taxonomy of Pelargonium species and cultivars, their origins and growth in the wild. Geranium and Pelargoniums: The genera Geranium and Pelargonium, In M. Lis-Balchin (Ed.), Medicinal and aromatic plants-industrial profiles, London: Taylor and Francis
Nakatani N., 1994, Antioxidative and antimicrobial constituents of herbs and spices, Charalambous, G. (Ed.), Spices, Herbs and Edible Fungi, Elsevier Science, New York, 251–271
Newman D.J., Cragg G.M., 2007, Natural products as sources of new drugs over the last 25 years, J Nat Prod, 70:477
Okigbo R.N., Eme U.E., Ogbogu S., 2008, Biodiversity and conservation of medicinal and aromatic plants in Africa, Biotechnol.Mol.Biol.Rev., 3(6):127-134
Pietta P.G., 2000, Flavonoids as antioxidants, J. Nat. Prod., 63, 1035-1042
Pohlit A.M., Lopes N.P., Gama R.A., Tadei W.P., Neto V.F.D., 2011, Patent literature on mosquito repellent inventions which contain plant essential oils - A review, Planta Medica, 77, 598-617
Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C.A., 1999, Antioxidant activity applying an improved ABTS radical cation decolorization assay, FreeRadBiol.Med., 26, 1231-1237
Saraswathi J., Venkatesh K., Baburao N., Hilal M.H., Roja Rani A., 2011, Phytopharmacological importance of pelargonium species, J. Med. Plants Res., 5:2587–2598
Seo S.M., Kim J., Lee S.G., Shin C.H., Shin S.C., Park I.K., 2009, Fumigant antitermitic activity of plant essential oils and components from ajowan (Trachyspermum ammi), allspice (Pimenta dioica), caraway (Carumcarvi), dill (Anethum graveolens), geranium (Pelargonium graveolens), and litsea (Litsea cubeba) oils against Japanese termite, JAgricFChem, 57:6596-6602
Shahidi F., Janitha P.K., Wanasundara P.D., 1992, Phenolic antioxidants, Crit.ReV.Food Sci. Nutr., 32, 67-103
Shan B., Cai Y.Z., Sun M., Corke H., 2005, Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents, JAgricFoodChem, 53, 7749–7759
Silva N.C.C., Fernandes A.J., 2010, Biological properties of medicinal plants: a review of their antimicrobial activity, J.Ven.An.Tox.incl.Trop.Dis., 16:402-413
Slezák F., 2007, Conservation of the antioxidant elements in wines occurring Little Carpation region: research report. Modra: Biocentrum Modra a VÚP Bratislava
Tezcan F., Kolaylı S., Sahin H., Ulusoy E , Erim B.F., 2011, Evaluation of organic acid, saccharide composition and antioxidant properties of some authentic Turkish honeys, J. Food Nutr.Res., 50:33-40
Thaipong K., Boonprakob U., Crosby K., Cisneros-Zevallos L., Byrne D.H., 2006, Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts, J.FoodComp.Anal., 19, 669–675
Uhl S.R., 2000, Handbook of spices, seasonings, and flavorings, Boca Raton, FL: CRC Press
Velioglu Y.S., Mazza G., Gao L., Oomah B. D., 1998, Antioxidant activities and total phenolics in selected fruits, vegetables, and grain product, J. Agric. Food Chem., 46, 4113-4117
Verma R.S., Verma R.K., Yadav A.K., Chauhan A., 2010, Changes in the essential oil composition of rose–scented geranium (Pelargonium graveolens L'Herit. ex Ait) due to date of transplanting under hill conditions of Uttarakhand, IndianJNatProdRes, 1:367-370
Wu C.Q., Chen F., Wang X., Kim H.J., He G.Q., Haley-Zitlin V., Huang G., 2006, Antioxidant constituents in feverfew (Tanacetum parthenium) extract and their chromatographic quantification, FoodChem, 96, 220–227
Zheng W., Wang S.Y., 2001, Antioxidant activity and phenolic compounds in selected herbs, JAgricFChem, 49(11), 5165–5170

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MINERAL COMPOSITION OF PODS, SEEDS AND FLOUR OF GRAFTED CAROB (CERATONIA SILIQUA L.) FRUITS

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Abstract

Carob pods are the fruits of the carob tree (Ceratonia siliqua L. Fabaceae). They are used for various purposes, including pharmaceutical industry, decoration, for human nutrition and for animal feed. Grafted carob fruits and one of the traditional products, produced from it- carob flour, were evaluated for their mineral composition. The carob consists of two parts, including pulp and seeds. The pulp represents 90% of the fruit. Carob powder is used as an ingredient in cakes and cookies and as cocoa substitute. Among the analyzed major minerals, Mg (859.0 mg/kg dry weight) was the most abundant element, and the pulp and seeds were also rich in Fe and Zn. Carob flour contained these elements in high amounts. This study has corroborated the notion that carob fruit and flour are rich sources of minerals and the seeds generally have higher amount of macro and micro minerals than the pulp.

Key words*: mineral composition; carob; Ceratonia siliqua L.*

INTRODUCTION

Carob fruits are among the most important tree fruit crops in the Mediterranean region and their production and consumption have increased considerably in recent years. The carob (Ceratonia siliqua) is slowly growing, woody and widespread plant. It is in general currency in Arabia and Oman, while it was introduced in California, Mexico and Southern Australia (Marakis et al., 1988) and is also distributed in some parts of Bulgaria - Black Sea coast, the Balkan Mountains, south central and northeaster Bulgaria. *Ceratonia siliqua* is considered a phylogenetically primitive species of tropical origin that has been cultivated in the Mediterranean area since historic times (Zohary, 2002). It is an economically important plant (Biner et al., 2007; Makris and Kefalas, 2004; Ozcan et al., 2007), which has been used for afforestation in half-withered regions (Catarino, 1993; Tous et al., 2009). The pod is light to dark brown, oblong, flat, straight or slightly curved, with a thick margin, and range from 10 to 20 cm in length and 1.5–2 cm in width. The unripe pod is green, moist and very astringent, but the ripe pod is sweet. The broken pod has a characteristic odour caused by its 1.3% isobutyric acid content (Morton 1987). Carob fruit generally consists of 80–90% pulp and 10–20% seed (Naghmouchi et al., 2009; Tetik et al., 2011a) and also contains 50–65% sugars (mainly composed of sucrose), 1–5% proteins and 0.2–08% lipids as well as crude fiber (11%) and significant amount of minerals (1–6%) (Avallone et al., 1997; Ayaz et al., 2007; Ozcan et al., 2007; Yousif and Alghzawi, 2000). Considering the mineral content of fruit, calcium, potassium, magnesium, sodium, phosphorus and iron are abundant (Eksi and Artik, 1986; Ozcan et al., 2007).

For many centuries, carob pods have been used in many countries for both human and animal nutrition. The use of carob pods in food dates back to ancient times, where the pulps are reported to have been consumed in raw form (Brandt, 2002; Haber, 2002; Owen et al., 1987). The fruit of the carob tree has recently become a valuable commodity and has been evaluated for multipurpose uses such as gum, syrup, powder, biofertilizer, d-pinitol, ethanol, mannitol, lactic and citric acid (Batu, 2005; Baumgartner et al., 1986; Carvalheiro et al., 2011; El-Shatnawi and Ereifej, 2001; Medeiros and Lannes, 2009; Parrado et al., 2008; Petit and Pinilla, 1995; Roukas, 1998; Tetik et al., 2011b;Turhan et al., 2010a,b, 2006).

They are widely used in the boiled juice (syrup) production.

Carob seeds are extremely hard and carob endosperm contains 30–40% by weight of galactomannan that is a polysaccharide

molecule composed mannose and galactose sugar units. So, the seeds (10%) are used industrially for carob bean gum or locust bean gum production.

The other major food source derived from carob is the powder with high protein content. Its structure is very suitable for cabinet making, and to make utensils as well as charcoal. It is also used as fodder and material in the tannery industry. Carob powder is a natural sweetener with flavour and appearance similar to chocolate; therefore it is often used as cocoa substitute. The advantage of using carob as a chocolate substitute is that carob powder ingredient free from caffeine and theobromine. In Europe several carob commercial products can be found as form of roasted carob flour used as a cocoa substitute in baking, cereal bars, chocolate confectionery, ice creams and light products. Other products, such as carob germ flour contains high protein content, almost 50%, with a high content of lysine and arginine. Carob germ flour is used as dietetic human food (Dakia et al., 2007) or as a potential ingredient in cereal-derived foods for celiac people (Feillet & Roulland, 1998). This low-cost product is highly rich in valuable compounds, particularly polyphenols, which have been described to present antioxidant activity and potential health benefits in humans. Therefore, the present study was undertaken to examine the nutritive properties as determine the mineral composition of grafted carob pods, seeds and flour.

MATERIALS AND METHODS

In the study were used grafted carob fruits which were collected from Mersin, Turkey and they were harvested at the end of the summer season of 2014. Carob fruits were randomly chosen and used in the analyses. The samples were broken into pulps and seeds with a pair of secateurs and were stored at 4^0C before analysis. It was studied a raw carob flour, obtained from carob pods, which was provided also from region of Mersin, Turkey.

Macro (magnesium-Mg) and micro (iron- Fe, copper-Cu, manganese-Mn, zinc-Zn, selenium-Se) minerals of carob samples were determined according to validated laboratory method (in Food Research and Development Institute Plovdiv). For this purpose, samples were washed with tap water and deionized water.

The method used - ICP-OES is based on the pulverizing of acid mineralization of studied products in inductively coupled plasma, where is carried out the excitation of chemical elements' atoms and subsequent measurement of their specific emission at specific wavelengths. The measured intensities are compared with the intensity of series of standard solutions containing determined elements measured under the same conditions.

Protocol of analysis: it was weighted to 1 g of samples and they put to microwave mineralization. It is carried out with microwave system in hermetically sealed pressure vessel. In Teflon vessel are added the samples, 2 to 3 ml of 0.2% solution of HNO_3 and 2 to 3 ml H_2O_2. Mineralized sample is filtered through a paper filter into a volumetric flask of 10 cm^3 and fill up with 0.2% solution of nitric acid.

RESULTS AND DISCUSSIONS

The chemical composition and mineral content of carob fruits have been studied. According to Ozcan et al. (2007), carob fruit (pulps and seeds) and flour are rich in carbohydrates, proteins and also are a good source of K, Ca, Na, Fe, and Mg. According to the literature data many factors affect the chemical composition of the fruit as well as its mineral content, for example, temperature, dryness (Nunes et al., 1992), irrigation and fertilization (Correia and Martins-Loucao, 1997) and salinity (El-Dengawy et al., 2011). The distribution of the macro and micro minerals obtained from the analysis of the carob samples is summarized in Table 1.

Table 1. Mineral content of carob pulps, carob seeds and carob flour

№	Mineral content (mg/kg)	Carob-pulps	Carob-seeds	Carob flour
1.	Selenium	1.60	1.60	1.60
2.	Magnesium	265.0	859.0	346.0
3.	Iron	16.80	82.0	76.60
4.	Copper	5.60	20.2	6.80
5.	Manganese	4.42	50.8	8.90
6.	Zinc	10.80	21.0	11.90

When carob samples (pulps, seeds, flour) were compared in terms of minerals, the seeds of the grafted samples generally contained higher mineral concentrations. Magnesium was the mineral with the highest concentration (between 265 mg/kg and 859 mg/kg) in all samples. Among the micro minerals, Fe had the highest concentration (between 16.80 mg/kg and 82.0 mg/kg) in the grafted carob fruit seeds and also they generally contained higher macro and micro minerals than the fruit pulp (Fig. 1 and Fig.2). Ayaz et al. (2007) studied the chemical composition of Anatolian carob pods and found the mineral concentration in the samples to be: 60 mg/100 g Mg, 1.88 mg/100 g Fe, 1.29 mg/100 g Mn and 0.85 mg/100 g Cu. Ozcan et al. (2007) studied carob fruits supplied by a local carob processing plant in Antalya and analyzed the chemical composition and mineral content of the fruits and their different products.

According to their results, the concentrations of minerals were lower to those in this study. But, they also reported higher concentrations of Fe (40.02 mg/kg), besides lower concentrations of Cu (2.29 mg/kg) and Zn (0.25 mg/kg) compared to our results. Youssef et al. (2013) also reported the concentration of macro and micro elements (9.69 mg/kg Mn, 4.58 mg/kg Cu) in carob powder produced by carob fruits collected in Egypt. Gubbuk et al. (2010) studied with wild and grafted (Etli and Sisam genotypes) carob fruits grown in Antalya.

Thus, our results are compatible to those in the literature.

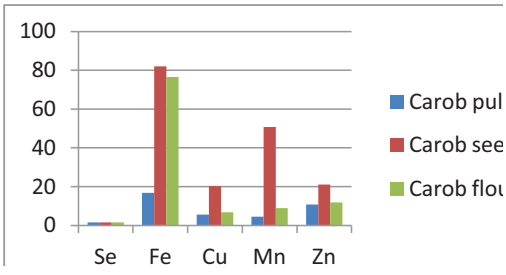

Figure 1. Micro minerals (mg/kg) of pulps, seeds and flour of *Ceratonia siliqua.*

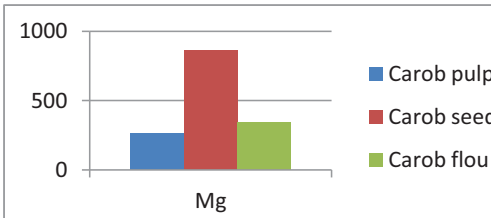

Figure 2. Magnesium content (mg/kg) of carob pulps, seeds and flour.

CONCLUSIONS

The results presented here show significant differences between fruit parts (pulp or seed) and raw carob flour. According to the results; the seeds of the grafted types had higher concentrations of the analyzed macro and micro minerals and all the samples are good sources of Se. The highest Mg concentration was observed in the seed of carob fruit (859 mg/kg). Furthermore, Fe, Mg and Zn levels as well as the Cu were higher in the seeds of the grafted carob types. The results obtained of this study prove that the carob fruits and flour, actually could be used as a good supplement in the healthy human diet.

REFERENCES

Avallone, R., Plessi, M., Baraldi, M., Monzani, A., 1997. Determination of chemical composition of carob (Ceratonia siliqua): protein, fat, carbohydrates, and tannins. J. Food Compos. Anal. 10, 166–172.

Avallone, R., Cosenza, F., Farina, F., Baraldi, C., Baraldi, M., 2002. Extraction and purification from Ceratonia siliqua of compounds acting on central and peripheral benzodiazepine receptors. Fitoterapia 73 (5), 390–396.

Ayaz, F.A., Torun, H., Ayaz, S., Correia, P.J., Alaiz, M., Sanz, C., Gruz, J., Strnad, M., 2007. Determination of chemical composition of anatolian carob pod (Ceratonia siliqua L.): sugars, amino and organic acids, minerals and phenolic compounds. J. Food Quality 30, 1040–1055.

Batu, A., 2005. Production of liquid and white solid pekmez in Turkey. J. Food Quality 28, 417–427. Bartsch, H., et al. (2003). Isolation and structure elucidation of the major individual polyphenols in carob fibre. Food and Chemical Toxicology, 41, 1727–1738.

Baumgartner, S., Genner-Ritzmann, R., Haas, J., Amado, R., Neukom, H., 1986. Isolation and identification of cyclitols in carob pods (Ceratonia siliqua L.). J. Agric. Food Chem. 34, 827–829.

Biner, B., Gubbuk, H., Karhan, M., Aksu, M., Pekmezci,

M., 2007. Sugar profiles of the pods of cultivated and wild types of carob bean (Ceratonia siliqua L.) in Turkey. Food Chem. 100, 1453–1455.
Brandt, L.A. (2002). Carob fibre offers health benefits. Prepared foods, 171(1), 51.
Carvalheiro, F., Moniz, P., Duarte, L.C., Esteves, M.P., Girio, F.M., 2011. Mannitol production by lactic acid bacteria grown in supplemented carob syrup. J. Ind. Microbiol. Biotechnol. 38, 221–227.
Catarino, F., 1993. The carob tree: an exemplary plant. Naturopa 73, 14–15.
Correia, P., Martins-Loucao, M., 1997. Leaf nutrient variation in mature carob (Ceratonia siliqua) trees in response to irrigation and fertilization. Tree Physiol. 17, 813–819. Cruz, C., Lips, S.H., Martins-Loucao, M.A., 1993. Effect of root temperature on carob growth: nitrate versus ammonium nutrition. J. Plant Nutr. 16, 1517–1530.
Dakia, P.A., Wathelet, B., Paquot, M., 2007. Isolation and chemical evaluation of carob (Ceratonia siliqua L.) seed germ. Food Chem. 102, 1368–7134.
Eksi, A., Artik, N., 1986. Harnup (kec iboynuzu) meyvesi ve pekmezinin kimyasal bilesimi. Ankara Universitesi Ziraat Fakultesi Yıllı˘gı 36, 77–82.
El-Dengawy, E.-R.F., Hussein, A.A., Alamri, S.A., 2011. Improving growth and salinity tolerance of carob seedlings (Ceratonia siliqua L.) by Azospirillum inoculation. Am.-Eurasian J. Agric. Environ. Sci. 11, 371–384.
El-Shatnawi, M.K.J., Ereifej, K.I., 2001. Chemical composition and livestock ingestion of carob (Ceratonia siliqua L.) seeds. J. Range Manage. 54, 669–673.
Feillet, P. and Roulland, I. M., (1998). Caroubin: A gluten-like protein isolate from carob bean germ. Cereal Chem., 75. 488 -492.
Gubbuk, H., Kafkas, E., Guven, D., Gunes, E., 2010. Physical and phytochemical profile of wild and domesticated carob (Ceratonia siliqua L.) genotypes. Span. J. Agric. Res. 8, 1129–1136.
Haber, B.(2002). Carob fiber benefits and applications. Cereal foods world, 47 (8), 365-369.
Makris, D.P., Kefalas, P., 2004. Carob pod (Ceratonia siliqua L.) as source of polyphe-nolic antioxidants. Food Technol. Biotechnol. 42, 105–108.
Marakis, S., Kalaitzakis, J., Mitrakos, K., 1988. Criteria for recognizing carob tree varieties. In: Proceedings of the II. International Carob symposium, Valencia, Spain, pp. 195–208.
Medeiros, M.L., Lannes, S.C.D., 2009. Chemical evaluation of cocoa substitutes and study of formulated chocolate drink powder. Cienc. Tecnol. Aliment. 29, 247–253.
Morton, Miami, FL. Ozcan, M.M., Arslan, D., Gokcalik, H., 2007. Some compositional properties and mineral contents of carob (Ceratonia siliqua) fruit, flour and syrup. Int. J. Food Sci. Nutr. 58, 652–658.
Naghmouchi, S., Khouja, M.L., Romero, A., Tous, J., Boussaid, M., 2009. Tunisian carob (Ceratonia siliqua L.) populations: Morphological variability of pods and kernel. Sci. Hortic.—Amsterdam 121, 125–130.
Nunes, M.A., Ramalho, C., Domingos, J., Silva Rijo, P.d., 1992. Seasonal changes in some photosynthetic properties of Ceratonia siliqua (carob tree) leaves under natural conditions. Physiol. Plant. 86, 381–387.
Owen, R. W., Haubner, R., Hull, W. E., Erben, G., Spiegelhalder, B., MORTON, J. F. 1987. Carob. In *Fruits of Warm Climates*. (C.F. Dowling, ed.).
Parrado, J., Bautista, J., Romero, E.J., Garcia- Martinez, A.M., Friaza, V., Tejada, M., 2008. Production of a carob enzymatic extract: potential use as a biofertilizer. Bioresour. Technol. 99, 2312–2318.
Petit, M.D., Pinilla, J.M., 1995. Production and purification of a sugar syrup from carob pods. Food Sci. Technol.—Leb 28, 145–152.
Roukas, T., 1994. Solid-state fermentation of carob pods for ethanol production. Appl. Microbiol. Biotechnol. 41 (3), 296–301.
Roukas, T., 1998. Carob pod: A new substrate for citric acid production by Aspergillus niger. Appl. Biochem. Biotechnol. 74, 43–53.
Roukas, T., 1999. Citric acid production from carob pod by solid-state fermentation.Enzyme Microb. Technol. 24 (1–2), 54–59.
Tetik, N., Turhan, I., Oziyci, H.R., Gubbuk, H., Karhan, M., Ercisli, S., 2011a. Physical and chemical characterization of Ceratonia siliqua L. germplasm in Turkey. Sci. Hortic.—Amsterdam 129, 583–589.
Tetik, N., Turhan, I., Oziyci, H.R., Karhan, M., 2011b. Determination of d-pinitol in carob syrup. Int. J. Food Sci. Nutr. 62, 572–576.
Tous, J., Romero, A., Hermoso, J.F., Ninot, A., Plana, J., Batlle, I., 2009. Agronomic and commercial performance of four Spanish carob cultivars. HortTecnology 19, 465–470.
Turhan, I., Bialka, K.L., Demirci, A., Karhan, M., 2010a. Enhanced lactic acid production from carob extract by Lactobacillus casei using invertase pretreatment. Food Biotechnol. 24, 364–374.
Turhan, I., Bialka, K.L., Demirci, A., Karhan, M., 2010b. Ethanol production from carob extract by using Saccharomyces cerevisiae. Bioresour. Technol. 101, 5290–5296.
Turhan, I., Tetik, N., Aksu, M., Karhan, M., Certel, M., 2006. Liquid-solid extraction of soluble solids and total phenolic compounds of carob bean (Ceratonia siliqua L.). J. Food Process Eng. 29, 498–507.
Yousif, A.K., Alghzawi, H.M., 2000. Processing and characterization of carob powder. Food Chem. 69, 283–287.
Youssef, M.K.E., El-Manfaloty, M.M., Ali, H.M., 2013. Assessment of proximate chemical composition, nutritional status, fatty acid composition and phenolic compounds of carob (Ceratonia siliqua L.). Food and Public Health 3, 304–308.
Zohary, M., 2002. Domestication of the carob (Ceratonia siliqua L.). Israel J. Plant Sci. 50, 141–145.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ASSESSMENT OF SOME NUTRIENTS IN BAKERY PRODUCTS

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Abstract

Bakery products constitute an important part of the human diet, providing a high quantity of carbohydrates, but also proteins, dietary fibres, B group vitamins and minerals. Consumption of whole grain foods means high intake of dietary fibre and micronutrients, which has been associated with a reduced risk of coronary heart disease, diabetes, obesity and some forms of cancer. The aim of the present study was to appreciate the nutritive value of some bakery products by assessment of content in thiamine and α-tocopherol. The researches were performed on common products made with wheat or rye flour and on some enriched products obtained by adding different ingredients of vegetal origin, such as olives, onion, garlic or seeds. These supplementary ingredients were used to improve the taste and to diversify the range of bakery products. In the same time, their addition results in obtaining healthier food. α-Tocopherol content was analysed by HPLC method and thiamine content was determined by spectrofluorometric method. The obtained results showed a higher content of thiamine in the enriched products comparing to the traditional ones. Moreover, the analysis indicated an improvement of α-tocopherol content, although the bakery products usually contain low amounts of this vitamin.

Key words*: bread, biscuits, thiamine, α-tocopherol.*

INTRODUCTION

Bakery products constitute an important part of the human diet, widely accessible, providing a high quantity of carbohydrates, proteins, dietary fibres, vitamins of B group and minerals.

Lately, there is an increased concern for incorporating bioactive ingredients in popular foods such as bread and bakery products, because of growing interest of consumers for healthier food (Sivam et al., 2010). As result, there is a high demand for functional foods with large amounts of antioxidants and dietary fibres, especially because they are associated with health benefits (Pelucchi et al., 2004; Arts et al., 2005; Scott et al., 2008). Since the endogenous fibre polysaccharide content in white wheat flour is only 2-4%, using whole grains as source of dietary fibre in bread would raise the health profile of the final products (Pelucchi et al., 2004; Scott et al., 2008, Dikeman et al., 2006).

Vitamin B_1, also known as thiamine, plays a major role in the obtaining of energy from dietary carbohydrates and fats, so that deficiency of this nutrient may influence the functions of most of body organs. Severe and prolonged deficiency has been reported to affect the nervous system, the heart, and digestive function, among other areas. Most foods contain small amounts of thiamine, but good dietary sources of thiamine include whole-grain or enriched cereals and rice, legumes, wheat germ, bran, brewer's yeast. Thiamine is known to occur in the outer integuments and germ of cereal grain. In wheat grain, the endosperm represents 80–85% of grains dry mass, but contains only 3% of the total thiamine (Kalnina et al., 2014). The highest proportion (about 80%) of thiamine is found in the external layers of wheat grain. Unfortunately these are missing in white flour (Batifloulier et al., 2006). Whole wheat flour, one of the most common and important whole grains, retains wheat bran and germ and acts as a rich source of

dietary fibre, vitamins, minerals and antioxidants (Wang et al., 2014, Liyana-Pathirana and Shahidi, 2007).

Cereal grains contain also intrinsic phenolic antioxidants. Free radicals derived from a wide range of biological reactions in the body can damage essential biomolecules. For example, lipid peroxide radicals have been associated with chronic degenerative diseases such as cancer, inflammatory, aging, cardiovascular and neurodegenerative disease (Shahidi et al., 1995; Arts et al., 2005). Natural antioxidants such as flavonoids, tocopherols, and phenolic acids may inhibit lipid peroxidation in food and improve food quality (Fan et al., 2007; et al., 2007; Wojdyło et al., 2007).

Vitamin E is the term for a group of tocopherols and tocotrienols, of which α-tocopherol exerts the highest biological activity and is exclusively obtained from the diet. Nuts, seeds, vegetable oils are among the best sources of vitamin E and significant amounts are available in wheat germ oil, green leafy vegetables and fortified cereals. Vitamin E is the major lipid-soluble antioxidant in the cell antioxidant defence system which protects tissues against the damaging effects of free radicals. Due to the potent antioxidant properties of tocopherols, their impact in the prevention of chronic diseases believed to be associated with oxidative stress has been studied and beneficial effects have been demonstrated (Brigelius-Flohe et al., 1999).

Consumption of whole grain foods means high intake of dietary fiber and micronutrients, which has been associated with a reduced risk of coronary heart disease, diabetes, obesity and some forms of cancer (Anderson et al., 2009).

The aim of the present study was to appreciate the nutritive value of some bakery products by assessment the content in thiamine and α-tocopherol. The researches were performed on traditional products made with wheat or rye flour and on some enriched products obtained by adding different ingredients of vegetal origin, such as olive oil, onion, garlic or different seeds. These supplementary ingredients were used to improve the taste and to diversify the range of bakery products. In the same time, due to their content in thiamine and α-tocopherol, their addition results in obtaining healthier food.

MATERIALS AND METHODS

Samples

The determinations were performed on traditional products, made with wheat or rye flour, and on some enriched products obtained by adding different ingredients of vegetal origin. Thirteen samples of such bakery products were analysed in order to determine the content in thiamine and α-tocopherol. The determinations were performed in triplicate for some type of bread (made of white or whole-wheat flour, of rye flour and of wheat flour enriched with different vegetal ingredients) and four types of biscuits. The extractions of analysed compounds were conducted according to the protocol used for each determination.

Methods

Determination of thiamine content

Sample preparation. The bakery products were dried and then finely grounded. For extraction of thiamine, the sample was digested with diluted sulphuric acid on a boiling water bath for 15 min and subsequently treated with an enzymatic mixture, containing phosphatase and protease, in order to release the thiamine from the natural ester and protein bonds.

Analysis method. Thiamine content was analysed using a fluorometric method based on the oxidation of thiamine with oxidizing reagent (potassium ferricyanide in alkaline solution) to fluorescent thiochrome. The thiochrome obtained is extracted with isobutyl alcohol. The intensity of fluorescence of the isobutyl alcohol extract is compared with that of the standard solution (100 μg/ml thiamine). Also a blank (control) is prepared by adding of sodium metabisulphite in the sample. The intensity of fluorescence is measured with a JASCO FP-6300 spectrofluorometer and fluorescence value of the blank test is subtracted from that of the sample extract.

Determination of α-tocopherol content

Sample preparation. The first step for extraction was consisted in saponification with 11% KOH ethanolic solution at 80°C, for 15 minutes. Ascorbic acid was used for preventing vitamin E oxidation. α-Tocopherol was extracted with 4 ml iso-octane by vortexing for 2 minutes. After separation of the two phases 1 ml iso-octane extract was evaporated using a centrifugal evaporator and the fatty residue was reconstituted in 0.5 ml methanol. All the samples were prepared and analysed in triplicates.

Analysis method. Separation of tocopherols was carried out by RP-HPLC method, using a Waters Alliance system, with UV detection. The separation was performed with C8 Symmetry column and a mobile phase consisting in methanol + acetonitrile + water solution (45:45:10, v/v/v), with 1 ml/min flow rate. The data were achieved and processed with EMPOWER 2.0 specialized software. Calibration curve was obtained using 56.22 µg/ml α-tocopherol (Sigma-Aldrich) standard solution and the results were calculated and expressed as mg α-tocopherol in 1 g product.

RESULTS AND DISCUSSIONS

In its natural state, wheat is a good source of vitamins B_1 and E. However, because most of these biocompounds are concentrated in the outer layers of the wheat grain, a smaller proportion is found in the flour at the end of the milling process. Moreover, the stability of vitamins in processed products is reduced because its sensitivity to heat, oxidising and reducing agents, light, and other kinds of physic and chemical factors. Yet it seems that during cooking the temperature inside the bakery product is significantly lower although baking temperatures are high (over 200°C), therefore over 70 % of the vitamins remain unaltered (Cort et al., 1976).

The biochemical analysis performed to determine the B_1 vitamin content (figure 1) revealed high value in rye bread (4.67 mg/kg) followed by whole-wheat bread (4.59 mg/kg) and whole-wheat mixed seeds rolls (3.19 mg/kg). By comparison, lower thiamine content was determined in the white bread (3.05 mg/kg), as expected due to the removal of grains outer layers by milling. These results are supported by researches performed by other authors. According to the scientific literature, higher B_1 vitamin content was obtained in whole grain flour (4.8 mg/kg) comparing with wheat flour 550 type (2.5 mg/kg); similar vitamin B_1 content was found in whole grain rye (4,2 mg/kg) (Kalnina et al., 2014).

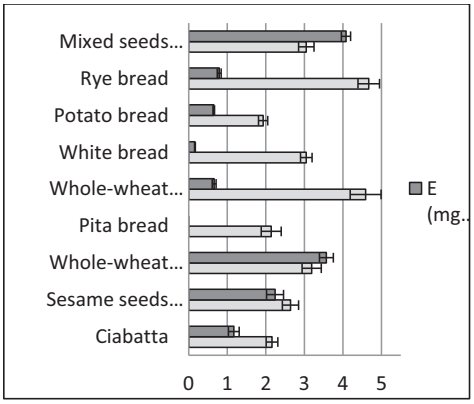

Figure 1. Amounts of vitamins B_1 and E in the analyzed bakery products

Smaller values of thiamine content were measured in ciabatta (2.16 mg/kg) and pita bread (2.14 mg/kg), some bakery specialties used in many Mediterranean, Balkan and Middle Eastern cuisines (figure 1). Ciabbatta is an Italian white bread made from wheat flour, water, salt, olive oil and yeast, while pita is a soft, slightly leavened flatbread baked from wheat flour. It seems that using highly refined white flour for obtaining of bakery products resulted in decreasing of vitamin B_1 content.

The addition of some vegetal ingredients had a different influence: the sesame seeds contributed to the enrichment in B_1, while potatoes in bread determined a reduction of vitamin B_1 amount in the finished product. So, the measured values of vitamin B_1 ranged between 2.64 mg/kg in sesame seeds rolls and 1.93 mg/kg in potato bread. Indeed, the scientific literature reports higher values of thiamine in the whole sesame seeds (7.1-

8.3 mg/kg) (Makinde et al., 2013) compare to those determined in potatoes (0.29-1.32 mg/kg) (Goyer et al., 2011). Although a part of the thiamine content losses occur during baking, it appears that ingredients containing large amounts of B_1 contribute at enrichment of finished bakery products.

Also the biscuits made by dough supplementation with potato flakes registered lower amounts of vitamin B_1 (1.95 mg/kg) compared to sweet biscuits, containing 2.33 mg/kg B_1 (figure 2). However, significant increase of the B_1 amount in biscuits (3.76 mg/kg B_1) was registered by addition of garlic, beside potato flakes.

The present results correspond to the data reported regarding the content in B_1 of the added ingredients: potatoes (0.29-1.32 mg/kg), garlic (3.42-15.28 mg/kg) (Al-Timimia et al., 2013).

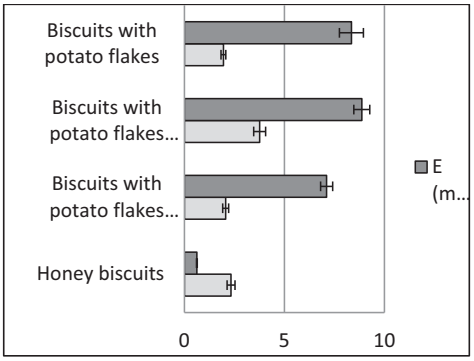

Figure 2. Amounts of vitamins B_1 and E in some types of biscuits

Although vitamin E is present in high amounts in wheat germ, the scientific literature indicates only small amount remained in the wheat flour due to the absence of the germ and bran fractions in the refined flour. Furthermore, the content of vitamin E in wheat flour decreases in time: about one-third of vitamin E amount is lost after one year of storage (Nielsen et al., 2008).

As concerning the vitamin E content in the analysed samples (figure 1), small values were registered in the bakery products made with highly refined flour: 0.16 mg/g vitamin E in white bread, while in pita bread it was not detectable by the used analysis method.

Higher value of vitamin E content were measured in whole-wheat bread (0.66 mg/g), as expected given the data from cited literature which indicate that the germ fraction contained by this type of flour is riched in vitamin E (16 times higher amount of vitamin E than in any other fraction) (Engelsen et al., 2009). Besides, also the total lipids content in these bakery products are correlated with vitamin E amounts: it was determined a higher value in whole-wheat bread (1.81 % lipids) compared to the one measured in white bread (1.46 % lipids) or pita (1.42 % lipids).

Nevertheless significant increase of α-tocopherol was registered in the bakery products supplemented with different ingredients. Thus, addition of olive oil in ciabatta resulted not only in a higher content of total lipids (2.45 g %), but in a higher amount of vitamin E determined in this bakery specialty (1.17 mg/g) (figure 1). These results correspond with the high concentrations of vitamin E found in most of the samples of Greek virgin olive oils selected from various regions, values ranging between 98-370 mg/kg (Psomiadou et al., 2000; Escuderos et al., 2009). Also, the different kind of seeds added in some bakery product resulted in a rich content in total lipids (between 5-6 %), and, in the same time, contributing to enrichment in vitamin E of the finished products. According to figure 1, large amount of vitamin E were detected: 4.08 mg/g in mixed seeds bread, 3.57 mg/g in whole-wheat mixed seeds rolls, 2.64 mg/g in sesame seeds rolls.

The analysed varieties of salted biscuits (fig.2) showed higher values of vitamin E content in comparison with the mentioned bakery products, ranging between 7.11 mg/g in potato flakes and onion biscuits and 8.87 mg/g in potato flakes and garlic biscuits. As expected, the total lipids content was higher in biscuits (between 18.33 and 18.66 %), considering the dough recipe containing fat and eggs, which also can provide vitamin E. However, some of the vegetal ingredients

added in biscuits (garlic) brought a contribution to increasing the amount in vitamin E, which was lower in the potato flakes biscuits (8.35 mg/g).

CONCLUSIONS

Bakery products made by using highly refined white flour (white bread, pita bread, ciabatta) contained lower amounts of vitamin B_1 and vitamin E compared with those containing whole-wheat flour or rye flour. However, ciabatta is an exception regarding the vitamin E content, which is high due to the olive oil added.

Addition of supplementary ingredients (olive oil, garlic, onion, mixed seeds) to some types of bakery products resulted in obtaining B_1 and E vitamins enriched food.

These supplementary vegetal ingredients were used to improve the taste and to diversify the range of bakery products, aiming to be an encouragement and an orientation for healthier food consumption.

ACKNOWLEDGEMENTS

This work was supported by MADR 2020 Sectorial Plan, project ADER 8.1.3/2013.

REFFERENCES

Al-Timimia F.M.A., Al-Ganib M.J.A., Al-Dulimyi E.M.K., 2013, Determination of some water-soluble vitamins in different species of garlic extracts by using high performance liquid chromatography, Journal of Chemical and Pharmaceutical Research, 5(10):15-20

Anderson J.W., Baird P., Davis J.R.H., Ferreri S., Knudtson M., Koraym A., 2009, Health benefits of dietary fiber. Nutrition Reviews, No. 67, p.188–205

Arts I.C.W., Hollman P.C.H., 2005, Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr.: 81(1):317S–25S

Batifloulier F., Verny M.A., Chanliaud E., Demigne C., 2006, Variability of B vitamin concentrations in wheat grain, milling fractions and bread products. European Agronomy, Vol.25, p.163–169.

Brigelius-Flohé R., Traber M., 1999, Vitamin E: function and metabolism, FASEB Journal, vol. 13, no. 10, p.1145-1155

Dikeman C.L., Murphy M.R., Fahey G.C., 2006, Dietary fibers affect viscosity of solutions and simulated human gastric and small intestinal digesta. J Nutr.;136:913–19.

Fan L., Zhang S., Yu L., Ma L., 2007, Evaluation of antioxidant property and quality of breads containing Auricularia auricula polysaccharide flour. Food Chem.;101:1158–63

Goyer A., Haynes K., 2011, Vitamin B_1content in potato:effect of genotype, tuber enlargement and storage and estimation of stability, and broad-sense heritability, Am. J. Pot. Res.88:374-385

Engelsen M.M., Hansen A., 2009, Tocopherol and Tocotrienol Content in Commercial Wheat Mill Streams, Cereal Chemistry Journal, Vol. 86, nr. 5, p. 499-502

Escuderos M.E., Sayago A., Morales M.T., Aparicioc R., 2009, Evaluation of α-tocopherol in virgin olive oil by a luminiscent method, Grasas y aceites, 60 (4), p. 336-342

Kalnina S., Rakcejeva T., Gramatina I., Kunkulberga D., 2014, Investigation of total dietary fiber, B1 and B2 vitamin content of flour blend for pasta production, Foodbalt, p. 133-137

Liyana-Pathirana C.M., Shahidi F., 2007, The antioxidant potential of milling fractions from breadwheat and durum. J Cereal Sci.;45:238–47 Makinde, F.M., Akinoso, R., 2013, Nutrient composition and effect of processing treatments on anti nutritional factors of Nigerian sesame (*Sesamum indicum Linn*) cultivars, International Food Research Journal 20(5): 2293-2300

Nielsen M.M., Hansen A., 2008, Stability of Vitamin E in Wheat Flour and Whole Wheat Flour During Storage, Cereal Chem; 85:6, p. 716 - 720

Pelucchi C., Talamini R., Galeone C., Negri E., Franceschi S., Dal Maso L., Montella M., Conti E., La Vecchia C., 2004, Fibre intake and prostate cancer risk. Int J Canc.;109:278–80

Psomiadou E., Tsimidou M., Boskou D., 2000, α-Tocopherol Content of Greek Virgin Olive Oils, *J. Agric. Food Chem.*, *48*(5), p. 1770–1775

Scott K.P., Duncan S.H., Flint H.J., 2008, Dietary fibre and the gut microbiota. Nutr Bull.;33:201–11.

Shahidi F., Naczk M., 1995, Food phenolics: Sources, chemistry, effects and applications. Lancester, Pa.: Technomic Publishing Company, Inc; Nutritional and pharmacological effects of food phenolics; pp. 171–91.

Sivam A.S., Sun-Waterhouse D., Quek S.Y., O Perera C., 2010, Properties of Bread Dough with Added Fiber Polysaccharides and Phenolic Antioxidants: A Review, J Food Sci. 75(8), p.163-174.

Wang L., Deng L., Wang Y., Zhang Y., Qian H., Zhang H., Qi X., 2014, Effect of whole wheat flour on the quality of traditional Chinese Sachima. Food Chemistry, Vol. 152(1), p.184–189

Wojdyło A., Oszmianski J., Czemerys R., 2007, Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem.;105:940–49.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

POLYPHENOLS CONTENT AND ANTIOXIDANT ACTIVITIES IN INFUSION AND DECOCTION EXTRACTS OBTAINED FROM *FRAGARIA VESCA* L. LEAVES

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Abstract

Fragaria vesca L. (wild strawberry) belongs to the Rosaceae family. The leaves and roots from wild strawberry are herbal materials applied in traditional medicine. Fragaria vesca are rich source of biologically active substances like tannins, procyanidins, anthocyanidins, flavonoids and phenolic acids. The aim of this study was to compare the value of phytochemical compounds and antioxidant activities in infusion and decoction obtained from the wild strawberry leaves. The extracts were analyzed regarding their secondary metabolite content (total polyphenols, total flavonoids and total proanthocyanidins) and antioxidant activities (DPPH and CuPRAC methods). The analysis of decoction extracts from the leaves harvested in blooming period revealed the highest level of total polyphenols (46.1 mg GAE/g DW), total flavonoids (4.7 mg QE/ g DW), total proantocyanidines (22.3 mg/ g DW) and antioxidant activities – radical scavenging activity (DPPH – 325.0 mM TE/ g DW) and metal reducing ability (CuPRAC – 1257.9 mM TE/ g DW). The results showed that the water extracts from leaves of Fragaria vesca are appropriate additives for preparation of functional foods and natural cosmetic products with improved biological activity.

Key words*: Fragaria vesca L., phytochemistry, decoction, infusion, antioxidants.*

INTRODUCTION

Fragaria vesca is commonly called as wild strawberries, is a plant that grows naturally throughout the northern hemisphere (Folta and Gardiner, 2009). The leaves, roots and fruits are herbal materials used in traditional medicine. Plants material are collected during the flowering season and prepared as infusion, decoction or tincture. Different extracts from *Fragaria vesca* possess antioxidant, anti-inflammatory, antibacterial, astringentic, antidiarrheic and antidysenteric acrivities (Kiselova et al., 2006; Cheel et al., 2007; Neves et al., 2009; Sarić-Kundalić et al., 2010; Kanodia et al., 2011, Buricova et al., 2011, Borah et al., 2012, Liberal et al., 2014,). Leaves from wild strawberry are rich source of bioactive compounds such as flavonoids, ellagitannins, procyanidines and phenolic acids (Mudnic et al., 2009; Buendia et al., 2010; Buendia et al., 2010, Buricova et al., 2011, Liberal et al., 2014).

Therefore, the aim of this study is to reveal the bioactivity and polyphenols content of water extracts obtained from *Fragaria vesca* leaves collected from different areas.

MATERIALS AND METHODS

Plant material

Aerial parts (leaves) by several random chosen plants of *F. vesca* L., were collected from their natural habitats nearby "Zdravec" hut and "Vruhovruh" hut – Rhodopa mountain all in May and October 2013. The samples were dried in shade at ambient temperature for 7 days, and finely ground by homogenizer. The powder was used for different extraction.

Extraction procedure

Two aqueous extracts (infusion and decoction) were prepared according to Pistón et al., 2014.

Briefly, for decoction preparation, the dried leaves (1 g) were added to 50 mL of hot ultrapure water, than heated, kept in boiled water for 15 min and after that the mixture was removed from the heat, stood for 20 min and filtered through filter paper. Infusion was prepared by adding 50 mL of ultrapure hot water at 95°C to 1 g of dried leaves and the mixture was left to stand for 20 min to be also filtered using filter paper. Both the infusion and decoction extracts were analysed for polyphenol content and antioxidant activity

Total proanthocyanidins assay
Acid butanol was used for assaying proanthocyanidins, according to Porter et al. (1986). Six milliliters of the acid butanol reagent (950 mL of n-butanol with 50 mL concentrated HCl), 0.5 mL aliquot of the fraction, and 0.1 mL of the iron reagent (2 % ferric ammonium sulphate in 2 mol/L HCl) were added to 10 mL screw cap tube and then vortexed. The tube was capped loosely and put in a boiling water bath for 15 min. The absorbance of formed colored complex was read at 550 nm. Condensed tannins were expressed as leucocyanidin equivalent (Hagerman, 2011).

Determination of total flavonoids
Total flavonoids were determined spectrophotometrically by the method described by Kivrak et al., (2009). 0.2 ml of each obtained extract was added to test tubes containing 0.1 ml 10 % aluminium nitrate (Sigma), 0.1 ml 1M potassium acetate (Sigma) and 3.8 ml ethanol (Merck). The reaction time was 40 min at ambient temperature. The absorbance was measured at 415 nm. The results were expressed in mg equivalent of quercetin per g dry weight (DW).

Determination of total polyphenolic compounds
The total phenolic contents were measured using a Folin-Ciocalteu assay. Folin-Ciocalteu reagent (1mL) (Sigma) diluted five times was mixed with 0.2 mL of sample and 0.8 mL 7.5 % Na_2CO_3. The reaction was 20 min at room temperature in darkness. After reaction time, the absorption of sample was recorded at 765 nm against blank sample, developed the same way but without extract. The results were expressed in mg equivalent of gallic acid (GAE) per g dry weight (DW), according to calibration curve; build in range of 0.02 - 0.10 mg gallic acid used as a standard.

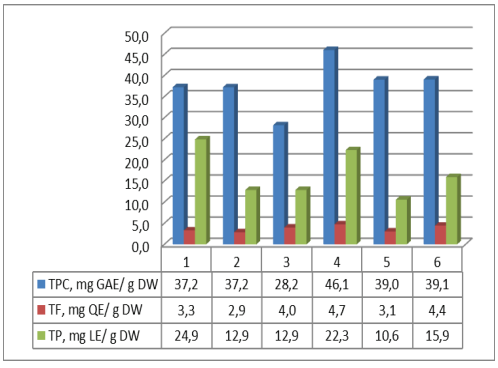
Antioxidant activity (AOA)
DPPH radical scavenging activity: Investigated extract (150 µl) were mixed with 2850 µl freshly prepared DPPH solution (0.1 mM in methanol). The mixtures were incubated for 15 min at 37 °C in darkness and the reduction of absorbance at 517 nm was measured by spectrophotometer. A standard curve was created with Trolox in concentration between 0.005 and 1.0 mM. The results are expressed in mM Trolox® equivalents (TE) per g dry weight (DW).

Cupric ion reducing antioxidant capacity: The reaction was started by mixing of 1 ml $CuCl_2xH_2O$ (10 mM in dd H_2O), 1 ml Neocuproine (7.5 mM in methanol), 1 ml ammonium acetate buffer (0.1 M; pH 7.0), 100 µl of investigated extract and 1 ml dd H_2O. The reaction time was 20 min at 50 °C. After cooling, the absorbance (450 nm) was read against a reagent blank, developed on the same way but the extract was replaced with methanol. A standard curve was created with Trolox. The results are expressed in mM Trolox® (TE) per g DW.

RESULTS AND DISCUSSIONS

Decoction extract presented the highest phenolic content (39 - 46 mg GAE /g DW), followed by the infusion (28-37 mg GAE /g DW) (Figure 1) as these content account for 10.8%, 7.3% and 6.3% of dry weight of each extract, respectively (Figure 2). The phenolic contents obtained in this study is similar to the naturally found in strawberry leaves extracts as report by Mudnic et al., (2009) and Buendia et al., (2010). Many authors detailed description and identified of phenolic compounds from strawberry leaves and other extracts (Buendia et al., 2010, Buricova et al., 2011, Liberal et al., 2014). Through HPLC–MS, GC-MS methods ware described and separated, quantificated of phenolic compounds such as flavonoids (quercetin-3-glucuronide kaempferol-3-glucoside), procyanidines (procyanidin B1, epigallocatechin, catechin, (epi)afzelechin-(epi)catechin), ellagitannins (sanguiin H-6, castalagin,

lambertianin C, galloyl-bis-HHDP-glucoseb and ellagic acid glucosides). In the present study the total amount of investigated polyphenols has been quantified. On this base the total flavonoid and total proanthocyanidins contain in water extracts has been analyzed (Figure 1) The highest content of total proanthocyanidins was obtain from leaves collected in May (blooming period) from natural habitats near "Vruhovruh" hut – 24.9 mg LE/g DW and 22.3 mg LE/g DW infusion and decoction, respectively. Similar results for the concentration of total proanthocyanidins were reported by Buendia et al., (2010) and Ivanov et al (2014). Population from "Vruhovruh" hut (24.9 mg LE/g DW) accumulated twice times more proanthocyanidins in leaves than population growth in blooming period nearby "Zdravec" hut (12.9 mg LE/g DW). High concentration of flavonoids was detected in infusion and decoction extracts obtained from natural population from "Zdravec" hut collected in October – 4.0 and 4.4 mg QE/ g DW (Figure 1).



	1	2	3	4	5	6
TPC, mg GAE/ g DW	37,2	37,2	28,2	46,1	39,0	39,1
TF, mg QE/ g DW	3,3	2,9	4,0	4,7	3,1	4,4
TP, mg LE/ g DW	24,9	12,9	12,9	22,3	10,6	15,9

Figure 1. The total polyphenolics, flavonoids and proanthocyanidins in different water extracts from *Fracaria vesca* L. leaves (from 1 to 3, infusion, and from 4 to 6 decoction); 1 and 4 from natural habitats near "Vruhovruh" hut collect in May; 2 and 5 from natural habitats near "Zdravec" hut collect in May; 3 and 6 from natural habitats near "Zdravec" hut collect in October.

In our study, we decided to evaluate antioxidant activities of water extracts of *F. vesca* by application of two methods, based on mixed hydrogen atom transfer (HAT) mechanisms (DPPH) and a method, based only on and single electron transfer SET mechanism (CUPRAC). To evaluate antioxidant activities of investigated water extracts, their abilities to scavenge DPPH radicals, as well as their power to reduce cupric (CUPRAC) ions were investigated (Figure 3). Decoction extract from Vruhovruh's hut population was the extract with the highest antioxidant activity in all used extracts (325.0 and 1257.9 mM TE/g DW for DPPH and CUPRAC methods, respectively). The extract obtained from population grown neat "Zdravec" hut collected from October was the extract with the lowest activity (164.8 and 487.5 mM TE/g DW for DPPH, and CUPRAC methods, respectively) (Figure 3).

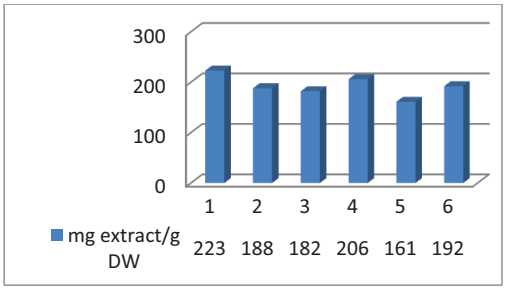
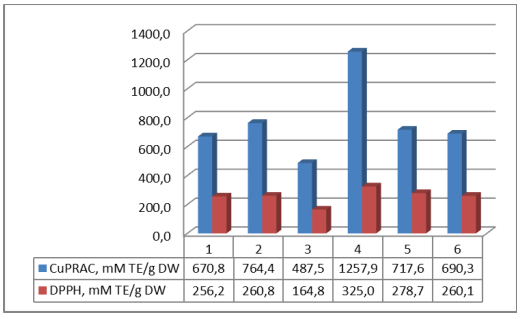


Figure 2. Total extract in different water extracts from leaves of *Fracaria vesca* L. (from 1 to 3, infusion, and from 4 to 6 decoction); 1 and 4 from natural habitats near "Vruhovruh" hut collect in May; 2 and 5 from natural habitats near "Zdravec" hut collect in May; 3 and 6 from natural habitats near hut "Zdravec" collect in October.



	1	2	3	4	5	6
CuPRAC, mM TE/g DW	670,8	764,4	487,5	1257,9	717,6	690,3
DPPH, mM TE/g DW	256,2	260,8	164,8	325,0	278,7	260,1

Figure 3 Antioxidant activity obtain from different water extracts from leaves of *Fracaria vesca* L. (from 1 to 3, infusion, and from 4 to 6 decoction); 1 and 4 from natural habitats near "Vruhovruh" hut collect in May; 2 and 5 from natural habitats near "Zdravec" hut collect in May; 3 and 6 from natural habitats near hut "Zdravec" collect in October.

The results showed the correlation between total polyphenolics in investigated extracts and their antioxidant activities 89 % and 98 % for CuPRAC and DPPH methods, respectively. Correlation between total proanthocyanidins and antioxidants activity was 45%. These results suggest that antioxidant activities were

obtained mainly from hydrolysable tannins, which were included in the total polyphenolics analysed with Folin-Ciocalteu reagent.

CONCLUSIONS

The current report detailed information for phenolic content and antioxidant activity of edible strawberry *Fragaria vesca* L leaves grown in Bulgaria. The antioxidant potential of aqueous extracts of *Fragaria vesca* L leaves, shown positively correlation with total polyphenolic contents, are important source of proanthocyanidins and tannins with potential application as radical scavengers and metal reducing activity. Therefore, this complex of biologically active substance offers many future applications in field of herbal medicine and nutrition for production of healthy food with well-pronounced healthy effect.

REFERENCES

Borah M., Ahmed S., Das S. 2012. A comparative study of the antibacterial activity of the ethanolic extracts of *Vitex negundo* L., *Fragaria vasca* L., *Terminalia arjuna* and *Citrus maxima*, Asian Journal of Pharmaceutical and Biological Research 2(3), 183-187

Buendia B., Gil M.I., Tudela J.A., Gady A.L., Medina J.J., Soria C., Lopez J.M., Tomas-Barberan F.A. 2010. HPLC-MS analysis of proanthocyanidin oligomers and other phenolic in 15 strawberry cultivars J. Agric. Food Chem., 58(7), 3916-3926

Buricova L., Andjelkovic M., Cermakova A., Reblova Z., Jurcek O., Kolehmainen E, Verhe R., Kvasnicka F., 2011. Antioxidant capacity and antioxidants of strawberry, blackberry, and raspberry leaves. Czech. J. Food Sci., 29(2), p. 181-189

Cheel, J., Theoduloz, C., Rodríguez, J.A., Caligari, P.D.S., Schmeda-Hirschmann,G., 2007. Free radical scavenging activity and phenolic content in achenes and thalamus from *Fragaria chiloensis* ssp. chiloensis, *F. vesca* and *F. x ananassa* cv. Chandler. Food Chemistry 102, 36–44.

Folta, K.M., Gardiner, S.E. (Eds.), 2009. Genetics and Genomics of Rosaceae. Springer, New York, New York (doi: 10.1007/978-0-387-77491-6).

Hagerman, A., 2011. The Tannin Handbook http://www.users.muohio.edu/hagermae/tannin.pdf.

Kanodia I., Petkova N., Pavlov A., Denev P. 2014, Optimization of proantocyanidine extraction process from Fragaria vesca L. leaves. Scientific Bulletin. Series F. Biotechnologies, 18, 115-118

Kanodia, L., Borgohain, M., Das, S., 2011. Effect of fruit extract of *Fragaria vesca* L. on experimentally induced inflammatory bowel disease in albino rats. Indian Journal of Pharmacology 43, 18–21.

Kiselova, Y., Ivanova, D., Chervenkov, T., Gerova, D., Galunska, B., Yankova, T., 2006. Correlation between the in vitro antioxidant activity and polyphenol content of aqueous extracts from Bulgarian herbs. Phytotherapy Research 20, 961–965.

Kivrak I., Duru M., Öztürk M., Mercan N., Harmandar M., Topçu G., 2009. Antioxidant, anticholinesterase and antimicrobial constituents from the essential oil and ethanol extract of *Salvia potentillifolia*. Food Chem., 116, 470-479

Liberal J., Francisco V., Costa G., Figueirinha A., Amaral M.T., Marques C.M., Girao H., Lopes M.C., Gruz M.T., Batista M.T. 2014. Bioactivity of *Fragaria vesca* leaves through inflammation, proteasome and autophagy modulation Journal of Ethnopharmacology, 158, 113-122

Mudnic I., Modun D., Brizic I., Vukovic J., Generalic I., Katalinic V., Bilusic T., Ljubenkov I., Boban M. 2009. Cardiovascular effects in vitro of aqueous extract of wild strawberry (*Fragaria vesca* L.) leaves. Phytomedicine, 16, 462-469

Neves, J.M., Matos, C., Moutinho, C., Queiroz, G., Gomes, L.R., 2009. Ethnopharmacological notes about ancient uses of medicinal plants in Trás-os-Montes (northern of Portugal). Journal of Ethnopharmacology 124, 270–283

Pistón M., Machado I.,. Branco C.S., Cesio V., Heinzen H., Ribeiro D., Fernandes E., Chisté R.C., Freitas M. 2014. Infusion, decoction and hydroalcoholic extracts of leaves from artichoke (*Cynara cardunculu*s L. subsp. *cardunculus*) are effective scavengers of physiologically relevant ROS and RNS Food Research International 64, 150–156

Porter L.J, Hirstich L.N., Chan B.G., 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochem., 25, p. 223–230

Sarić-Kundalić, B., Dobes, C., Klatte-Asselmeyer, V., Saukel, J., 2010. Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina. Journal of Ethnopharmacology 131, 33–55.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

EVALUATION OF CONSUMERS' TENDENCY TO DRY AGED BEEF MEATS

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Abstract

The survey was undertaken to determine the consumers' tendency to dry aged beef meats in Campus of Selcuk University at Konya between October-November 2014 with a total number of 155 consumers. As a result of the research, the followings were found out: 53,5% of participants had information on dry aging applications, 46,5% had no information on this. 20% of participants were consuming dry aged meat products, 80% had never consume these products. 54.8% of participant did not know the product, 30,8% did not find them attractive for consumption, 14,4% did not prefer these foods because of their expensive price. When the participants were taken into consideration with income levels changing from 4001 to 5000 TL (Turkish lira), the group was observed with the highest (63,6%), while 1000-2000 TL group was observed the lowest recognition rate (44,4%). When the way of getting information about the dry aged meat products were taken into consideration, post-graduate education was playing an important role on recognation of the products ($p<0.05$). Thus, social-mass media and educatio level, may have a significant impact on spread of this product and may become easily accessible.

Key words: *dry aging, beef meat, consumers' tendency, survey.*

INTRODUCTION

The changes in the socio-economic structure of society are reflected in their consumption habits. In other words, the alimentation culture of a society is ranging and developing, it is influenced by various factors such as geography, climate, agriculture, industrialization and the spread of mass media (Gift and Özdoğan 2005; Baysal, 2002; Lohr 2003; Brown et al., 2000).

Red meat consumption in Turkey can be affected by many factors such as economic reasons; the annual population growth rate and the changes in structure of the population, consumer preferences, quality of the products, distribution of the products, consumer education, hygienic characteristics of meat, religious beliefs, health problems, climate conditions, traditions and food-related ads (Şeker et al, 2011; İçöz 2004; Stefanikova et al, 2006).

A particular age group, social class, income and education levels have affects on the consumption choice of consumer to belong to a specific rural or urban environment. In addition, the rapid increase in the number and types of foodstuffs and social-mass media are effective in consumer preferences (Kızılarslan and Kızılarslan 2008).

Dry aging applications are new concepts for Turkey. To become widespread of these applications, the socio-economic structure of the country, nutrition culture, the impact of social media and mass media, as well as climate and industrialization are undeniable factors. This study was conducted to identify public awareness and consumer trends related to the dry-aging treated meat products.

MATERIALS AND METODS

The material of this study was composed of data obtained from the survey. The survey had been implemented according to the voluntary basis among people over 18, between the dates of October-November 2014 in University of Selcuk campus in Konya-TURKEY. At the first part, 5 questions were asked to determine the independent variable (age, gender, education level, marital status, income); In the second part of the survey 6 closed-ended questions were asked to the participants to determine their consumption behavior. Results and percentage frequency of the research were determined by using the SPSS 21.0 software package. Chi-square (Pearson's Chi-Square) test was applied to investigate the effect of "income" and "level of education" on consumer preferences.

RESULTS AND DISCUSSIONS

The findings of the research consisted of demographic characteristics of the consumers, awareness of the dry aged meat products, consumers purchasing preferences of these products and future-oriented attitudes.

Data are related to the demographic characteristics of the participants are shown in Table 1. In the study, 58 women (37.4%) and 97 men (62.65%); totaly 155 people participated. Most participants (40%) were in 19-30 age group. 40% of participants were determined that, their monthly average income level were between 2001 and 3000 TL (Turkish lira). It is found that, the 50% of participants have postgraduate education level.

Table 1. Characteristics of the surveyed consumers

Factors	Groups	n	%
Gender	Female	58	37.4
	Male	97	62.6
Marital Status	Married	100	64.6
	Single	55	35.4
Age	19-30	62	40
	31-40	39	25.2
	41-50	36	23.2
	51-60	17	11
	More than 60	1	0.6
Mountly Income (TL)	1000-2000	27	17.4
	2001-3000	62	40
	3001-4000	21	13.5
	4001-5000	22	14.3
	More than 5000	23	14.8
Education Level	Postgraduate	78	50.3
	Undergraduate	28	18.1
	Secondary Education	49	31.6

TL:TurkishLira

Table 2. The evaluation of the survey questions.

Survey Questions		n	%
1. Are you aware of dry aging concept?	Yes	83	53.5
	No	72	46.5
2. Have you ever consumed dry aged meat products?	Yes	31	20.0
	No	124	80.0
3. Why do not you prefer eating dry aged meat products?	Not know the product	57	54.8
	Very expensive	15	14.4
	Not attractive to consumption	32	30.8
4. How did you get information about dry aged products?	Education	40	35.1
	Social media and mass media	33	28.9
	Milieu	41	36.0
5. Do you think consuming these products in the future?	Yes	76	49.0
	No	18	11.6
	I am not sure	61	39.4
6. Would you recommend these products to people around you?	Yes	70	45.2
	No	22	14.2
	I am not sure	63	40.6

The data are obtained from the survey results without taking into account gender, education level and overall evaluation, the average monthly income is given in Table 2. In the study, it was observed that, 53.5% of participants had information about the application of dry aging and these products, but 46.5% of participants had not.

Considering the prevalence of consumption the products, 20% of participants had

consumed dry aged meat products but 80% of participants had not. Evaluating the reasons of the preference for the products, results were observed that; 54.8% of the respondents had never heard of this concept, 30.8 of them did not find it attractive for consumption, 14.4% found it very expensive.

Table 3.Survey findings according to the participants' income levels

Are you aware of dry aging concept ?						
Mountly Income (TL)		**Yes**	**No**	**Total**	**p**	**Chi Square**
1000-2000	**n**	12	15	27	0.76	1.87
	%	44.4	55.6	100		
2001-3000	**n**	34	28	62		
	%	54.8	45.2	100		
3001-4000	**n**	11	10	21		
	%	52.4	47.6	100		
4001-5000	**n**	14	8	22		
	%	63.6	36.4	100		
More than 5000	**n**	12	11	23		
	%	52.2	47.8	100		
Total	**n**	83	72	155		
	%	53.5	46.5	100		

TL: Turkish Lira

Looking at participants; awareness of the product according to the income levels (Table 3) revealed that; the group with 4001-5000 TL income level has the highest level (63.6%),the group with 1000-2000 TL income has the lowest level (44.4%). Differences between income levels were found statistically insignificant (Table 3; $p>0.05$). This showed that, income levels were not important criteria for the recognition of the product.

Table 4. Survey findings according to the participants income levels

Have you ever consumed dry aged meat products?						
Mountly Income (TL)		**Yes**	**No**	**Total**	**p**	**Chi square**
1000-2000	**n**	3	24	27	0.24	5.50
	%	11.1	88.9	100		
2001-3000	**n**	11	51	62		
	%	17.7	82.3	100		
3001-4000	**n**	5	16	21		
	%	23.8	76.2	100		
4001-5000	**n**	8	14	22		
	%	36.4	63.6	100		
More than 5000	**n**	4	19	23		
	%	17.4	82.6	100		
Total	**n**	31	124	155		
	%	20	80	100		

TL: Turkish Lira

The most consumption of dry aged meat products (36.4%) have been observed in the group with 4001-5000 TL income level (Table 4). But there is no statistically significant difference between consumption and income levels ($p>0.05$; Table 4)

Table 5. Survey findings according to the participants' income levels

Why do not you prefer to dry aged meat products?							
Mountly Income (TL)		**Not know the product**	**Very expensive**	**Not attractive to consumption**	**Total**	**p**	**Chi square**
1000-2000	**n**	13	4	2	19	0.71	5.41
	%	68.4	21.1	10.5	100		
2001-3000	**n**	19	6	14	39		
	%	48.7	15.4	35.9	100		
3001-4000	**n**	10	1	5	16		
	%	62.5	6.3	31.3	100		
4001-5000	**n**	6	2	3	11		
	%	54.5	18.2	27.3	100		
More than 5000	**n**	9	2	5	16		
	%	56.3	12.5	31.3	100		
Total	**n**	57	15	29	101		
	%	56.4	14.9	28.7	100		

Taken into consideration to the participants' income; rate of 56.4% due to lack of knowledge, 14.9% found the products too expensive, 28,7% found them not attractive for consumption were determined. These differences were determined to be statistically insignificant (Table 5; $p> 0.05$). However, significant effects on consumption are assumed to be provided by increasing the product's presentation.

Table 6. Survey findings according to the participants' education levels

How did you get information about dry aged products?							
Education Level		**Education**	**Social media and Mass media**	**Milieu**	**Total**	**P**	**Chi Square**
Post graduate	n	28	8	7	43	0.001	18.16
	%	65.1	18.6	16.3	100		
Under graduate	n	5	5	6	16		
	%	31.3	31.3	37.5	100		
Secondary Education	n	7	4	17	28		
	%	25	14.3	60.7	100		
Total	n	40	17	30	87		
	%	46.0	19.5	34.5	100		

The way of getting information about the dry aged meat products have been assessed, postgraduate education has been found to play an important role on recognition of the product (Table 6; $p < 0.05$).

Considering the way of getting information on product, the participants with the secondary education has been identified as having the most significant impact on the milieu, also the participants with undergraduate, education, social media, mass-media and social environment has the same impact degree.

CONCLUSIONS

The research was conducted by researchers own equity and was not supported by any organization. Therefore, the study sample was limited only for Konya Selcuk University Campus. This condition may weaken the power of representation of the research and the results achieved through the research should only be considered to represent the students and the faculty members of Selcuk University. On the other hand, the survey was made with voluntary basis by the participants, there has been a cluster towards to the high education level. The results of research could be affected on favor of postgraduates as stated by Şeker et al. (2011).

Production-marketing-consumption chain are essential in a society. For the development of community, conscious, responsible and trained producers, marketers and consumers are needed. Educated and informed consumers will affect the mold of production and the marketing sector and they will provide the change in the economy and society.

In this research, consumers' awareness, attitudes and knowledge levels to dry aged meat products were determined. The high prices of the this type of meat products and the lack of accessibility though them prevent the spread of the product and also make them less consumable by people with all levels of income. The main problem is considered inadequacy of the new products release. According to the research results, education level and social media-mass have important effects on this product to be widespread and to be easily accessible.

REFERENCES

Baysal, A. 2002. Beslenme kültürümüz. 3th edition. T.C. Kültür ve Turizm Bakanlığı Geleneksel El Sanatları, Ankara,TURKEY.

Lohr, L. 2003. Factors Affecting International Demand And Trade in Organic Food Products. Economic Research Service/USDA. P, 67-79. http//:www.USDA/EconomicResearchService/Changing Structure of Global Food Consumption and Trade/WRS-01-1.

Browne, A.W., Haris P.J.C., Hofny-Collins A.H., Pasiecznik N., Wallace R.R.,2000. Organic Production And Ethical Trade: Definition, Practice and Links. Food Policy. 25: 69-80.

Cevger Y, Aral Y, Demir P, Sarıözkan S., 2008. The situation of animal products consumption and consumer preferences among intern students at the Ankara University Faculty of Veterinary Medicine, Ankara Üniv Vet Fak Derg, 55, 189-194.

İçöz Y.,2004. Talep kavramı, kırmızı et ve et ürünlerinde talebi etkileyen faktörler. Tarımsal Ekonomi Enst, *T.E.A.E-BAKIŞ*, 7(1):1-4. http://www.tepge.gov.tr/Dosyalar/Yayinlar/f730417d23e84174b2a323e10f1a170a.pdf

Stefanikova Z, Sevcikova L, Jurkovicova J, Sobotova L, Aghova L., 2006. Positive and negative trends in university students' food intake. *Bratisl Lek Listy*, 107: 217-220.

Kızılaslan N, Kızılaslan H,2008.Knowledge Level and Attitudes of the Consumers Related to the Food.

Products They Buy (Tokat City Example). Journal of Agricultural Faculty of Uludag University, 22(2):67-74.

Armağan G,Özdoğan M., 2005 .Consumption Patterns of Ecological Egg and Chicken Meat and Determining the Consumer Preferences. Hayvansal Üretim, 46(2): 14-21.

Şeker İ., Özen A., Güler H., Şeker P., Özden İ., 2011. Red Meat Consumption Behavior in Elazığ and Consumers' Opinion in Animal Welfare. Kafkas Univ Vet Fak Derg.17(4):543-550.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PHYSICAL-CHEMICAL PROPERTIES OF MILK FAT GLOBULE MEMBRANE AT DIFFERENT STAGES OF ISOLATION

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Abstract

Fat globules in milk are present in form of emulsion stabilized by milk fat globule membrane (MFGM) which originates in mammary gland. Buttermilk which is rich source of *MFGM finds application as an ingredient in dairy and food industry (emulsions, infant formulas & low fat products) for its emulsifying and stabilizing properties which are associated to polar lipids. Phospholipids & some proteins have also shown some medicinal properties. So, MFGM received much attention in recent years due to its health-beneficial & technological properties. This work has been carried out with the objective to characterize MFGM at different stages of extraction. MFGM fractions were isolated from fresh cream and collected at different steps during processing to study its physical-chemical characterization. The fractions were characterized for their chemical composition to have an idea at which stage which components are isolated. The sample were also characterized for their surface properties which included study of mechanical properties of monolayer using Langmuir film balance, surface tension and zeta potential. Physical-chemical studies of fractions suggest that each fraction of MFGM have different chemical composition and surface properties which could be of interest for use in different food products depending on the requirement. The choice of these fractions could be done for their use as food additive/ingredient in food industry depending on the application. It may even have an extended application in pharmaceutical industry.*

Key words**:** *Composition, Milk Fat Globule Membrane, Surface properties, Valorization.*

INTRODUCTION

Milk fat exists as an emulsion of tiny, spherical oil globules (Jensen, 2002). The diameter of milk fat globules ranges from 0.2 to 15 µm which is surrounded by a 4 to 10 nm milk fat globule membrane (MFGM) (Danthine *et al*., 2000; Singh, 2006). This MFGM (2-6% of Fat globules) is the protective coat which ensures structural integrity of milk fat. It has tripartite structure. The primary monolayer surrounding the fat globule is rich in proteins and is derived from endoplasmic reticulum membrane. The secondary bilayer is rich in polar lipids and is derived from plasma membrane (Vanderghem *et al*., 2010).

Over last two decades, many studies revealed composition & nutritional aspects of MFGM. Models of structure & organization have been continuously up-dated (Danthine *et al*., 2000; Vanderghem *et al*., 2011). Work related to the physical-chemical properties have also been carried out. These work mainly focused on studying the role of various components of native MFGM towards surface and techno-functional properties (Shimizu *et al.*, 1980; Innocente *et al.*, 1997; Karray *et al.*, 2006; Danthine & Blecker, 2014; Kanno *et al*., 1991; Phan *et al*., 2014; Corredig and Dalgleish, 1998; Dzul-Cauich *et al*., 2013).

In present work MFGM fractions were collected at different stages of isolation. The effects of compositional differences of MFGM fractions on the surface properties were studied.

MATERIALS AND METHODS

Milk Fat Globule membrane extraction- MFGM was isolated from fresh raw cream following the procedure described by

Vanderghem *et al*. (2008) with slight modifications. A detailed scheme for extraction of MFGM is presented in Figure 1. Cream was subjected to centrifugation between washes in Avanti J-E (Beckman Coulter Inc., Belgium). Fractions were freeze dried and stored at -50 °C. MFGM fraction 3 (F3) represents the complete MFGM extracted.

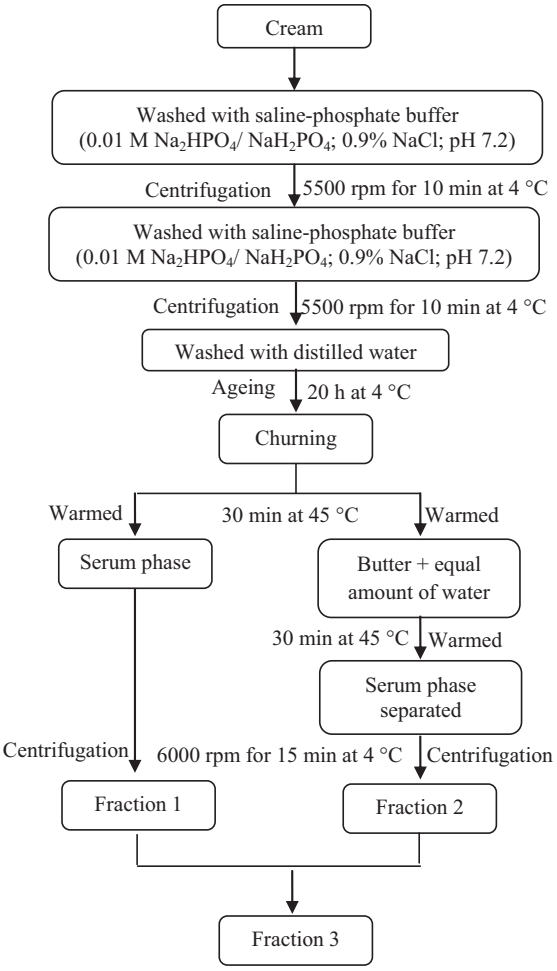

Figure 1 - Scheme for extraction of MFGM fractions

Determination of protein and lipid content of MFGM- The nitrogen content was estimated using Rapid N (Elementar Analysensysteme GmbH, Germany) and multiplied by 6.38 to get the protein content.

Lipid extraction was performed according to the method of Rombaut *et al*. (2005) with slight modifications. One g of sample was taken and washed three times with 25ml chloroform/methanol (2:1) solution (10 min handshaking and centrifugation for 5 min at 3000 rpm). The sample was subjected to centrifugation in Avanti J-E (Beckman Coulter Inc., Belgium). This supernatant was filtered (5µm filter paper) and collected in 250 ml separation funnel after every washing. Following this 40 ml 0.57% NaCl solution was added in the funnel and thorough mixed. The mixture was allowed to stand overnight and then the lower phase was collected. 40 ml chloroform was added to the funnel and after 5 hours the lower non-aqueous phase was removed. The solvent from this non-aqueous phase was evaporated by rotary vacuum evaporator RE-121 Rotavapor (BUCHI Labortechnik GmbH, Netherlands). The lipids were dissolved in 10 ml of 2:1 chloroform: methanol and stored at 4 °C.

Compression isotherm measurements- Isotherms measurements were carried out using a Langmuir film balance FW2 (Lauda, Lauda-Königshofen, Germany). Deionised water (MilliQ water; Millipore Corp., Bedford, MA, USA) was used as sub phase. The temperature of the trough was maintained at 20.0 ± 0.5 °C by external circulating water. The MFGM fractions were spread at the air water interface (80 µL, 5 mg/ ml) with a Hamilton micro syringe just at the air-water interface. A time period of 15 min was allowed for the formation of the film. The compression isotherms (Surface pressure/ Surface area) were obtained by compressing the spread monolayer at a constant rate of 90 cm^2/ min.

Zeta Potential Titration curve measurements- These measurements were carried out by using Delsa nano Zeta potential analyser (Beckman Coulter, Belgium). MFGM fractions were dispersed in Milli-Q water at the rate of 0.5% and kept overnight at 4 °C before measurement.

Zeta Potential Titration curve and isoelectric point were obtained by electrophoretic light scattering method. MFGM fractions were subjected to titration with 0.1N HCl in the pH range of 6.5 to 2.

Dynamic Surface Tension measurements- Dynamic surface tension measurements were determined by an automated drop volume tensiometer (TVT1, Lauda, Germany) at the air/water interface at 25 °C. MFGM fractions were dispersed in Milli-Q water at the rate of 0.5% and kept overnight at 4 °C before measurement.

RESULTS AND DISCUSSIONS

Yield and composition of MFGM fractions

The amount of cream used for extraction of MFGM was 1200g. A total of 9.70 g of MFGM was recovered, of which Fraction 1 was 4.70 g and Fraction 2 was 4.97 g.

The average percentage value of protein recorded for MFGM F1 and MFGM F2 were 44.19% and 28.76% respectively on wet basis. The average percentage value of fat recorded for MFGM F1 and MFGM F2 were 31.22% and 63.88% respectively on wet basis. These values were in accordance with the values reported in literature (Danthine *et al*., 2000; Dewettinck *et al*., 2008).

Study of monolayers

Langmuir film balance allows determination of the surface pressure as a function of surface area which reflects the packing of monolayer (lipid-protein interactions) at air-water interface.

Figure 2 shows the compression isotherms (π-A isotherm) of three fractions of MFGM. When monolayers were compressed, two transition phases were identified for MFGM F3 and one transition phase was identified for MFGM F1 and MFGM F2. Phase one appears when it undergoes compression; the surface pressure increases until an inflection point (A_{t1}, π_{t1}). Upon further compression, the second transition phase is characterized by an increase in the slope in the π-A isotherm until an inflection point (A_{t2}, π_{t2}). Finally, the horizontal break in the isotherm can be considered as the collapse of the monolayer and is characterized by maximum surface pressure (π_c). The values of the characteristics points of the π-A isotherm are presented in table 1.

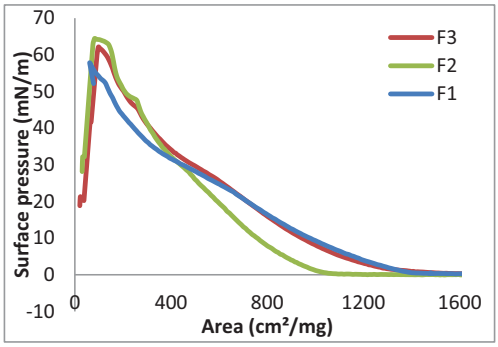

Figure 2 - Compression isotherm of a MFGM fractions monolayer

Table 1 - Characteristic points and film elasticity of the compression isotherm for MFGM fractions

Characteristics	F3	F2	F1
A_{t0} (cm^2/mg)	1270.15	986.13	1349.64
A_{t1} (cm^2/mg)	637.47	-	632.57
A_{t2} (cm^2/mg)	254.76	259.61	-
π_{t1} (mN/m)	23.84	-	23.55
π_{t2} (mN/m)	45.75	47.34	-
π_c (mN/m)	62.11	63.19	52.53
Film elasticity (mN/m)	23.33	30.06	18.17

The first transition phase of MFGM F3 coincides with transition phase of MFGM F1 and second transition phase of MFGM F3 coincides with MFGM F2. These differences

could be attributed to the compositional differences. MFGM F1 had a higher protein content compared to MFGM F2 and MFGM F2 had a higher lipid content compared to MFGM F1.

The π-A isotherm profile of MFGM is more or less similar to previous published work. Innocente *et al*. (1997) reported the two transition phases for MFGM compression isotherm at surface pressure of 22 mN/ m and 48 mN/ m. Similarly Danthine and Blecker (2014) reported the two transition phases for MFGM compression isotherm at surface pressure of 28.5 mN/ m and 49 mN/ m. In our work the two transition phases for MFGM F3 compression isotherm were recorded at surface pressure of 24 mN/ m and 46 mN/ m. A little difference was observed for values of surface pressure at the transition phase. These differences observed could be attributed to the difference in the membrane composition which could certainly modify the phase behaviour of the monolayers.

The change in surface pressure per unit change in film interfacial area is termed as Film elasticity (ε). Thus it measures resistance to change in film area (Graham and Phillips, 1980). F2 had a better film elasticity as compared to other fractions. A better film elasticity indicates better emulsion and foam stability. Thus compression isotherms could be used to indicate the stability behaviour of the fractions.

Zeta Potential Titration curve and Isoelectric point measurements

The stability of the dispersing particle is influenced by their surface charge. Zeta potential is an index of dispersion stability of particles. If zeta potential is high, particles are stable due to high electrostatic repulsion. On the contrary, if zeta potential is low (nearing zero), particles have a tendency to form an aggregate which is referred to as isoelectric point (Greenwood and Kendall, 1999).

Figure 3 shows the zeta potential titration curve for the three MFGM fractions. The isoelectric pH of MFGM F1, MFGM F2 and MFGM F3 is 4.24, 3.73 and 4.06 respectively. This difference could be attributed to difference in their protein composition. All fractions were found to be stable in the pH range above 5.5 and below 3.0 (Table 2).

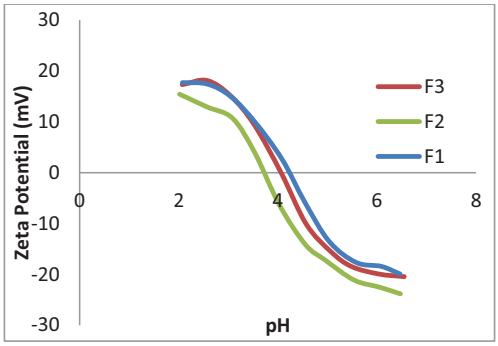

Figure 3 - Zeta Potential Titration curve for MFGM fraction

Table 2- Zeta potential (mV) values in pH range 6.5-2.0

pH	MFGM F1	MFGM F2	MFGM F3
6.5	-19.93	-23.83	-20.45
6.0	-18.44	-22.54	-19.94
5.5	-17.65	-21.11	-18.46
5.0	-13.51	-17.20	-15.57
4.5	-5.75	-14.16	-10.14
4.0	2.65	-6.39	-0.11
3.5	9.92	3.93	9.42
3.0	15.02	10.69	15.07
2.5	17.42	12.93	18.12
2.0	17.71	15.43	17.31

Thus, the MFGM fractions could be used in food products which are in this pH range without affecting the stability of the products.

Study of Dynamic Surface Tension

The dynamic surface tension gives the surface tension at particular surface age. Surface active substances cause a lowering of surface tension. MFGM fractions were characterized by the drop volume tensiometer in the dynamic mode at 25 °C.

Surface tensions of MFGM F1, F2 and F3 at concentration of 5 mg MFGM/ ml at 60 sec were recorded to be 51 mN/m, 53.6 mN/ m and 53.3 mN/ m respectively (Figure 4). Surface tension of MFGM at concentration of 1 mg MFGM/ml and 13.5 mg MFGM/ml at 60 sec were reported as 57 mN/ m and 51 mN/ m respectively (Vanderghem, 2009). A slight difference in the values could be attributed to the different concentration and compositional difference of the MFGM.

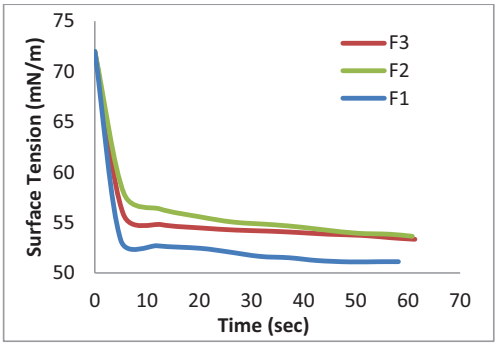

Figure 4 - Dynamic surface tension curves for MFGM fractions

The differences in the adsorption kinetics curves of different MFGM fractions could be explained by the different protein and lipid composition of different fractions. Equilibrium in surface tension value was attained within 30 sec for both MFGM F1 and MFGM F2 and within 15 sec for MFGM F3. The dynamics of adsorption has importance in determining the emulsifying stability. The time required for obtaining equilibrium in surface tension indicates the emulsifying stability of the substance.

CONCLUSIONS

Each fraction of MFGM had different chemical composition which contributed towards different surface properties. The surface properties can be used as a tool to study the techno-functional properties and the stability behavior of the MFGM fraction. Further it could be interesting to verify these results by studying the techno-functional properties of these fractions.

ACKNOWLEDGEMENTS

This research was made possible from the financial support of CURAGx, Gembloux Agro-Bio Tech, University of Liege, Belgium.

REFERENCES

Corredig M., Dalgleish D.G., 1998. Characterization of the interface of an oil-in water emulsion stabilized by milk fat globule membrane. Journal of Dairy Research, 65:465-477.

Danthine S., Blecker, C., 2014. Interactions of lipases with milk fat globule membrane monolayers using a Langmuir film Balance. International Dairy Journal, 35:81-87.

Danthine S., Blecker C., Paquot M., Innocente N., Deroanne, C., 2000. Evolution des connaissances sur la membrane du globule gras du lait: synthèse bibliographique. Lait, 80:209-222.

Dewettinck K., Rombaut R., Thienpont N., LE T.T., Messens K., Van Camp J., 2008. Nutritional and technological aspects of milk fat globule membrane material. International Dairy Journal, 18:436-457.

Dzul-Cauich J.G., Lobato-Calleros C.., Pérez-Orozc J.P., Alvarez-Ramirez J., Vernon-Carter E.J., 2013. Stability of water-in-oil-in-water multiple emulsions: influence of the interfacial properties of milk fat globule membrane. Revista Mexicana de Ingeniería Química, 12(3):425-436

Graham D. E., Phillips M. C., 1980. Proteins at liquid interfaces: dilatational properties. Journal of Colloid Interface Science, 76:227-231.

Greenwood R., Kendall K., 1999. Electroacoustic studies of moderately concentrated colloidal suspensions. Journal of the European Ceramic Society, 19(4):479–488.

Innocente N., Blecker C., Deroanne C. & Paquot M, 1997. Langmuir film balance study of the surface properties of a soluble fraction of milk fat-globule membrane. Journal of Agricultural & Food Chemistry, 45: 1559-1563.

Jensen R.G., 2002. The composition of bovine milk lipids. January 1995 to December 2000. Journal of Dairy Science, 85(2):295-350.

Kanno C., Shimomura Y., Takano E., 1991. Physicochemical properties of milk fat emulsions stabilized with bovine milk fat globule membrane. Journal of Food Science, 56:1219-1223.

Karray N., Danthine S., Blecker C., Attia H., 2006. Contribution to the study of camel milk fat globule membrane. International Journal of Food Science & Nutrition, 57(6/7):382-390.

Phan T.T.Q., Le T.T., Van der Meeren P., Dewettinck, K., 2014. Comparison of emulsifying properties of milk fat globule membrane materials isolated from different dairy by-products. Journal of Dairy Science, 97:4799–4810.

Rombaut R., Camp J.Y., Dewettinck K., 2005. Analysis of phospbo- and sphingolipids in dairy products by a new HPLC method. Journal of Dairy Science. 88:482-488.

Singh H., 2006. The milk fat globule membrane – a biophysical system for food applications. Current Opinion in Colloid & Interface Science, 11:154–163.

Shimizu M., Yamauchi K., Kanno C., 1980. Effect of proteolytic digestion of milk fat globule membrane proteins on stability of the globules. Milchwissenchaft, 35:9-12.

Vanderghem C., 2009. Etudes des properties physico-chimiques de la membrane du globule gras du lait. Doctoral dissertation, Gembloux Agro Bio-Tech, University of Liege, Belgium.

Vanderghem C., Blecker C., Danthine S., Deroanne C., Haubruge E., Guillonneau F., Pauw E.D., Francis F., 2008. Proteome analysis of the bovine milk fat globule: enhancement of membrane purification. International Dairy Journal, 18:885-893.

Vanderghem C., Bodson P., Danthine S., Paquot M., Deroanne C., & Blecker C., 2010. Milk fat globule membrane and buttermilks: from composition to valorization. Biotechnology Agronomy Society Environment, 14:485-500.

Vanderghem C., Francis F., Danthine S., Deroanne C., Paquot M., De Pauw E. & Blecker C., 2011. Study of the susceptibility of the bovine milk fat globule membrane proteins to enzymatic hydrolysis & organization of some of the proteins. International Dairy Journal, 21:312-318.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ANTIOXIDANT ACTIVITY AND BIOACTIVE COMPOUNDS OF *ROSA CANINA* L. HERBAL PREPARATIONS

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Abstract

The aim of the present research was to make a comparison between two Bulgarian commercial forms of Rosa canina L. for herbal preparations in terms of the possible benefits by oral intake. The plant samples were investigated for their antioxidant activity and the bioactive substances. The total phenolic content of the extracts was evaluated as well. Four reliable methods (DPPH, ABTS, FRAP and CUPRAC assays) for antioxidant activity assessment were applied. The highest results were recorded by the FRAP assay.The plant Rosa canina is known as wealthy source of carotenoids and organic acids, therefore fat soluble β-carotene, lycopene and lutein in addition to water soluble malic, citric, fumaric and ascorbic acids were evaluated by HPLC-methods. According the conducted assays the tested samples have similar composition. Lutein concentration in both tested extracts was calculated as 6.9 μg/g DW. Lycopene and β-carotene were determined to be 13.91 and 12.18 μg/g DW and 27.14 and 22.83 μg/g DW, respectively. HPLC determination of organic acids showed that the amount of citric acid in the extracts was 7343 and 6583 μg/g DW and fumaric acid content in both samples was 30 μg/g DW. The size of the plant particles used for the extraction seems to contribute significantly to the exhibited activity.

Key words*: herbal tea; Rosa canina; fruits; antioxidant activity; bioactive compounds.*

INTRODUCTION

Currently there is considerable interest in new natural antioxidants to replace the synthetic ones that are used in foods and therapeutic regimens.

Rosa canina L. (the dog rose) is a shrub of the *Rosaceae* family, native to Europe, western Asia and north-eastern Africa. Fruits (hips) have been used in the traditional prevention and therapy of common cold and other infections, as a diuretic agent and for the treatment of various inflammatory diseases for a long time. Clinical efficacy has been demonstrated only for osteoarthritis (Chrubasik et al., 2006; Christensen et al., 2008; Chrubasik et al., 2008). Based on the results of other authors Kiliçgun and Dehen (2009) stated that the hips display an anti-inflammatory, antioxidant and anti-mutagen effect. Recently the potential of nutritional and therapeutic benefits among natural antioxidants was revealed based on traditional knowledge and western science (Aresenescu, 2008). The constituents of dog rose fruit (hips) are endowed with vitaminisant, astringent, colagogue, choleretic, diuretic, antidiarrhoea, antioxidant properties, etc. (Yi et al., 2007). In addition, Orhan et al. (2009) reported that the rose hips also have antidiabetic properties.

Several authors reported that the nutritive and therapeutical value of the mature dog rose fruit (*Cynosbati* fructus) is due to their content of sugars, organic acids, pectins, flavonoids, tannins, carotenoids (β-carotene, licopene, and isomeres of rubixanthin), vitamins (especially vitamin C, but also vitamins B1, B2, K, PP, D, and E), macro- and microelements etc. (Pârvu, 2000; Demir şi Ozcan, 2001; Tiță, 2003;

Stănescu et al., 2004; Arsenescu et al., 2008; Orhan et al., 2009). The dog rose seeds contain oil and minerals; the fatty acids within the dog rose oil are mainly represented by the linoleic, oleic, linolenic, palmitic, stearic, and arachidonic acid, (Ozcan, 2002). The varied content of the fruit (hips) confers the next properties: antiscorbutic, anti-inflammatory, even anti-mutagene; it also increases the biosynthesis of colagene, stimulates the immune system, improves the body resistance to sustained effort, (Pârvu, 2000; Tiță, 2003; Kiliçgun and Dehen, 2009; Orhan et al., 2009). Some properties of the dog rose hips are attributed to some hypothetical compounds of silicium. At the same time, *Rosa canina* L. is useful to prevent soil erosion, serves as a stock for roses, etc. (Arsenescu, 2008).

The *Rosa canina* L. fruits have constituted an important source of food and medicine for many cultures. Common food preparations using rose hips include juice, wine, tea, jelly, jam, as well as mixed with dried salmon eggs (Moerman, 2002).

The aim of the present study was to compare two typical *Rosa canina* commercial products used in the everyday life of people. Based on the results a most suitable tea form could be recommended for an oral intake.

MATERIALS AND METHODS

Plant material

Two different commercial available and widely used in everyday life *Rosa canina* L. forms were obtained from a local pharmacy (Plovdiv, Bulgaria). The sample A – rosehip tea in ready to use paper bags and the sample B - Rosa whole fruits, which were blended and stored at ambient temperature in the dark, until use.

Preparation of the plant extract

For the purposes of the present study different extraction procedures were applied.

In order to evaluate the total phenolic content and the antioxidant potential of the both plant samples two extraction techniques with water were conducted as described. Water was chosen as solvent based on its simple and traditional use.

- *infusion* – extracts were obtained by allowing 2 g of the plant material to remain suspended in the boiled water for 5 min and then the solution was filtered;
- *decoction* – extracts were obtained by boiling of the 2 g plant material for 30 min with 40 ml of water; The resulting solution was then filtered.

In order to evaluate chemical composition of the two investigated rose hips forms in terms of organic acids and carotenes contents, the samples were subjected to extraction as follow:

Extaction of organic acids - 0.1 g plant material was extracted with 1ml of 3 % meta - phosphoric acid (HPO_3) as previously described by Georgieva et al. (2013a).

Extraction of carotenes **-** 2 ml of methanol was added to 0.1 g plant paterial (20:1) followed by addition of 5 ml of carbon tetrachloride and methanol mixture in ratio 3:1, the solution should contain 0.5 % BHT. The extraction procedure was carried out according Georgieva et al. (2013b).

Determination of total phenolics (TPC)

A modified Kujala et al. (2000) method with Folin – Ciocalteu`s reagent was used for the determination of the total polyphenolic content (TPC). Gallic acid was employed as a calibration standard and the results were expressed as mg gallic acid equivalents (mg GAE) per gram of plant dry weight (DW).

Determination of antioxidant activity (AOA)

$DPPH^{\bullet}$ radical scavenging assay

Antioxidant activity was described as having activity against the stable form of the synthetic product $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazil) by the method of Brand-Williams et al. (1995) with slight modifications. A freshly prepared 4.10^{-4} M solution of $DPPH^{\bullet}$ (in methanol) was mixed with the sample in a ratio of 2:0.5. The unit of Trolox equivalent antioxidant capacity (TEAC) defined the concentration of Trolox having equivalent antioxidant activity expressed as μM TE/g DW.

$ABTS^{\bullet+}$ radical scavenging assay

The radicals scavenging activity of the ultrasound extract against radical cation ($ABTS^{\bullet+}$) was estimated according to a previously reported procedure with some modifications (Re et al., 1999). $ABTS^{\bullet+}$ was produced by reacting 7 mM of $ABTS^{\bullet+}$ solution with 2.45 mM of potassium persulphate, and the mixture was kept in the dark at room temperature for 12-16 h. At the moment of use,

the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30 °C. 1 ml of $ABTS^{\bullet+}$ solution was added to each sample (0.01 ml) was vigorously mixed. After reacting at 30 °C temperature for 6 min, the absorbance at 734 nm was measured. The TEAC value was defined as the concentration of Trolox having equivalent antioxidant activity expressed as μM TE per gram dry weight (μM TE/g DW).

Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of Benzie and Strain (1999) with slight modification. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe (II)-tripyridyltriazine compound from colorless oxidized Fe (III) form by the action of electron donating antioxidants. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. 150 μl of plant extracts were allowed to react with 2850 μl of the FRAP reagent solution for 4 min at 37 °C and the absorbance of the reaction mixture was recorded at 593 nm. The results were expressed as μM TE/g DW.

CUPRAC assay

The CUPRAC assay was carried out according to the procedure of Ak and Gülcin (2008). To a test tube were added 1 ml of $CuCl_2$ solution (1.0×10^{-2} M), 1 ml of neocuproine methanolic solution (7.5×10^{-3} M), and 1 ml NH_4Ac buffer solution (pH 7.0), and mixed; 0.1 ml of herbal extract (sample) followed by 1 ml of water were added (total volume = 4.1 ml), and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of herbal extracts was measured as μM TE/g DW.

Determination of organic acids and carotenes

Analyses were performed on HPLC system Waters 2487 using dual λ absorbance detector and Waters 1525 binary pump (Waters, Milford, USA).

Organic acids

Chromatographic separation was accomplished with Discovery® SH C18 column (25 x 4.6 mm) RP (5 μm) (Supelco), UV detection at 244 nm and 210 nm, column temperature 30 ^{0}C and sample volume injection 20 μl. For elution of the sample 6.0 mM aqueous solution of phosphoric acid (pH = 2.1) was used. The applied flow rate was as reported by Georgieva et al. (2013a).

Carotenes

Chromatographic separation was accomplished with Symmetry® C18 column (5μm, 15 cm x 4,6 mm), UV detection at 270 nm and 290 nm, column temperature 30 ^{0}C and sample volume injection 20 μl. Elution was performed by gradient system of mobile phase A - methanol: acetonitrile in a ratio of 8:2, and mobile phase B - MTBE (methyl tert-butyl ether). HPLC analysis was conducted with a flow rate previously described by Georgieva et al. (2013b).

Statistical analysis

All measurements were carried out in triplicates. The results were expressed as mean ± SD using MS-Excel software.

RESULTS AND DISCUSSIONS

Total polyphenolic content

The total phenolic content was determined using Folin-Ciocalteu method, reported as gallic acid equivalents by reference to a standard curve. The total phenolics in the samples ranged from 12.07 ± 0.17 to 37.17 ± 0.17 mg GAE/g DW (Table 1). The values for the good grounded tea bags in commercial form seemed to be with better phenolic content, based probably of the relatively equal plant sample particles. However, the decoction technique show better extractability compared to the infusion. The TPC for samples A and B after decoction were 37.17 ± 0.17 and 25.64 ± 5.06 mg GAE/g DW, respectively.

Dietary antioxidant capacity is related to the total polyphenol content of fruits and vegetables (Hanson et al., 2004; Leccese et al., 2007; Kubola and Siriamornpun, 2008; Beltran et al., 2009). Polyphenols clearly improve the status of different oxidative stress biomarkers

(Williamson and Manach, 2005). In this respect, it is well understood that some polyphenols, administered as supplements with food, have the ability to improve health status, and this is indicated by several biomarkers of cardiovascular risk (Keen et al., 2005).

Antioxidant activity

The results from the DPPH, ABTS, FRAP and CUPRAC assays are presented in Table 1. The values between the different methods varied widely. The authors therefore strongly suggested that, when analyzing the antioxidant activity of samples, it is better to use at least two methods due to the differences between the test systems (Ou et al., 2002).

The highest results were recordered by the FRAP assay (from 344.85 ± 7.25 to 771.86 ± 5.25 μM TE/g DW).

It has to be noted that the values of all conducted methods were in favor of the both decoction extracts. This statement is in agreement with the total phenol assay results.

Table 1. Total phenol content (mg GAE/g DW) and *in vitro* antioxidant activity (μM TE/g DW) of *Rosa canina* water extracts

Samples/ Analyses	TPC	DPPH	ABTS	FRAP	CUPRAC
Sample A infusion	18.93 ± 0.34	3.02 ± 0.02	213.38 ± 0.54	367.35 ± 3.23	115.43 ± 4.59
Sample A decoction	37.17 ± 0.17	6.32 ± 0.05	518.78 ± 0.78	771.86 ± 5.25	514.25 ± 7.19
Sample B infusion	12.07 ± 0.17	2.66 ± 0.03	283.56 ± 4.18	344.85 ± 7.25	93.15 ± 17.14
Sample B decoction	25.64 ± 5.06	3.66 ± 0.03	370.67 ± 8.34	571.15 ± 5.48	125.09 ± 20.35

Among the investigated extracts the decoction extract of sample A showed the higher CUPRAC value – 514.25 ± 7.19 μM TE/g DW, while the infusion of sample B the lowest - 93.15 ± 17.14 μM TE/g DW (Table 1).

The results of the antioxidant potential of the investigated samples by DPPH and ABTS assays correspond well to the already mentioned results obtained to the other methods. The both decoctions were with the highest antioxidant activity.

Noticeable the correlation among all results is high. This confirmed the better effectiveness of extraction accomplished by the decoction technique. This result confirmed the two investigated plant samples as a natural source of antioxidants. The investigated ready to use rose hip paper tea bags (sample A) revealed as more potent according all performed assays.

HPLC

The established amounts of the organic acids and carotenes in the investigated extracts of *R. canina* were presented in Table 2. The content of citric and fumaric acids in tested two samples was demonstrated in contrary to traces of both malic and ascorbic acids. On the other hand, Bozan et al. (1998) reported citric and ascorbic acid contents in growing in the Central Asian region, hips and described citric acid as the main organic acid in *R. canina* fruits. Pereira and co-workers (2013) investigated

Table 2. Chemical composition of *Rosa canina* extracts (μg/g DW)

Samples	Organic acids				Carotenes		
	Malic acid	Citric acid	Ascorbic acid	Fumaric acid	Lutein	Lycopene	β-carotene
Sample A	Trace	7343±24.5	Trace	31.25±2.5	6.9±0.2	13.91±0.7	27.14±2.1
Sample B	Trace	6583±20.3	Trace	27.8±1.3	6.89±0.1	12.18±0.1	22.83±3.4

malic, citric, ascorbic and fumaric acid content in several fruits including *R. canina* and confirmed their presence in the sample. The detected amounts of ascorbic and malic acids in the present study were outside the sensitivity of the applied HPLC assay which maybe due to

the sample preparation and probable losses of the substances.

In our research, we investigated and confirmed the presence of lutein, lycopene and β-carotene. The lutein concentration was established to be 6.9 μg/g DW for both extracts.

In general, the demonstrated results were relatively similar especially concerning lutein and lycopene contents. However, in sample A the amounts of all detected chemical components were higher compared to sample B.

Although additional research work is required in order to evaluate all potential activities of the investigated plant samples and the complete chemical composition, Rose hips could be considered as a functional food due to the reported in the literature health effects. In their review, Fan at al. (2014) reported the functional, medical, and physiological properties of *R. canina* as they confirmed the presence of lutein, lycopene and β-carotene.

CONCLUSIONS

The present work investigated the potential beneficial effect of the commercial varieties of *Rosa canina* used in daily life for tea preparation. The evaluated antioxidant activity and total phenolic content of two water extracts revealed the capacity in favour of the rose hip sample, which was more homogeneously grounded.

The chemical composition of the tested samples show similar results in terms of several organic acids and carotenes. The predominat compounds were established to be citric acid and β-carotene. In general, sample A showed better results concerning the tested chemical compounds in accordance with the other conducted assays.

Based on the results the intake of *Rosa canina* extracts can be recommended as antioxidant supplement in addition to the other known positive effects.

REFERENCES

Ak T., Gülçin I., 2008. Antioxidant and radical scavenging properties of curcumin. Chemico-Biological Interactions, 174: 27–37.

Aresenescu A., 2008. Pharmacognostical research on the species *Rosa canina* L. (in Romanian): UMF Cluj-Napoca.

Arsenescu-Popa A., Mladin P., Popescu H., 2008. Studii pentru actualizarea monografiei produsului medicinal *Cynosbati* fructus (fruct de măceş). Craiova medicală, 10(2): 121–124.

Beltrán-Orozco M. C., Oliva-Coba G. T., Gallardo-Velázquez T., Osorio-Revilla G., 2009. Ascorbic acid, phenolic content and antioxidant capacity red, cherry, yellow and white types of pitahaya cactusfruit (*Stenocereus stellatus* Riccobono). Agrociencia, 43: 153–162.

Benzie F. F., Wai Y., Strain J. J., 1999. Antioxidant (reducing) efficiency of ascorbate in plasma is not affcted by concentration. Journal of Nutritional Biochemistry, 10: 146–150.

Bozan B., SagdullaevT. B., Koþar M., Aripov N. K., Baþer C. H. K., 1998. Comparison of ascorbic and citric acid contents in *Rosa canina* L. fruits growing in Central Azian Region. Khim. Prir. Soedin.768–771.

Brand-Williams W., Cuvelier M. E., Berset C., 1995. Use of a free radical method to evaluate antioxidant activity. LWT- Food Science and Technology, 28: 25–30.

Christensen R., Bartels E. M., Altman R. D., Astrup A., Bliddal H., 2008. Does the hip powder of *Rosa canina* (rosehip) reduce pain in osteoarthritis patients?-a meta-analysis of randomized controlled trials. Osteoarthritis and Cartilage, 16: 965–972.

Chrubasik C., Duke R. K., Chrubasik S., 2006. The evidence for clinical efficacy of rose hip and seed: a systematic review. Phytotherapy Research, 20: 1–3.

Chrubasik C., Wiesner L., Black A., Müller-Ladner U., Chrubasik S., 2008. A one-year survey on the use of a powder from *Rosa canina* lito in acute exacerbations of chronic pain. Phytotherapy Research, 22: 1141–1148.

Demir F., Ozcan M., 2001. Chemical and technological properties of rose (*Rosa canina* L.) fruits grown wild in Turkey. Journal of Food Engineering, 47: 333–336.

Fan C., Pacier C., Martirosyan D.M., 2014. Rose hip (*Rosa canina* L): A functional food perspective. Functional Foods in Health and Disease, 4 (11): 493-509.

Georgieva L., Marchev A., Ganeva D., Bojinov B., Pavlov A., 2013a. Improved HPLC methods for determination of organic acids from Bulgarian sorts of tomatoes. SCIENTIFIC WORKS VOLUME LX „FOOD SCIENCE, ENGINEERING AND TECHNOLOGIES – 2013“ Plovdiv, 60: 626–631.

Georgieva L., Marchev A., Ivanov I., Ganeva D., Bojinov B., Pavlov A., 2013b. Improved HPLC methods for determination of carotenoids and tocopherols in different varieties of tomatoes. SCIENTIFIC WORKS VOLUME LX „FOOD SCIENCE, ENGINEERING AND TECHNOLOGIES – 2013“ Plovdiv, 60: 632–637.

Hanson P. M., Yang R-Y., Wu J., Chen J-T., Ledesma D., Tsou S. C. S., Tung-Ching L., 2004. Variation for antioxidant activity and antioxidants in tomato. Journal of the American Society for Horticultural Science, 129(5): 704–711.

Keen C., Holt R., Oteiza P., Fraga C., Schmitz H., 2005. Cocoa antioxidants and cardiovascular health.The American Journal of Clinical Nutrition, 81(1): 298S–303S.

Kilicgun H., Dehen A., 2009. *In vitro* antioxidant effect of *Rosa canina* in different antioxidant test systems. Pharmacognosy Research, 1: 417-420.

Kubola J., Siriamornpun S., 2008. Phenolic contents and antioxidant activities of bitter gourd (*Momordica charantia* L.) leaf, stem and fruit fraction extracts *in vitro*. Food Chemistry, 110(4): 881–890.

Kujala T. S., Loponen J. M., Klika K. D., Pihlaja K., 2000. Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. Journal of Agricultural and Food Chemistry, 48: 5338–5342.

Leccese A., Bartolini S., Viti R., 2007. Total antioxidant capacityand phenolics content in apricot fruits. International Journal of Fruit Science, 7(2): 3–16.

Moerman D. E., 2002. Native American ethnobotany. Portland, OR: Timber Press, 482-486.

Orhan N., Aslan M., Hosbas S., Deliorman O., 2009. Antidiabetic effect and antioxidant potential of *Rosa canina* fruits. Pharmacognosy Magazine, 5: 309–315.

Ou B. X., Hunag D. J., haMPsCh- WooDill M., Flanagan J. A., DeeMer E. K., 2002. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. Journal of Agricultural and Food Chemistry, 50(11): 3122–3128.

Ozcan M., 2002. Nutrient composition of rose (*Rosa canina* L.) seed and oils. Journal of Medicinal Food 5(3): 137–140.

Pârvu C., 2000. Universul plantelor. Mică Enciclopedie. Edit. „Enciclopedică", Bucureşti: 360–362.

Pereira C., Barros L., Carvalho A-M., Ferreira I., 2013. Use of UFLC-PDA for the Analysis of Organic Acids in Thirty-Five Species of Food and Medicinal Plants. Food Analytical Methods, 6: 1337–1344.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C. A., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine, 26: 1231–1237.

Stănescu U., Miron A., Hăncianu M., Aprotosoaie C., 2004. Plantele medicinale de la A la Z. Monografii ale produselor de interes terapeutic. I. Edit. „Gr. T. Popa", Iaşi: 176–177.

Tiţă I., 2003. Botanică farmaceutică. Edit. Did. şi Ped., Bucureşti: 680-681.

Williamson G., Manach C., 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. The American Journal of Clinical Nutrition, 81(1): 243S–255S.

Yi O., Jovel E. M., Towers G. H. N., Wahbe T. R., Cho D., 2007. Antioxidant and antimicrobial activities of native *Rosa* sp. from British Columbia, Canada. International Journal of Food Sciences and Nutrition, 58(3): 178–189.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BIOTECHNOLOGICAL PROCESSES FOR OBTAINING HERBAL ANTIOXIDANTS USEFUL IN FOOD INDUSTRY

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Abstract
A systematic scientific research for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceutical, drug research and food. A sustainable development of extractive biotechnological processes from herbal species can be achieved by classical method and also by supercritical fluid extraction(SFE). This paper is presenting the obtaining of some selective extracts from Rosmarinus officinalis L. species, with dual function of ingredients and antioxidants for food products. The fresh and dried leaves of Rosmarinus officinalis are frequently used as a food preservative and in traditional Mediterranean cuisine as a flavoring agent. Classical Soxhlet method and supercritical fluid extraction (SFE) method with CO_2 and ethanol cosolvent were applied for obtaining the Rosemary officinalis L. extracts. The herbal extracts have been investigated by UV-Vis spectroscopy for quantitative determination of the total polyphenols and flavonoids content, polyphenolcarboxylic acids and rosmarinic acid, according to Romanian and European Pharmacopoeia. In addition to this, four SFE extracts and four Soxhlet extracts were screened for their radical-scavenging capacities and antioxidant activities by various in vitro, non cellular assays, respectively chemiluminescence method in an luminol hydrogen peroxide system, and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging. The results revealed excellent correlation between the antioxidant capacity and the amount of active constituents of extracts (rosmarinic acid, total polyphenols and flavonoids content, polyphenolcarboxylic acids).

Key words:*Rosmarinus officinalis, Antioxidant activity; Supercritical CO_2 extraction.*

INTRODUCTION

Natural antioxidants are frequently used in the pharmaceutical and food industry, and obtaining these biocompounds by innovative biotechnological processes is a continuing challenge to replace synthetic food additives. Synthetic antioxidants such as butylhydroxy-anisole (BHA) and dibutylhydroxytoluene (BHT) are often and quite efficiently used in food processing. It is important to consider that there are some restraints to their use because of the evidence that they may be harmful to human health. Due to these considerations, obtaining of natural antioxi-dants represents a priority not only to prevent food degradation, but also to achieve additives and ingredients non toxic for use in the pharmaceutical and cosmetic industries (Kikuzari and Nikatani 1993), (P. F. Leal, et al. 2003).

Rosemary (*Rosmarinus officinalis* L.) is a spontaneous shrub, growing in all Medite-rranean countries. It is a herbal species frequently used as a food preservative and, in traditional Mediterranean cuisine, as a flavoring agent (Angioni, et al. 2004), Sotelo - Félix, et al. 2002). Also, *Rosmarinus officinalis* L. has been used as a medicinal herb due to its pharmacological actions: hepatoprotective (Sotelo - Félix, et al., 2002), antimicrobial (Del Campo, Amiot and Nguyen-The, 2000; Bozin, et al., 2007), antithrombotic (Yamamoto, et al. 2005), diuretic (Haloui, et al. 2000), antidiabetic (Bakirel, et al. 2008), anti-inflammatory (Altinier, et al. 2007), antioxidant (Perez-Fons, Garzon and Micol 2010) and anticancer (Lo, et al. 2002; Dörrie, Sapala and Zunino, 2001; Huang, et al., 2005; Visanji, Thompson and Padfield 2006).

These potent biological activities, including antioxidant properties have been assigned to the presence of many bioactive compounds in its composition. Phenolic acids, flavonoids, hydroxycinnamic acid derivatives and polyphenol-carboxilic acids are considered as the main dietary phenolic compounds. In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids (Manach, et al. 2004). These compounds exhibit a wide series of therapeutical properties, such as antimicrobial, anti-atherogenic, anti-inflammatory, antithrombotic, anti-allergic, cardioprotective, vasodilatory effects and antioxidant(N. Balasundram 2006).The major types of compounds found in rosemary are phenolic diterpenes including: carnosic acid, carnosol or rosmanol; flavonoids such as genkwanin, cirsimaritin or homoplantaginin; and triterpenes such as ursolic acid (Bai, et al. 2010; Bicchi, Binello and Rubiolo 2000; Del Baño, et al. 2004). The most well-studied bioactive compounds of *Rosmarinus officinalis*L, are carnosic acid (Figure 1a), caffeic acid (Figure 1b) and its derivative, rosmarinic acid (α-o-caffeoyl-3,4-dihydroxyphenyllactic acid) (Figure 1c). These compounds are thought to have biological and antioxidant significant properties and are under investigation as potential therapeutics for different illness. (Perez-Fons, Garzon and Micol 2010).

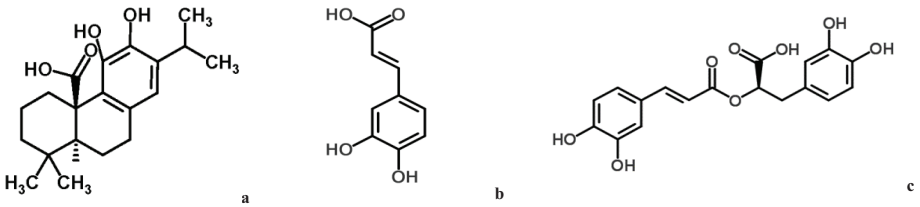

Figure 1. Chemical structure a) carnosic acid, b) caffeic acid. c) rosmarinic acid

In this regards the present paper is focused on key trends in the biotechnological production of antioxidants obtained by processing hydroalcoholic extracts of *Rosmarinus officinalis* L. It presents a new experimental design, applying classical Soxhlet extraction and supercritical fluid extraction SFE, and processing hydroalcoholic extracts, in order to achieve some antioxidants useful in the food industry. Bioactive compounds such as polyphenols, flavonoids and phenolic diterpenes from plant sources have been traditionally extracted by a conventional solid-liquid extraction or Soxhlet. Several studies shows new promising extraction methods are arising, which introduce some form of additional energy in order to facilitate extraction process of chemical compounds in a faster way (Garcia-Salas, et al. 2010). Such a method is the supercritical fluid extraction (SFE) using different types of co solvent at high pressure, or supercritical fluids such as CO_2. SFE has received much attention in the past several years, especially in food, pharmaceutical and cosmetic industries, because it presents an ecological alternative for conventional processes which use toxic organic solvents. An important disadvantage in the use of supercritical CO_2 is its low polarity, making the extraction of polar analytes quite difficult. However this limitation can be solved by adding of solvents such as methanol or ethanol which have the function of polar modifiers, to increase supercritical CO_2 solution power (Jian Bo Xiao 2007.)

MATERIALS AND METHODS

Plant materials. The *Rosmarinus officinalis* L. species are commercial samples, obtained from FARES: S.C. Romania. The dried leaves were stored in dark at 4ºC, for 10 days. Just before the extraction process by Soxhlet and supercritical fluid extraction (SFE) method, the leaves were ground in a blender, to produce a powder with an approximate size of 0.5 mm.

Chemicals. Aluminium chloride, sodium acetate, Folin-Ciocalteu phenol reagent, Arnow reagent, 1-diphenyl-2-picrylhydrazyl, 3-aminophthalhydrazide, hydrogen peroxide, rutin, quercetin, gentisic acid, caffeic acid,

syringic acid, gallic acid, rosmarinic acid, ascorbic acid were purchased from Sigma-Aldrich.

Solvents: Ethanol, Methanol, Acetone, Ethyl acetate, HCl, DMSO (Merck, analytical grade), ultrapure water (Millipore water system). Carbon dioxide 99.8% Linde Group Romania.

Equipments. Soxhlet extraction system, Supercritical Fluid Extraction System SFT-150, (Supercritical Fluid Technology, SUA)

Spectrophotometer UV-Vis, Jasco, Japan V-570 for DPPH method and quantitative determination of flavonoids, polyphenols, polyphenolcarboxylic acids and total hydroxycinnamicderivates, (Romanian Pharmacopoea, edition X 1993; Ciulei, et al. 1995)

Chemiluminometer (Sirius Luminometer Berthelot - GmbH Germany): for *antioxidant activity* measurements by chemiluminescence tehnique (CL).

Soxhlet extraction (SHE)

Plant material was extracted in a Soxhlet extraction apparatus, using 100g of dried and ground herbs (approximate size of 0.5 mm) in 1000 ml solvent1:1 (v/v) mixture of ethanol (99.8%, P.A., Merck) and ultrapure water (Millipore water system). The heating power was set to 50°C and the extraction was achieved within 3 h.

Supercritical fluid extraction (SFE)

Rosmarinic extracts were obtained using CO_2 (99.8%, food grade, Linde Group Romania), 20% [wt] of co-solvent, a 1:1 (v/v) mixture of ethanol (99.8%, P.A., Merck), and ultrapure water (Millipore water system) at 50 °C, and pressures of 200 and 300 bars. The CO_2 was admitted into the system at a flow rate of $6x\ 10^{-5}$ kg/s, up to the point where no solute was observed at the exit of the column (60 min). Considering that CO_2 behaves as a supercritical fluid above its critical temperature (304.25 K) and critical pressure (72.9 atm or 73.8659 bar/ 7.39 MPa), the experiment was run at pressures between ~~of~~ 200 and 300 bars and temperatures of 50 °C (Rodrigues, et al. 2002; Leal, et al. 2003).

The technological processing for antioxidants obtaining

The crude extract solutions obtained by Soxhlet and SFE method were processed, through a succession of technological steps consisting in vacuum concentration until obtaining a residue which was passed through successive precipitations with polar and non-polar solvents, centrifugation, filtering at low pressure and purification. Operational parameters specific to each stage mentioned above are presented in Table 1,2.

Table 1. Processing the extract of *Rosmarinus officinalis* L. obtained by the Soxhlet method (SHE)

Samples	RH1	RH1	RH3	RH4
Temperature of concentration (°C)	30	40	50	60
Speed of concentration (rpm)	100	200	300	400
Solvent of precipitation	Acetone	Ethanol	Ethyl acetate	Acidulated ethanol
Ratio of precipitation (w/v)	1:10	1:15	1:2	1:10
Time of centrifugation (min)	20	30	40	45
Speed of centrifugation (rpm)	3000	6000	3000	6000

Table 2. Processing the extract of *Rosmarinus officinalis* L. obtained by Supercritical fluid extraction method (SFE)

Samples	RS1	RS2	RS3	RS4
Temperature of concentration (°C)	30	40	50	60
Speed of concentration (rpm)	100	200	300	400
Solvent of precipitation	Acetone	Ethanol	Ethyl acetate	Acidulated ethanol
Ratio ofprecipitation (w /v)	1:10	1:15	1:2	1:10
Time of centrifugation (min)	20	30	40	45
Speed of centrifugation (rpm)	3000	6000	3000	6000

It may be noted that manufacturing processes are similar and the corresponding operational parameters of each stage are identical, the only difference between the two sets (RH and RS) of samples is the extraction method: SHE respectively SFE.

Powders obtained in the processing steps were dried at room temperature, washed successively with ethanol, to remove toxic compounds and then allowed to completely dry. The washing operation was repeated three times. Each of the eight obtained yellowish - white powders, was milled to a very fine consistency (RH1, RH2, RH3, RH4 – Soxhlet method and RS1, RS2, RS3, RS4- Supercritical fluid extraction method)

Chemical analysis of the samples

The quantitative determinations of the flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivativeswere done according to the FR X and the

European Pharmacopoeia Ed. 6.0, Rosemary leaf Monography (Romanian Pharmacopoea, Edition X, 1993). All measurements were repeated three times.

Antioxidant activities

CL method. The antioxidant activity (AA%) of the samples (RH1, RH2, RH3, RH4 – Soxhlet method and RS1, RS2, RS3, RS4- Supercritical fluid extraction method) has been determined and compared with that of pure standards: rutin, quercetin, gentisic acid, caffeic acid, syringic acid, gallic acid, rosmarinic acid, ascorbic acid. Chemiluminescence method (CL) was applied using luminol-H_2O_2 as generator system, in TRIS-HCl, buffer pH= 8.4, using Sirius Luminometer Berthelot - GmbH Germany. The antioxidant activity of samples was calculated using the following relation (Iftimie Badea N., 2004; Del Baño, et al. 2004)

$$AA\,\% = \frac{I_0 - I}{I_0} \cdot 100$$

where: I_0 = the maximum CL for standard at t=5 s; I = the maximum CL for sample at t =5 s.

DPPH Method. Quantitative evaluation of radical scavenging abilities (SR%) of the samples was carried out by an adapted DPPH method (Sanja Matić 2013). Sample stock solutions (1.0mg/mL) were diluted to final concentrations of 250, 125, 50, 25, 10 and 5 g/mL, in methanol. One mL of a 0.3 mM solution of DPPH in methanol was added to 2.5 mL of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm and converted into the percentage of radical-scavenging activity (SR%) using the following formula:

$$SR\% = 100\left(1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right)$$

Methanol (1.0 mL) plus herbal extract solution (2.5 mL) was used as a blank. DPPH solution (1.0 mL; 0.3 mM) plus methanol (2.5 mL) was used as a negative control.

RESULTS AND DISCUSSIONS

The application of Soxhlet extraction is ordinary, but supercritical solvent extraction for the preparation of natural antioxidants has, until now, been limited. The procedure was used for the extraction of rosemary and sage leaves. Propane, butane, methanol, ethanol may be used as co-solvents for improving yield or selectivity. Supercritical fluid extraction allows a continuous modification of dissolution power and selectivity by changing the solvent density. An essential drawback in the use of supercritical CO_2 is its low polarity, making the extraction of polar analytes difficult. This limitation may be overcame by adding small amounts of polar modifiers, such as methanol or ethanol to the supercritical CO_2, in order to increase its solution power. In the present study, the modifier ethanol enhanced the solubility of solutes in supercritical CO_2 and thus the efficiency of extraction increased, as demonstrated by the phytochemical characterization of the obtained bioproducts. (Mühlnickel T 1992; Djarmati Z 1991; Jian Bo Xiao 2007; Mühlnickel 1992).

Chemical analysis

Phytochemical analysis showed that SFE method is more efficient for extraction of the compounds of therapeutic interest. Thus, there is a significant increase (46.2%-57%) of the amount of flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivatives (Figure 2,3,4,5). All the extract samples obtained by SFE, and further processed in the same conditions as the extract samples obtained by Soxhlet method, led to much higher amounts of biological active compounds, comparing to those obtained from the Soxhlet extracts.

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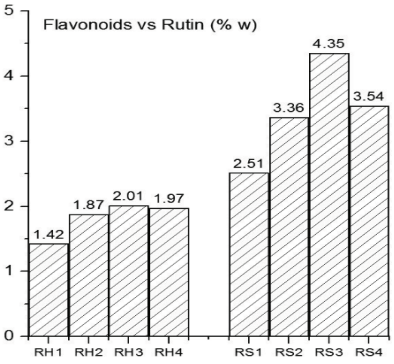

Figure 2. Flavonoids content, mass % (as rutin)

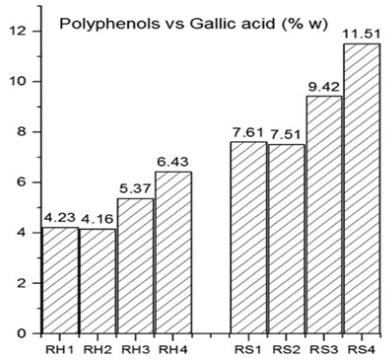

Figure 3. Polyphenols content, mass % (as gallic acid)

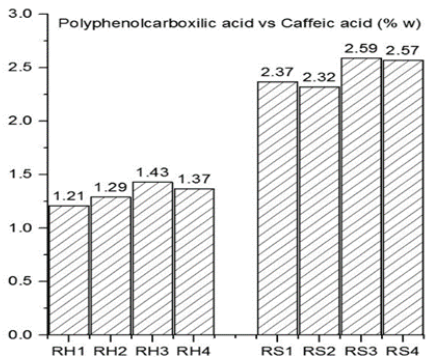

Figure 4. Polyphenolcarboxilic acids content, mass % (as caffeic acid)

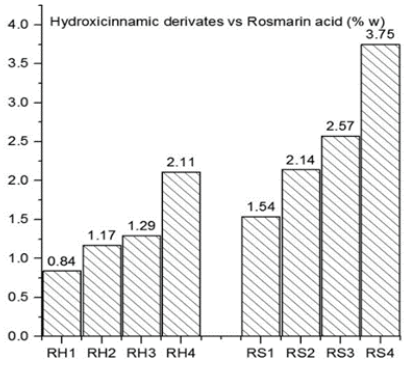

Figure 5.Hydroxycinnamic derivatives content, mass % (as rosmarinic acid)

Antioxidant activities

Antioxidant activity for the two sets of analyzed samples (RH set - Soxhlet extraction and RS set - supercritical fluid extraction with CO_2 solvent), demonstrated by both methods, DPPH and CL, reached high values, ranging from 92.78% - 97.01% for DPPH and 94.23% - 98.94% for CL.

The choice of optimal processing parameters, in conjunction with the method of extraction (SFE), led finally to obtaining of selective extracts, enriched in active principles (flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivatives) which present a very high antioxidant activity (98.94% $_{CL}$, 97.01% $_{DPPH}$).

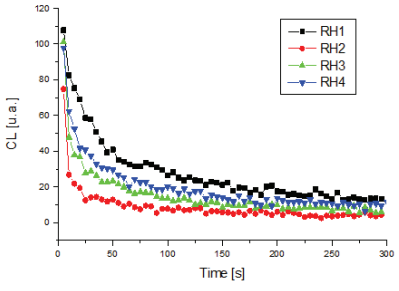

Figure 6. CL evolution in time of RH1, RH1, RH1, RH4 samples

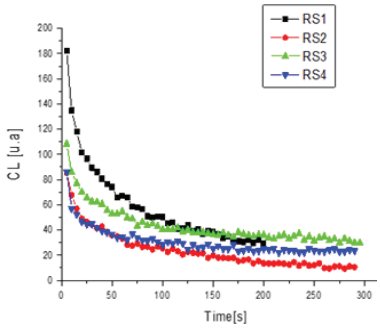

Figure 7. CL evolution in time of RS1, RS2, RS3, RS4 samples

Table 3.Antioxidant activity and kinetics parameters

No.crt	Samples code	Extraction method	$k_i(s^{-1})$ [1]	$v_i\ (s^{-1})$ [2]	AA%
1	RH1	SHE	0.089	243.87	94.23
2	RH2	SHE	0.101	119.15	97.22
3	RH3	SHE	0.097	115.72	96.14
4	RH4	SHE	0.064	480.27	96.92
5	RS1	SFE	0.098	432.74	97.28
6	RS2	SFE	0.115	342.96	98.94
7	RS3	SFE	0.086	427.64	96.81
8	RS4	SFE	0.109	456.11	98.17
9	R[a]	-	0.114	291.80	78.61
10	Q[b]	-	0.107	114.83	96.36
11	GE[c]	-	0.079	398.42	58.93
12	CA[d]	-	0.067	317.14	83.17
13	SA[e]	-	0.112	609.18	64.31
14	GL[f]	-	0.057	169.27	85.60
15	RS[g]	-	0.098	283.42	89.11
16	AS[h]	-	0.094	467.18	99.50

[1] k_i: rate constant, [2] v_i: reaction rate indexed from the chemiluminescence curves; a: Rutin, b:Quercetin, c:Gentisic acid, d:Caffeic acid, e:Syringic acid, f:Gallic acid, g:Rosmarinic acid, h:Ascorbic acid

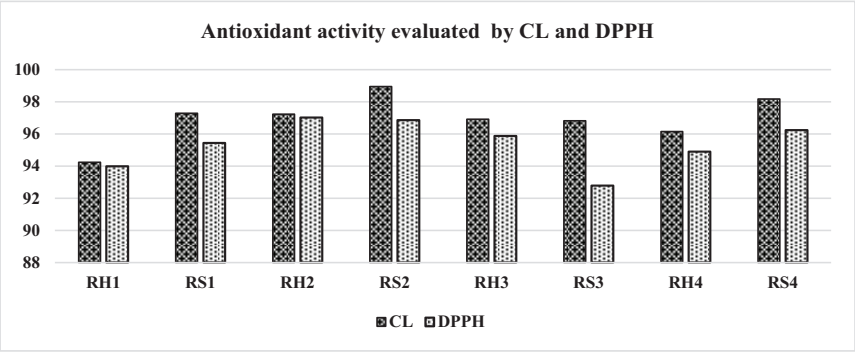

Figure 8. Evaluation of antioxidant activity by CL and DPPH method

CONCLUSIONS

In this work, two extraction methods were applied, SFE and Soxhlet, and the obtained extracts were processed by identical successive steps, obtaining herbal antioxidants with multiple functions: ingredients, flavors, additives and preservatives.

The study led to the development of an obtaining procedure of an antioxidant herbal product, including extraction and further processing steps, as a cost-effective, cleaner and more environmental friendly biotechnological processes than those based on conventional extraction methods. The antioxidants properties of the selective extracts, useful as food additives, are also emphasized. Investigation by CL and DPPH has revealed very high values of antioxidant capacity and phytochemical analysis revealed greater amounts of flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivates, when extraction is achieved by SFE method, which is a cost-effective technique in laboratory scale, with possibility to be extended at industrial scale. The advantages of SFE with CO_2, including: avoidance of toxic solvents, low operating temperature (no thermal degradation of most of the labile compounds), high selectivity and fast extraction (Jian Bo Xiao 2007), were demonstrated. Last, but not least, it should be pointed out the excellent correlation between the antioxidant capacity and the amount of active constituents of extracts (rosmarinic acid,

total polyphenol and flavonoids content, polyphenol carboxylic acids).

ACKNOWLEDGEMENTS

This paper was supported by the Romanian National Authority for Scientific Research, Project PN II PCCA No 113/2012 and PN II Partnership No 210/2014.

REFERENCES

Altinier G., Sosa S., Aquino R.P., Mencherini T., Loggia R.D., and Tubaro, 2007. Characterization of topical antiinflammatory compounds in *Rosmarinus officinalis* L,J. Agric. Food Chem.,55:1718–1723.

Angioni A., Barra A., Cereti E., Barile D, Coïsson J.D., Arlorio M., Dessi A., Coroneo V., and Cabras P., 2004. Chemical Composition, Plant Genetic Differences, Antimicrobial and Antifungal Activity Investigation of the Essential Oil of *Rosmarinus officinalis* L.J. Agric. Food Chem, X:3530-3535.

Balasundram N., Sundram K., Sammar S., 2006. Phenolic compounds in plants and agri-industrial by-products. Antioxidant activity, occurrence, and potential uses. Food.Chem., 1:191-203.

Bai N., He K., Roller M., Lai C., Shao X., Pan M., Ho C., 2010. Flavonoids and phenolic compounds from *Rosmarinus officinalis*. J. Agric. Food Chem., 58:5363–5367.

Bakirel T., Bakirel U., Keleş O.U., Ülgen S.G., Yardibi H., 2008. In Vivo Assessment of antidiabetic and antioxidant activities of rosemary (*Rosmarinus officinalis*) in alloxan-diabetic rabbits. J. Ethnopharmacol., 116:64–73.

Bicchi C., Binello A., Rubiolo.P., 2000. Determination of phenolic diterpene antioxidants in rosemary (*Rosmarinus officinalis* L.) with different methods of extraction and analysis. Phytochem. Anal., 11:236–242.

Bozin B., Mimica-Dukic N., Samojlik I., Jovin. E., 2007. Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., Lamiaceae) essential oils. J. Agric. Food Chem., 55:7879–7885.

Del Baño M.J., Lorente J., Castillo J., Benavente-García, Marín M.P., Del Río J.A., Ortuño A., Ibarra.I., 2004. Flavonoid distribution during the development of leaves, flowers, stems and roots of *Rosmarinus officinalis*. Postulation of a biosynthetic . J.Agric. Food Chem., 52:4987-4992.

Del Campo J., Amiot M., NguyenC., 2000. The Antimicrobial effect of rosemary extracts., J. Food Prot., 63:1359–1368.

Djarmati Z., Jankov R.M., Schwirtlich E., Djulina B., Djordjevic A.,1991. High antioxidant activity of extracts obtained from sage by supercritical extraction. J.Am Oil Chem Soc., 68:731-734.

Dörrie J., Sapala K., Zunino S.J., 2001. Carnosol-induced apoptosis and downregulation of Bcl-2 in B-lineage leukemia cells. Cancer Lett.,170:33–39.

Garcia-Salas P., Morales-Soto A., Segura-Carretero A., Fernández-Gutiérrez A., 2010. Phenolic-compound-extraction systems for fruit and vegetable samples. Molecules, 15: 8813–8826.

Haloui M., Louedec L., Michel J., Lyoussi.B., 2000. Experimental diuretic effects of Rosmarinus officinalis and Centaurium erythraea. J. Ethnopharmacol.,71:465–472.

Huang S., Ho C., Lin-Shiau S., Lin. J., 2005. Carnosol inhibits the invasion of B16/F10 mouse melanoma cells by suppressing metalloproteinase-9 through down-regulating nuclear factor-κB and c-Jun. Biochem. Pharmacol.,69:221–232.

Ciulei I., Istudor V., Palade M., Albulescu D., Gard.C.E., 1995. Pharmacognostic and phytochemistry analysis of vegetable products. Ed. Medicala, Bucharest, vol. 1.79-80

Iftimie (Badea) N., Herdan J.M., Giurginca M., Meghea A., 2004. Chemiluminescence technique for the evaluation of some mineral and vegetable oils protected by antioxidants. Rev.Chem., 55(7):512-514.

Sotelo - Félix J.I., Martinez -Fong D., Muriel P., Santillán R.L., Castillo D., Yahuaca P., 2002. Evaluation of the effectiveness of *Rosmarinus Officinalis* (Lamiaceae) in the alleviation of carbon tetrachloride-induced acute hepatotoxicity in the rat. J. Ethnopharmacol.,81: 145-154.

Jian Bo Xiao, Jing Wen Chen, Ming Xu., 2007. Supercritical fluid CO2 extraction of essential oil from *Marchantia convoluta*: global yields and extract chemical composition. Electronic Journal of Biotechnology, 10(1):141-148.

Kikuzari H., Nikatani N. J., 1993. Antioxidants Effects of Some Ginger Constituents. J. Food Sci.,58:1407-1410.

Lo A.,. Liang Y., Lin-Shiau S., Ho C., Lin. J., 2002. Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages. Carcinogenesis, 23:983–991.

Manach C., Scalbert A., Morand C., Rémésy C., Jiménez L., 2004. Polyphenols, food sources and bioavailability. Am. J. Clin. Nutr., 79: 727–747.

Mühlnickel T., 1992. Extraction with carbon dioxide manufacture of de-aromatized rosemary antioxidant. Food Mark Technol.,8:37-38.

Leal P.F.,. Braga M. E., Sato D N., Carvalho J. E., Marques M.O.M., Meireles. A. A., 2003. Functional Properties of Spice Extracts Obtained via Supercritical Fluid Extraction. J. Agric. Food Chem., 51:2520-2525.

Perez-Fons L., Garzon M.T., Micol V., 2010. Relationship between the antioxidant capacity and effect of rosemary (*Rosmarinus officinalis* L.) polyphenols on membrane phospholipid order. J. Agric. Food Chem., 58: 161–171.

Rodrigues, V. M., Sousa E. M. B. D., Monteiro A. R, Chiavone-Filho O., Marques M. O. M., Meireles M. A. A., 2002. Determination of the Solubility of Extracts from Vegetable Raw Material in Pressurized CO_2: a Pseudo-Ternary Mixture Formed by Cellulosic Structure+Solute+Solvent. Journal of Supercritical Fluids, 22(1): 21-26.

Romanian Pharmacopoea, ed. X. (1993), Bucharest, Ed. Medicala, 335, 779

Matić S., Stanić S., Bogojević D., Vidaković M., Grdović N., Dinić S., Solujić S., Mladenović M., Stanković N., Mihailović M., 2013. Methanol extract from the stem of Cotinus coggygria Scop., and its major bioactive phytochemical constituent myricetin modulate pyrogallol-induced DNA damage and liver injury. Mutation Research, 755:81-89.

Visanji J.M., Thompson D.G., Padfield P.J., 2006. Induction of G2/M phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and cyclin B1 levels. Cancer Lett., 237:130–136.

Yamamoto J., Yamada K., Naemura A., Yamashita T., Arai R., 2005. Testing various herbs for antithrombotic effect. Nutrition, 21:580–587.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

GC-MS HEADSPACE CHARACTERIZATION OF THE VOLATILE PROFILE OF GRAPE SKIN, PULP AND SEED EXTRACTS FOR THREE ROMANIAN VARIETIES

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Abstract

The Wine aroma is one of the most important factors in determining its character and quality. The aromatic compounds are accumulated in the peel and grape, and can pass to wine unmodified or by changing their form.The approach to determine certain markers which are depending mainly on grape variety and cultivation area is very innovative one. In this work, methanolic extracts of grape skin, pulp and seeds of three Vitis vinifera L. *varieties were assessed in order to differentiate among their volatile profiles. The grape samples were obtained from the Murfatlar vineyard (Romania): Cabernet Sauvignon, Merlot and Feteasca Neagra. The wines produced from these grapes are awarded the "Protected Designation of Origin" (PDO) label. The evaluation of the volatile profiles was done by gas chromatography/ mass spectrometric detection. The headspace analysis revealed several monoterpens, alcohols, acids and carbonyl compounds.*
The results are consistent with the methodology for the authentication of the grape variety according to the profile of the volatile compounds, in terms of semi-quantitative interpretation.

Key words*: grapes, GC-MS, headspace, aroma, quality.*

INTRODUCTION

Wine quality and identity are the result of different inter-correlated factors, especially terroir and grape variety, but also viticultural and winemaking techniques. Wine aroma is a key component of the former. It is comprised of certain volatile compounds that belong to different chemical families, arising from the grape metabolism, the yeast fermentation and the aging process (Domínguez and Eduardo, 2010).
The primary varietal flavours are accumulated in the skin and grape through specific processes of the metabolism. They are determined by the genetic nature of the varieties, and by the specific pedological and climatic factors. (Lengyel, 2012). The complex aroma of wine is derived from many sources. The components derived from grapes are responsible for the varietal character. The ability to monitor grape aroma compounds would allow for better understanding of how winemaking techniques influence the final volatile composition (Canuti et al., 2009). Grape aroma is comprised of a large number of volatile compounds including alcohols, esters, acids, terpenes, norisoprenoids, thiols and carbonyl compounds. These aroma compounds are predominantly localized in the skin and many are stored as conjugated in the vacuoles of the skin. The skins contain more than half of the total volatile compounds present in the grape berries. During winemaking, the "free" aroma compounds are released as a result of physical crushing and subsequent chemical and enzymatic hydrolysis enzymes (glycosidases or peptidases).
The volatile composition of grapes is one of the most important factors determining wine character and quality. However, there have been few studies linking volatile composition in grapes to the final volatile composition in the wine. These limitations are due, in part, to the lack of analytical methods that allow for rapid

screening or profiling of multiple volatile compounds that are present at a wide range of concentrations in both grapes and wine (Canuti et al., 2009).

MATERIALS AND METHODS

Sample preparation

The grape samples were collected from the Murfatlar vineyard (Romania): Feteasca Neagra, Cabernet Sauvignon and Merlot varieties. The grapes were sorted in terms of separating the skins, pulp and seeds, and subsequently subjected to solvent extraction, individually, in methanol (70% in distilled water) by maceration for 24h in the dark and at room temperature.

Gas chromatography/mass spectrometry analysis (GC-MS): ***Headspace*** - the mobile phase used was helium with a flow of 1 mL/min. The initial oven temperature was held at 40 °C for 5 min and then increased to 250 °C and held isothermally for 10 min at this temperature. The injection and ion source temperatures were 200 °C and 220 °C respectively, and the injection volume was 1µL in the split mode. Identification of volatile compound was achieved by comparing mass spectra found in the NIST2.0 MS library Database.

RESULTS AND DISCUSSIONS

Although headspace analysis has been widely used for analysis of grape and wine volatiles, static headspace analysis often comes with poor sensitivity for trace volatiles and dynamic headspace analysis suffers from interferences provided by water and ethanol.

Some grapes, like Cabernet Sauvignon, show significant analytical challenges due to the fact that the aroma compounds are present in low concentrations with norisoprenoids, esters, alcohols and aldehydes constituting the majority of the volatiles. There have been not so many applications of HSSPME for profiling aroma volatiles in Cabernet Sauvignon grapes (Canuti et al., 2009).

The main chemical constituents for the separate grape samples (skin, pulp, seeds) for the three grape varieties were determined by gas chromatography coupled with mass spectrometry.

The components identified by headspace screening of the extracts were: 1) butanoic acid, methyl ester; 2) tropilidene; 3) 2-ethyl heptanoic acid; 4) 3-ethylhexane; 5) 3-methyl-2-heptanol; 6) 2,4-dimethyl-1-heptene; 7) n-octane; 8) n-nonane; 9) 2-propyl-1-pentanol; 10) tridecane; 11) 3,5-dimathyloctane; 12) n-decane; 13) o-cymene; 14) undecane; 15) c-terpinen; 16) 3-methyldecane; 17) hexadecane; 18) estragole; 19) phenol,2,4-di-tert-butyl.

As can be seen from Table 1, the distribution of the identified compounds is diversified among the different parts of the grape.

The results are consistent with the findings of (Doneva-Sapceska et al., 2006), (Nechita, 2010), (Gomez et al., 1994), (Ashok Kumar and Vijayalakshmi, 2011), (Lamorte et al., 2007), (Nirmala and Narendhirakannan, 2011), (Welke et al., 2013), (Tamborra and Esti, 2010).

After the GC-MS screening of the volatile profile of the extracts, the same compounds for each of the three grape varieties were selected. The proportion of the peak area of each of these compounds is presented after the integration. The same 4 volatiles were taken into consideration in order to represent the ratio between them. From the tables there can be observed the difference between the certain ratios (Example: skin – the ratio between butanoic acid, methyl ester and estragole is 2.011 for Feteasca Neagra, as compared with the same ratio for Cabernet Sauvignon and Merlot varieties which are 11.373 and 7.572, respectively).

As long as the headspace volatile profiling is a qualitative one and, at the same time, a semi-quantitative one, it cannot be based on the simple comparison of the identified individual compounds, because the difference between the areas of the same component detected in separate samples is not conclusive and visible.

Thus, the ratio between the same two compounds supply a more clear distinction between the grape varieties.

The differences among the ratios can be successfully used in the attempt to authenticate wines, based on the profile of the volatile constituents.

The employed headspace technique is yet to be able to detect very well all the volatile compounds in grapes. An SPME-headspace (solid phase micro-extraction) method would cover a wider range of components and show a higher resolution.

Still, it can provide considerable results that can show a fine differentiation among grape samples.

Table 1. Tentatively identified volatile compounds of the skin, pulp and seeds extracts from Feteasca Neagra, Cabernet Sauvignon and Merlot grapes, respectively

	Feteasca Neagra			Cabernet Sauvignon			Merlot		
	% area								
Compound	skin	pulp	seeds	skin	pulp	seeds	skin	pulp	seeds
1	15.35	24.79	30.38	33.21	34.8	30.81	22.34	32.83	45.45
2		3.52	5.6				2.67		
3	6.76			7.18			2.24	6.24	
4		6.1	7.45	6.71	6.23	16.66	4.99	4.16	6.64
5		4.14	8.08	6.43	6.02		2.15		
6		2.27							
7		3.66	4.53	5.64	8.51	5.86	4.91	3.1	5.87
8	6.44	5.99	6.32	7.37	9.11	10.64	12.11	9.42	11.37
9	3.35		3.88						2.1
10	4.96	4.37		3.41	3.6		6.27	4.41	1.79
11	8.68	5.2	4.29		3.97	5.06	5.8	6.08	3.96
12	20.2	15.7	8.14	11.86	14.97	10.18	20.1	17.32	10.5
13	9.12	5.82	5.89	3.68	3.93	5.42	2.21	3.02	3.38
14	5.17	6.51		5.44		6.57			4.86
15	8.2	5.42	4.81	6.14	4.63	3.87	3.41	4.56	4.1
16	4.13	2.62					3.17	2.59	
17			1.97				1.99		
18	7.63	3.82	4.14	2.92	4.23		2.95	2.87	
19			4.51			4.93	2.69	3.41	

% area – the percentage of the peak area as proportion of the total area of the integrated peaks (100%)

Table 2. Ratios of the area percentages for the three grape varieties taken into study - ***skin***

Compounds	% Area		
	F.N.	C.S.	M.
1 butanoic acid, methyl ester	15.35	33.21	22.34
2. estragole	7.63	2.92	2.95
Ratio 1:2	**2.011**	**11.373**	**7.572**
3. o-cymene	9.12	3.68	2.21
4. c-terpinen	8.2	6.14	3.41
Ratio 3:4	**1.112**	**0.599**	**0.648**

Table 3. Ratios of the area percentages for the three grape varieties taken into study - ***pulp***

Compounds	% Area		
	F.N.	C.S.	M.
1 butanoic acid, methyl ester	24.79	34.8	32.83
2. estragole	3.82	4.23	2.87
Ratio 1:2	**6.489**	**8.226**	**11.439**
3. o-cymene	5.82	3.93	3.02
4. c-terpinen	5.42	4.63	4.56
Ratio 3:4	**1.073**	**0.848**	**0.662**

Table 4. Ratios of the area percentages for the three grape varieties taken into study - ***seeds***

Compounds	F.N.	C.S.	M.
1 butanoic acid , methyl ester	30.38	30.81	45.45
2. decane	8.14	10.18	10.5
Ratio 1:2	**3.732**	**3.026**	**4.328**
3. o-cymene	5.89	5.42	3.38
4. c-terpinen	4.81	3.87	4.1
Ratio 3:4	**1.224**	**1.400**	**0.824**

CONCLUSIONS

Headspace analysis (both static and dynamic) has been widely used for analysis of grape and wine volatiles.

The volatile composition of grapes is one of the most important factors determining wine character and quality.

The ratio between two or more same compounds provide a clear differentiation between grape varieties.

There have been few studies linking volatile composition in grapes to the final volatile composition in the wine.

Wine quality is the result of different inter-correlated factors, among which the grape variety plays an important role.

REFERENCES

Ashok Kumar, K., and Vijayalakshmi, K. (2011). GC-MS analysis of phytochemical constituents in ethanolic extract of Punica granatum peel and Vitis vinifera seeds. *International Journal of Pharma and Bio Sciences*, *2*, 461–468.

Canuti, V., Conversano, M., Calzi, M. L., Heymann, H., Matthews, M. a., and Ebeler, S. E. (2009). Headspace solid-phase microextraction-gas chromatography-mass spectrometry for profiling free volatile compounds in Cabernet Sauvignon grapes and wines. *Journal of Chromatography A*, *1216*, 3012–3022.

Domínguez, a. M., and Eduardo, A. (2010). Gas chromatography coupled with mass spectrometry detection for the volatile profiling of Vitis Vinifera CV. Carménère wines. *Journal of the Chilean Chemical Society*, *55*, 385–391.

Doneva-Sapceska, D., Dimitrovski, A., Milanov, G., and Vojnovski, B. (2006). Free and Potentially Volatile Monoterpenes in Grape Varieties. *Bulletin of the Chemists and Technologists of Macedonia*, *25*(1), 51–56.

Gomez, E., Martinez, a, and Laencina, J. (1994). Localization of free and bound aromatic compounds among skin, juice and pulp fractions of some grape varieties. *Vitis*, *4*, 1–4.

Lamorte, S. A., Gambuti, A., Genovese, A., and Moio, L. (2007). Volatile components of Vitis vinifera L. cvs. Uva di Troia, Aglianico and Fiano at different stages of ripening. *30th World Congress of Vine and Wine*, 1–6.

Lengyel, E. (2012). Primary aromatic character of wines. *Acta Universitatis Cibiniensis Series E: Food Technology*, *16*(1), 3–18.

Nechita, C.-B. (2010). *Contributions to the study of VOLATILES COMPOUND OF GRAPES AND wines obtained in Cotnari vineyard*.

Nirmala, J. G., and Narendhirakannan, R. T. (2011). in Vitro Antioxidant and Antimicrobial Activities of Grapes (Vitis Vinifera . L), *3*(4), 242–249.

Tamborra, P., and Esti, M. (2010). Authenticity markers in Aglianico, Uva di Troia, Negroamaro and Primitivo grapes. *Analytica Chimica Acta*, *660*, 221–226.

Welke, J. E., Manfroi, V., Zanus, M., Lazzarotto, M., and Alcaraz Zini, C. (2013). Differentiation of wines according to grape variety using multivariate analysis of comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection data. *Food Chemistry*, *141*(4), 3897–3905.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MONITORING OF ENZYMATIC COAGULATION OF COW'S MILK AT LOW TEMPERATURES BY AN OPTICAL METHOD

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Abstract

The kinetics of enzymatic coagulation of cow's milk was monitored by automated optical device. The optical method used for analysis and control of the coagulation process is non-destructive, which creates prerequisites for accurate analysis and forecasting process together with the applied numerical differentiation of the results obtained. The use of the optical device is determined by the relative change (%) of the intensity of light permeate flow during the process.
In cheese production, the process of the specific enzymatic coagulation of milk begins at temperatures below 20°C. Under these conditions, the coagulation occurs only in the enzymatic phase. In order to achieve the necessary energy levels to continue the process during the remaining phases, an increasing of the temperatures up to 35÷38°C was made.
The milk coagulants used in the experiment were: calf chymosin, microbial and camel enzyme, in quantities 30 $cm^3.10^{-2}.dm^{-3}$, with enzyme activity reduced to 1:10000.
Practical implementation of the optical method and device results in refinement of the technological process for production of cheeses, facilitating the examination and the control of the process of enzymatic coagulation, leading to improved quality and typification of the final product.

Key words*: optical method, enzymatic coagulation, milk-clotting enzymes, automatic control, relative absorbance.*

INTRODUCTION

The enzymatic coagulation of milk is a basic operation in the production of cheese. The temperature used to conduct the coagulation affects the rate of the process. At a temperature of coagulation below 10°C and over 65°C coagulation is not observed. In the temperature range of 10÷20°C, the rate of the coagulation is reduced, over 20°C it increases progressively, until temperatures of 40÷42°C – the optimal temperature for the used milk-clotting enzymes in cheese manufacturing (Baltadzieva, 1996).
The temperature has less impact on the enzymatic phase of the coagulation and significantly affects the course of the aggregation phase. The abovementioned trend was confirmed by the Stenne – Hutin process, called "coagulation at low temperatures" (Szadkovska, 1978), investigated also by Berridge (Berridge, 1952).
At temperatures between 20°C and 40°C, the dissociation constant of the enzyme-substrate complex vary slightly, until the rate of aggregation of the casein micelles is doubled (Lagaude et al., 2004).
When milk coagulants are placed in milk at 5°C, coagulation does not occur. After temperature increase a coagulum is formed faster than in the case of normal coagulation conditions. It was proved that at a low temperature only the enzymatic phase of hydrolysis of k-casein fraction of the casein micelles takes place. Therefore the casein micelles lose their stability and an increase of temperatures results in accelerated aggregation and faster rate of gelation (Dalgleish, 2009).
Morgenroth (Morgenroth, 1899) established that at low positive temperatures coagulation was not observed in milk. When the milk-clotting enzymes were left several hours in the mentioned conditions and then the milk was heated, it coagulates in a very short time.
Berridge (Berridge, 1952) has given a biochemical explanation of the processes occurring during "cold coagulation" - in the first stage of the coagulation, at low

temperature, part of the molecule of the k-casein fraction of the micelles was released, resulting in loss of its stabilizing properties. In the second stage begins general destabilization and aggregation of casein micelles. Further heating causes instant milk coagulation.

Based on Beridzh and Stenne – Hutin studies, methods and a technological equipment for the implementation of the continuous coagulation of milk were developed (coagulation in stream).

Different instruments and devices were developed for implementation of continuous coagulation of milk, based on the separation of enzyme and agregation phase - systems NIZO (the coagulation in stream covers the processes of coagulation up to placement in molds) and Sten-Hautin (two-stage coagulation using condensed milk concentrated to a ration 3:1) (Ramet, 1980).

Monitoring of the phases of the coagulation process is conducted using various methods and devices, differing in principle of action and constructive characteristics.

Dynamic and static instrumental techniques are mainly used, such as heuristics, rheological methods, "the hot wire method", ultrasonic methods, optical methods, microscopic methods and others. (Lucey, 2002; Hassan, 1995; Anderson, 2003).

The practical application of optical methods and systems leads to an optimization of the technological process in the production of cheeses, allowing precise analysis of the individual phases of enzymatic coagulation, thereby increasing the yield and improving the quality characteristics of the obtained finished product.

The aim of the conducted study was to monitor the enzymatic coagulation of cow milk, at low temperatures, using an automatic optical device.

MATERIALS AND METHODS

For the purposes of the experiment was used raw cow milk, obtained from the farm of the University of Agriculture, Plovdiv, cooled to 4 °C during the transportation, in order to preserve its quality characteristics.

The determination of the physicochemical parameters of the cow milk used for the experiments was performed by an ultrasonic milk analyzer. The values obtained are the following: protein – 3,36 %; fat – 4,9 %; non-fat solids – 8,75 %; density – 1,029 $g \cdot cm^{-3}$; titratable acidity – 18 °T; pH – 6,7.

Enzymes of different origins were used as milk coagulants - calf chymosin, with activity 1: 10000, enzyme of camel origin, strength 1: 70000 and microbial milk-clotting enzyme with activity 1:50000, produced and delivered by Cr. Hansen Company. To conduct the experiments, the enzyme activity was adjusted to 1:10000.

The amounts of the milk-clotting enzymes were 40 $cm^3.10^{-2}dm^{-3}$.

The monitoring of the process of rennet coagulation was performed by automatic optical device with an online application. Experimental data is archived by a system based on Data Loggers and software for their visualization by major information network - filing system MS DL 3.02.

The elements (optical fibers) of the optical device have a diameter d = 5 mm. The main beam of light was realized by a source with a wavelength corresponding to the absorption of the gel-forming structures (protein components) at 600 nm.

The process of coagulation at low temperatures was implemented using the following temperature values: 5, 10, 15, 20, and 25°C. Four multiple repetitions of the experiment were performed. For the statistical and mathematical analysis were used the average values obtained from the various experiences.

The volume of the samples was 500 $cm^3.10^{-2}dm^{-3}$.

The obtained experimental data were processed statistically by specialized mathematical software - Sigma Plot 11.0.

RESULTS AND DISCUSSIONS

An experiment was conducted to identify the amendment of the phases of enzymatic coagulation of cow milk, depending on the tested temperatures of coagulation, using an optical system.

An important element of the system is the optical part illustrated in Fig. 1.

The module of the light system is composed of a flexible optical fiber, firm optical fiber and a tip, presented in Fig. 2. The optical fibers end

with plugs, allowing connection to sensors and light source.

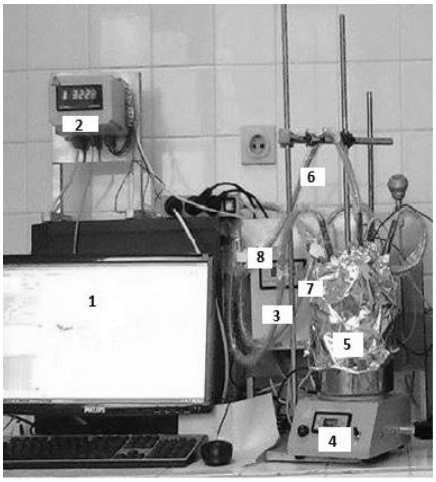

Figure 1. System for monitoring the coagulation of milk (1 – PC; 2 – electronic block of the optical device; 3 – device for measuring temperature; 4 – water bath with electronic thermostat; 5 – milk sample; 6, 7, 8 – optical fibers of the device)

The hardware of the optical laboratory device for monitoring the rennet coagulation is shown in Fig. 3.

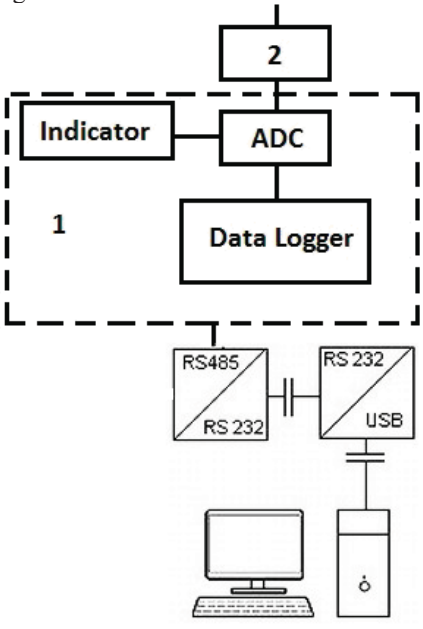

Figure 3. 1 – ADC, indicator and datalogger; 2 – compensating module; 3 – PC connection (RS 232)

The system can monitor the variation of the reflected and scattered light. The relative optical density (ROD, %) in the environment is monitored by establishing the variation of the intensity of the main beam (I_0), formed from a source of light in the visible part of the spectrum, and that of transmitted light (I_1).

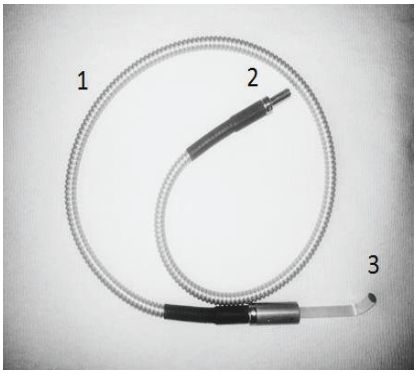

Figure 2. 1 – flexible optical fiber; 2 – tip; 3 – firm optical fiber

The influence of temperature on the course of enzymatic and aggregation phases of rennet coagulation was established and determined by the optical system of automatic control.

The temperature range investigated in the experiment include temperatures between 5÷25°C (considered as low temperatures of coagulation because the optimal temperature for conducting the process is 35÷37°C).

At each of these temperatures in the milk was added milk-clotting enzyme, then the sample was retained for a period of 30 min, followed by heating to a temperature of coagulation (35÷37°C). The duration of each of the conducted trials was 90 min.

The results obtained using calf chymosin are presented in Fig. 4.

A decrease in the values of the relative optical density was observed at temperatures 5÷15°C before heating the samples, which correlates with the theoretical statements of Stenne – Hutin and Berridge (Berridge, 1952; Szadkovska, 1978).

The negative values of the relative optical density are associated with the enzymatic phase of the coagulation process, related to the beginning of the hydrolysis of the k-casein

fraction of the protein micelle and the separation of glycomacropeptide residue. This reaction determines the destabilization of the protein micelle and the course of the enzymatic phase of the coagulation process.

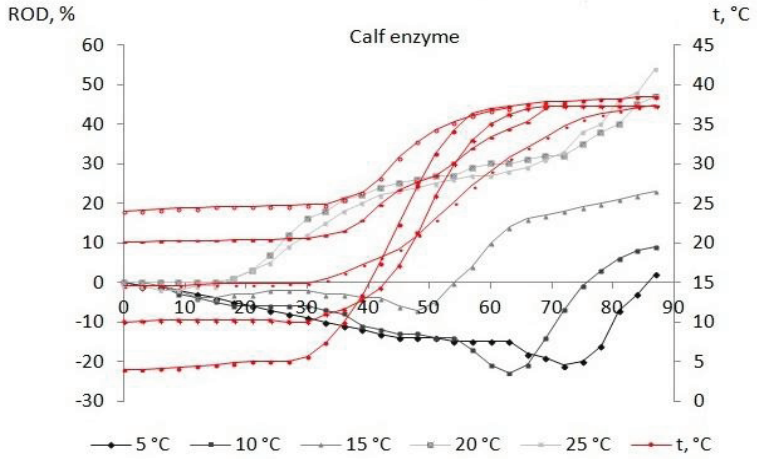

Figure 4. Monitoring of the enzymatic and aggregation phases of the coagulation process, using calf chymosin

The results presented in Figure 4 show that at temperatures 5÷15°C prevalence of enzymatic phase to that of aggregation is recorded, evidenced by the values of ROD, % remained negative until the start of heating. Increasing the temperature to 35÷37°C, the aggregation phase of the enzymatic coagulation begins.

As the temperature increases up to 35÷37°C (the optimal temperature for the coagulation process), the values of the relative optical density become positive, due to the preponderance of the aggregation phase and the flocculation reaction.

It was observed that in the temperature interval 5÷15°C, the enzymatic phase was prolonged depending on the temperature of the retention - at 5 °C the beginning of the aggregation phase was observed after reaching the temperature of coagulation, and by heating for a period of 25 min. The trend was similar for 10 and 15°C. Differences were observed in the aggregation time – for the first sample (10°C) the heating period is 10 min, while for the second (15°C) the aggregation phase occurs upon reaching 35°C.

Acceleration of the aggregation phase at temperatures of 20÷25°C was reported. The values of the relative optical density are positive, even before the heating starts i.e. the aggregation phase occurs before the optimal temperature of coagulation was reached.

Using bovine chymosin, intensive flocculation and increase of the degree of aggregation was observed, at a temperature of 16 ÷ 17°C.

Analogous experiment was conducted using an enzyme of camel origin. The results are presented in Fig.5.

The results presented on Figure 5, show similar trend, observed when calf chymosin was used.

At temperatures 5÷15°C prevalence of enzymatic phase to that of aggregation was marked, evidenced by the negative values of the relative optic density until the start of heating.

Differences, between the two enzymes (calf and camel), related to the aggregation time were noticed in the temperature interval 5÷15 °C, - when the camel enzyme was used, at 5°C and 10 °C, the same heating time was needed for the aggregation phase to start, after increasing the temperature to 37°C – approximately 25 min.

The aggregation phase in the sample kept at 15 °C occurs upon reaching 35°C, the same trend observed when the calf chymosin was used.

Reaching temperatures of 17÷18°C, an accelerated rate of aggregation by registering preponderance of the flocculation phase was established for the entire temperature range investigated.

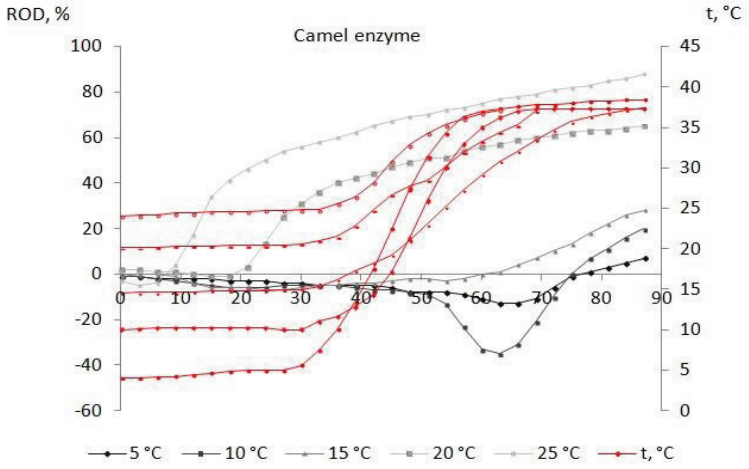

Figure 5. Monitoring of the enzymatic and aggregation phases of the coagulation process, using camel coagulant

The results obtained using an enzyme of microbial origin are presented in Fig. 6.

The results when the microbial enzyme was used differ significantly from the data obtained for the other two milk-clotting enzymes.

At temperatures 5÷15°C, lowest values of the relative optical density were reported before heating the samples, reaching – 50 relative optical units. At temperatures of 5°C the enzymatic phase was observed by heating to temperatures of coagulation for a period of 25 min, as in the case of the other two tested enzymes. Differences were established in relation to the aggregation phase - the flocculation was not observed by increasing the temperatures to 37°C, and the values of the optical density were negative by the end of the experiment. The trend observed for the samples kept in 10 and 15°C was similar to the other two examined coagulants.

Increasing of relative optical density values were recorded at a temperature of 25°C, associated with an increase in the rate of aggregation of casein structures and a formation of flocculates.

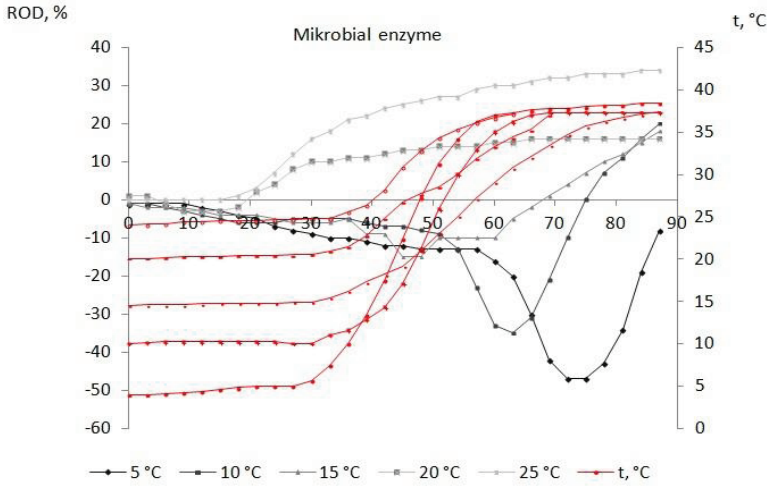

Figure 6. Monitoring of the enzymatic and aggregation phases of the coagulation process, using microbial coagulant

Depending on the type of enzymes and the values of the coagulation temperature, the phases of the coagulation process occurring at a different pace and with different characteristics. For all three milk coagulants, the enzymatic phase of the coagulation process was observed at low temperatures. However, using calf chymosin, the enzymatic phase was started without increasing the temperature and before heating.

Similar trend was established for the other two tested milk coagulants, different from the one observed for the calf chymosin: the course of the enzymatic phase and the decrease of the relative optical density are registered at increased temperatures, respectively 18÷20°C. These conditions provide the energy required for the implementation of the hydrolysis reaction of the k-casein.

However, when the calf chymosin was used, the temperature significantly affect the duration of the enzymatic stage, the time for aggregation and the rate of the flocculation of the protein component. The rate of aggregation is maximum when the sample was heated to a temperature of 20÷25°C, as similar trend was established when microbial enzyme was used. The fastest rate of the flocculation was observed when enzyme of camel origin was tested - aggregation was observed by increasing the temperatures to 17÷18°C, i.e minimum amount of energy was required for the beginning of the flocculation.

The results obtained using milk-clotting enzyme of camel origin are related to the action and characteristics of the enzyme - strong specificity for the k-casein fraction of the milk, resulting in accelerated aggregation and flocculation phase without significant influence of the temperature on the rate of the phases of the coagulation process.

CONCLUSIONS

The results of the conducted experiments establish the characteristics of the enzymatic and aggregation phases of the enzymatic coagulation, using three different genetic variants of milk-clotting enzymes.

Significant factor in the progress of enzymatic and aggregation phase of the coagulation process is the temperature of coagulation of milk. Low positive temperatures of the coagulation (5÷15°C) have a slight influence on the enzymatic phase and are significant factor in flocculation and aggregation phase - the acceleration of the aggregation phase was observed with increasing temperature for the three tested milk-clotting enzymes.

The developed automatic optical system used in the experiment can be applied in manufacturing practice for examination of the dynamics of the technological processes in the production of cheese, optimization and control of the coagulation process with the necessary practical accuracy.

The obtained results and dependencies are applicable in cheese production according to the conditions and types of enzymes used. They can be applied for a stream dosage of the coagulants, regulation of syneresis and preparation of technological schemes for the production of various types of cheeses.

REFERENCES

Andersen J.B., Friggens N.C., Sejrsen K., Sørensen M.T., Munksgaard L., Ingvartsen K.L., 2003. The effects of low vs. high concentrate level in the diet on performance in cows milked two or three times daily in early lactation Livestock Production Science, 81, 119–128.

Baltadzieva M., 1996. Technology of milk products. Sofia, Zemizdat (BG).

Berridge N.J., 1952. Some observations on the determination of the activity of rennet. The Analyst, 77, 57-62.

Dalgleish D.G., 2009. Coagulation of renneted bovine, casein micelles: dependence on temperature, calcium ion concentration, and ionic strength.Journal of Dairy Research, 50, 331-340.

Hassan H.A., 1995. Effects of crossing and environmental factors on production and some constituents of milk in Ossimi and Saidi sheep and their crosses with Chios. Small Rum. Res., 18, 165-172.

Lagaude A.,Fernandez L.,Cuq J.L., Marchesseau S., 2004. Characterization of curd formation during the rennet coagulation of milk by an optical microscopic method. International Dairy Journal,14,1033-1039.

Lucey J.A., 2003. Formation, structure, properties and rheologie of acid-coagulated milk gels. In cheese: Chemistry, Physics and Microbiology, vol. 1, General Aspects, 3rd edn (P.F.Fox, P.L.H. Mc Sweeney, T.M. Cogan and T.P. Guime), 105-122, London: Elsevier.

Morgenroth I., 1899. Centribl. Bakt. Par., i, 26, 349.

Ramet J.P., Weber F., 1980. Le lait, 60, 1–13.

Szadkovska et al., 1978. Method for coagulatin milk proteins. US Patent.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INFLUENCE OF THE TIME FOR INITIAL COAGULATION AND RENNET GEL COMPACTNESS ON THE PROTEIN LOSSES IN WHEY

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Abstract

Analyses of the possibilities for early cutting of rennet gel in the production of cheese were made, in order to achieve more efficient use of the technological equipment. The moment of cutting the coagulum was determined by studying the rheological properties of the gel (strength, compactness, elasticity). An increase in the losses of dry matter in whey was observed in early cutting of the coagulum, result of the fragile microstructure of the milk gel.
The influence of the time for initial coagulation and rennet gel compactness on protein (casein) losses in whey was examined during enzymatic coagulation of cow's milk. The amount of used milk-clotting enzymes of calf, camel and microbial origin was 30 $cm^3.10^{-2}.dm^{-3}$. Cutting of the gel was carried out after15, 22, 30 and 60 min. The final tested value was indicated as control.
For the three milk coagulants studied, inversely proportional alteration of protein losses was established, for a time of initial coagulation between 520-630 s. A tendency to minimize the protein losses in whey was monitored by increasing the compactness and the strength of the rennet gel, which was significantly influenced by the type of used coagulants.
The obtained results can be used to analyze, predict, and modificate the technological process in cheese practice and production, particularly for production of hard-type cheeses.

Key words*: rennet gel, mechanical processing, early cutting, enzymatic coagulation, milk coagulants.*

INTRODUCTION

The enzymatic coagulation of milk and the processing of the obtained rennet gel are basic technological operations determining the yield and quality of the final product in the production of hard cheeses. One of the factors that influence the amount of the final product is the losses of dry matter in whey. They are determined primarily by loss of total protein, casein and milk fat (Mona al., 2011).
The rheological properties of the curd (density, strength, elasticity) define the initial time of the mechanical treatment of the coagulum (cutting) (Castillo al., 2004).
The determination of the rheological properties is performed by various methods - penetrometric, ultrasonic, optical, viscometric, etc. (Storry, 1982; Richardson, 1985; James, 1996). The losses of protein in whey increase if the time of cutting is inaccurately determined. The protein losses originate mainly from the process of cutting and the separation of the whey from the obtained gel (draining). The losses are determined by the hardness and strength of the gel and the nature of the milk-clotting enzyme, when the same means of mechanical treatment (cutting and stirring rate) are used (Tunick, 2000; Law, 2010).
There is an increase of losses of casein in whey, depending on the time of accelerated initial coagulation (240 s) and in relation to the cutting time studied. For samples with initial coagulation at a moderate rate and characteristics (520÷630 s) was found inversely proportional alteration in protein losses, depending on the cutting time of the coagulum (Panayotov, 2012).
The cutting time affects the losses of dry matter in whey, the moisture content of the hard cheeses, the yield and the quality of the cheeses.
The rate of cutting and stirring affects the dimensions of the particles of the coagulum, which results in an increase of the losses of protein in whey (Johnston, 2001).
Early cutting of the rennet gel increases the impact of the mechanical operations on the

rennet gel, reduces the size of the particles of the coagulum and increases the loss of protein in whey (Johnston, 2001; Castillo, 2006).
The extension of the cutting time has a positive effect on increasing the yield in the production of hard cheeses. Performing later cutting results in the formation of dense and fragile gel that separates whey difficulty, leading to a high water content, hindering the process of ripening, resulting in deterioration of the final product quality (Macedo, 1997).
The aim of the research study was to determine the protein losses in whey depending on the time for initial coagulation and the thickness of the obtained rennet gel.

MATERIALS AND METHODS

For the purpose of the experiment was used cow milk, analyzed by ultrasonic analyzer, with the following physico-chemical parameters: protein–3.3%; fat–3.7%; non-fat solids–8.60%; density–1.028 $g \cdot cm^{-3}$; titratable acidity–17°T; pH–6.76. The milk was normalized at a fat content of 3.6% and dry matter of 12.5% was achieved.
Determination of the total protein content (casein and soluble proteins) was carried out by the Kjeldahl method, using H_2SO_4 0.1 n, with a relative density of 1.84 and 33% solution of NaOH 0.1 n.
The initial coagulation was determinate using the Berridge method (Berridge, 1952).
The density of the rennet gel was defined by a penetrometer, having a cylindrical shape with $F=2 \cdot 10^{-4}$ m^2, m=0.0139 kg and k = 0.5 $N \cdot kg^{-1}$, where F-working surface, m^2; m-mass, kg; k-constant, $N \cdot kg^{-1}$.
The strain of displacement Θ was calculated by moving the operating body (h): $\Theta = k \cdot m \cdot h^{-2} = 0.00695 \cdot h^{-2}, N \cdot m^{-2}$ (Todorov, 1975).
Milk coagulants used in the experiment (in amount of 30 $cm^3 \cdot 10^{-2}$ dm^{-3}): calf chymosin, pepsin and enzymes from microbial and camel sources (market preparations, produced by Chr. Hansen) with activity 1:10000. Each milk sample had a volume of 1000 cm^3.
Cutting of rennet gel was performed on 15, 22, 30 and 60 minutes from the introduction of the milk-clotting enzymes.
Statistical Data processing was carried out by mathematical software SigmaPlot 11.0.

RESULTS AND DISCUSSIONS

An experiment was conducted to study the protein losses in whey in correlation with the time for initial coagulation and the hardness of obtained rennet gel using various types of milk-clotting enzymes with different characteristics.
The quantities of the total protein and casein losses at different values of initial coagulation time for each tested milk-clotting enzymes were defined.
In Figures 1 and 2 are presented the results for the protein and casein losses according to the time for initial coagulation using four different genetic variants of milk coagulants.
The values for the initial coagulation with the use of calf chymosin, pepsin, and enzymes from microbial and camel sources vary in the range of 520÷650 s.
With the initial coagulation time of 648 s, when pepsin was used, high levels of protein losses were observed during the experiment, also found in previous studies of Panayotov al. (Panayotov al, 2012). The lowest values for the protein losses were marked for the enzyme from camel source, with time for initial coagulation 520 s. Similar correlation between the initial coagulation time and protein losses was presented by the other two tested milk-clotting enzymes. The obtained values ranged between 1.1 ÷ 1.2%.
Differences were observed between the studied parameters, for each of the milk-clotting enzymes applied, depending on the mechanical treatment of the gel, for cutting times respectively 15, 22, 30 and 60 min.
Minimum levels of protein losses were reported at cutting the gel after 60 min from adding the milk-clotting enzymes as also was found by Law (Law, 2010). For the pepsin preparation the losses at 15 and 60 min of cutting had similar values.
Regarding the losses of casein for the four tested cutting times, the trend was similar for all milk-clotting enzymes used. Maximum casein losses were observed for cutting time of 15 min and initial coagulation time of 648 s (0,43%), corresponding to the values registered for the pepsin preparation.
Minimum casein losses were established using milk-clotting enzyme from camel

source, varying in the range of 0.15÷0.20%, because of the specificity of the enzyme, described in studies of Kappeler (Kappeler, 2006).

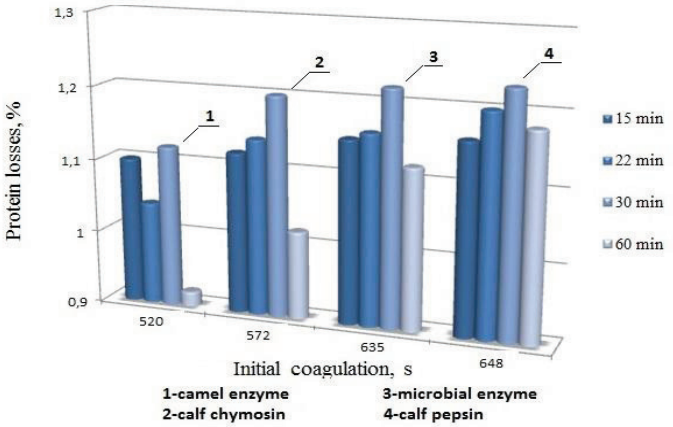

Figure 1. Total protein losses correlated with the time for initial coagulation, using enzyme of camel origin, calf chymosin, microbial enzyme and pepsin.

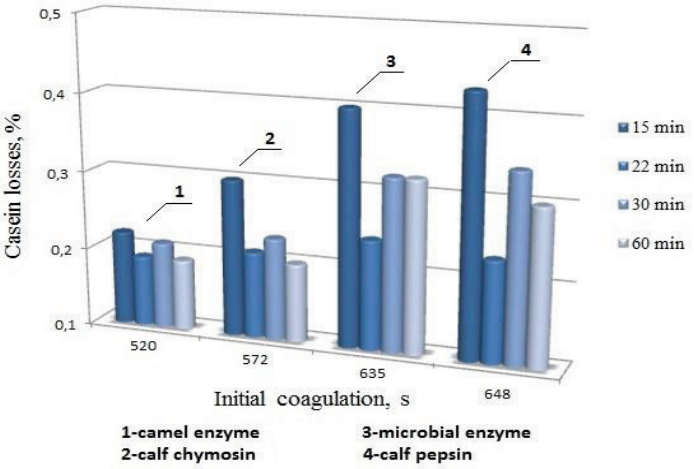

Figure 2. Casein losses correlated with the time for initial coagulation, using enzyme of camel origin, calf chymosin, microbial enzyme and pepsin

The results from the penetrometric study during the enzymatic coagulation are presented in Table 1. The values express the depth of immersion of the working body of the penetrometer using 30 $cm^3 \cdot 10^{-2} \cdot dm^{-3}$ of the milk-clotting enzymes for examination period of 60 s.

The average values of the strain displacement presented in Table 2 were calculated after mathematical processing of the data.

The results from Table 2 demonstrate that the values for the strain of displacement increase proportionally with the hardness and the strength of the obtained coagulum. Maximum values of the strain displacement (19.16 $N \cdot m^{-2} \cdot 10^{-6}$) were determinate for the camel enzyme, having a time for initial coagulation of 520 s.

The variation of the values of the strain displacement using calf chymosin and microbial enzyme was characterized by a similar rate and minimal differences in the strain of displacement during the experiment. The differences were established for cutting time 60 min after the enzymes were added in the milk, with variation of the values 18.96 ±

1.5 $N \cdot m^{-2} \cdot 10^{-6}$ for the calf chymosin and 12.07 ± 0.62 $N \cdot m^{-2} \cdot 10^{-6}$ for the microbial enzyme.
Because of the tendency of the pepsin to form labile and easily deformable rennet gel, the values for the strain of displacement at the end of the experiment using this enzyme were lower than those of the other milk coagulants tested - 9.12 ± 0.34 $N \cdot m^{-2} \cdot 10^{-6}$.

Table 1. Depth of penetration in rennet gel, using milk-clotting enzymes of microbial and camel origin, calf pepsin and chymosin

Milk-clotting enzyme type	Initial coagulation time, s	Depth of penetration (mm) in rennet gel for a cutting time (min) using milk-clotting enzymes in an amount of 30 $cm^3.10^{-2}.dm^{-3}$ for 60 s			
		15	22	30	60
Camel enzyme	520 ± 22	29,0 ± 1,8	25,0 ± 1,1	21,0 ± 1,8	18,4 ± 1,5
Calf chymosin	572 ± 23	35,2 ± 2,5	31,0 ± 2,3	27,0 ± 2,0	19,0 ± 1,8
Microbial enzyme	635 ± 22	36,5 ± 2,4	30,0 ± 2,8	27,0 ± 2,6	22,0 ± 2,1
Pepsin	648 ± 22	41, 3 ± 3,1	36,5 ± 2,4	31 ± 2,3	27 ± 2,6

Table 2. Strain of displacement in rennet gel, using milk-clotting enzymes of microbial and camel origin, calf pepsin and chymosin

Milk-clotting enzyme type	Initial coagulation time, s	Strain of displacement ($Nxm^{-2}.10^{-6}$) in rennet gel, obtained using milk-clotting enzymes in an amount of 30 $cm^3.10^{-2}.dm^{-3}$ for 60 s			
		15	22	30	60
Camel enzyme	520 ± 22	8,26 ± 0,18	9,97 ± 0,74	12, 13 ± 0,62	19,76 ± 1,64
Calf chymosin	572 ± 23	6,96 ± 0,16	8,15 ± 0,14	9,39 ± 0,42	18,96 ± 1,5
Microbial enzyme	635 ± 22	5,51 ± 0,18	8,26 ± 0,24	9,12 ± 0,34	12,07 ± 0,62
Pepsin	648 ± 22	3,88 ± 0,12	5,51 ± 0,18	8,15 ± 0,14	9,12 ± 0,34

Simultaneously with the determination of the strain of displacement were established the dependences: total protein and casein losses according to the thickness and the strength of the rennet gel.
The data obtained are presented in Figures 3-5.
The trend for the total protein and casein losses in whey, using the three milk-clotting enzymes, was similar. The highest values observed for the total protein losses was in the range 1.2 ÷ 1.4%, corresponding to a strain of displacement between 9.0÷12.13 $N \cdot m^{-2} \cdot 10^{-6}$ (or a time of cutting the rennet gel 30 min after addition of the coagulants). The losses of casein were in the range of 0.2÷0.4% (the highest values were observed at the lower values of the strain of displacement, corresponding to a cutting time 15 min after enzymes addition).
An increase of the protein losses was observed by increasing the thickness of the rennet gel, due to the formation of a gel with a friable and fragile structure. The results obtained using a calf chymosin showed differences, as the values for the total protein and casein losses were minimized at the highest values of the strain of displacement (gel with the highest hardness and strength). The process of cutting the rennet gel with higher density and strength showed a greater resistance to the cutting instruments, which leads to an increase of a total protein and casein losses in whey, resulting in a reduction in yield.
The most significant loss of protein and casein were registered using the enzyme of microbial origin, due to the rheological properties and characteristics of the formed rennet gel. The coagulum obtained in 40 min during the experiment was defined as fragile, easily deformable, with accelerated syneresis, resulting in decreasing of the values of the strain of displacement of 5.51÷12.07 $N \cdot m^{-2} \cdot 10^{-6}$.

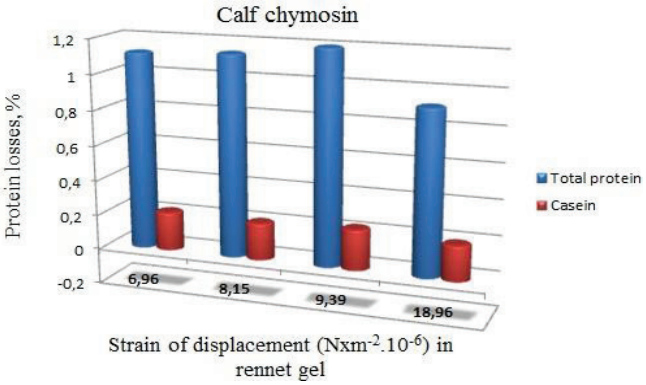

Figure 3. Total protein losses correlated with the strength of the rennet gel, using calf chymosin

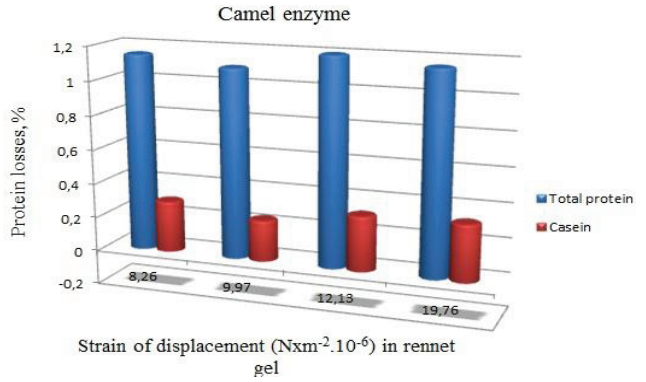

Figure 4. Total protein losses correlated with the strength of the rennet gel, using camel enzyme

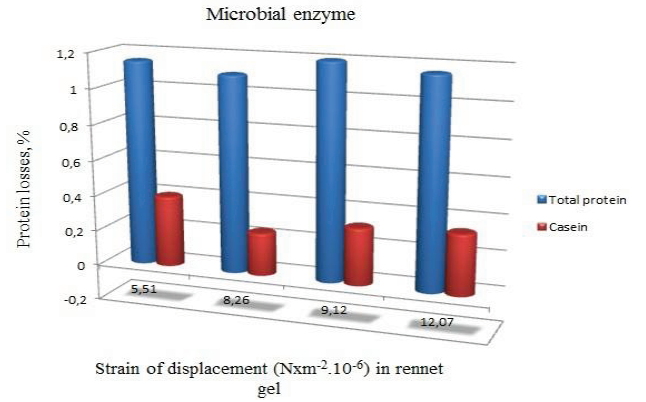

Figure 5. Total protein losses with the strength of the rennet gel, using microbial enzyme

The enzyme with camel origin leads to a formation of dense structure of the coagulum, with accelerated gelling and low syneresis. In comparison with the other tested milk-clotting enzymes, the values of the strain of displacement were maximum, which determines the minimization of the protein (1.02%) and casein (0.2%) losses.

The same pace of variation of the thickness and strength of the gels formed during the coagulation process was monitored for all the milk-clotting enzymes studied. This trend was explained by the insignificant differences in the data obtained for the total protein and casein losses in whey.

CONCLUSIONS

The conducted experiments and obtained results allow concluding that the three used milk-clotting enzymes had a similar trend in total protein and casein losses during the process of draining.

Inversely proportional variation in the values for the total protein and casein losses was monitored for the times of initial coagulation of 520÷650 s. Using enzyme with camel origin the losses were minimized, related to the acceleration of the phase of initial coagulation.

By increasing the compactness and strength of the rennet gel, the losses of protein in whey were decreased, influenced first by the milk coagulant used and second by the cutting time of the rennet gel. The late cutting of the coagulum, results in an increase of losses of dry matter in whey, due to the formation of a gel with a friable structure.

The obtained results can be used to analyze, predict and modify the technological process in cheese making practice, for the production of hard cheeses, using the three investigated enzymes.

To minimize the protein and casein losses the obtained coagulum can be cut in the earlier phase of the coagulation process, which provides minimal resistance to the cutting instruments and devices. The mentioned conditions create opportunities to maximize the protein component, increase yields and improve the quality characteristics of the final product.

REFERENCES

Berridge, N. J. (1952). Some observations on the determination of the activity of rennet. The Analyst, 77, 57–62.

Castillo M., González R., Payne F. A., Laencina J., López M. B., 2004. Optical monitoring of milk coagulation and inline cutting time prediction in murcian al vino cheese. Applied Engineering in Agriculture. American Society of Agricultural Engineers ISSN 0883−8542. Vol. 21, 3, 465-471.

Castillo M., Payne F.A, Wang T., Lucey J.A., 2006. Effect of temperature and inoculum concentration on prediction both gelation and cutting time. Cottage cheese-type gels. International Dairy Journal Vol. 16, 147–152.

Johnson M.E., Chen C.M., Jaeggi J.J., 2001. Effect of rennet coagulation time on composition., yield and quality of reduced-fat cheddar cheese. Journal of Dairy Science. Vol. 84, 5, 1027 - 1033.

Kappeler S.R., van der Brink H.J.M., Rahbek-Nielsen H., Farah, Z., Puhan, Z.Hansen, E.B., Johansen, E., 2006. Characterization of recombinant camel chymosin reveals superior properties for the coagulation of bovine and camel milk.Biochemical and Biophysical Research Communications.

Law A.B., Tamime Y.A., 2010. Technology of Cheesemaking. The Production, Action and Application of Rennet and Coagulants. Vol. 2, 100-102.

Macedo A.C., Malcata F.X., Oliveira J.C., 1997. Effect of production factors and ripening conditions on the characteristics of Serra cheese. International Journal of food science and technology, Vol.32, 6, 501-511.

Mona A.M., Gawad A.E., Nawal S.A., 2011. Cheese yield as affected by some parameters (Review). Acta Sci. Pol., Technol. Aliment. 10, 2, 131-153. ISSN 1644-0730.

Panayotov P., Boyanova P., Milenkov B., 2012. Influence of the time for initial coagulation and cutting time of rennet gel on the whey composition during enzymatic coagulation of cow and sheep milk. Journal MGUP., Vol. 2 (BG).

Richardson G. H., Okigbo L. M., Thorpe J. D., 1985. Instrument for measuring milk coagulation in the cheese vats. Journal of Dairy Science. Vol. 68, 32 – 36.

Steffe J.F., 1996. Rheological methods in food process engineering. Dept. of Food Process Engineering and Human Nutrition. Michigan State University, USA.

Storry J.E., Ford G.D., 1982. Development of coagulum firmness in renneted milk – a two phase process. J. Dairy Res. Vol. 49, 343–346.

Todorov L., 1975. Options for acceleration and mechanization of the process pressing in the manufacture of white brined cheese. Development and testing of the device for measuring the strength of the coagulum, obtained from cow milk. PhD thesis, 58-62 (BG).

Tunick M.H., 2000. Reology of dairy foods that gel, stretch, and fracture. J Dairy Sci., 83:1892–1898.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BIOLOGICALLY ACTIVE SUBSTANCES AND *IN VITRO* ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS FROM DANDELION (*TARAXACUM OFFICINALE*) ROOTS

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Abstract

Dandelion (Taraxacum officinale L. Weber ex F.H. Wigg.) roots were traditionally used in folk medicine worldwide due to its antidiabetic, choleretic, antirheumatic and diuretic properties. The aim of the current study was to determinate the biologically active substances in 95% ethanol and subsequent water extracts from dandelion roots and to evaluate their antioxidant activities. The carbohydrate composition was analyzed by the resorcinol assay, TLC and HPLC-RID methods. The total phenolic contents (TPC), total flavonoids (TF) and total dihydroxycinnamic derivatives contents were determined by Folin–Ciocalteau method, aluminium chloride colorimetric assay and Arnow's reagent, respectively. In vitro antioxidant activities of the extracts were estimated using ferric-reducing/antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assays. In spring harvested dandelion roots the total fructan content were established to be in range 17-19 % dw. The low molecular carbohydrate fraction presented by glucose, fructose, sucrose, 1-kestose and nystose dominated in 95 % ethanol extracts. The subsequent water extracts demonstrated the highest inulin content (12% dw), while TPC, TF and total dihydroxycinnamic derivatives contents were reported to be 9.2 mg GAE/g, 1.7 mg QE/g and 13.7 mg chlorogenic acid derivates/g dw, respectively. These extracts showed the highest antioxidant activity for both the DPPH (82.1 mM TE/g) and FRAP (52.9 mg mM TE/g) assays. Therefore, the subsequent water extracts possessed the highest antioxidant capacity, which positively correlated with the phenolic content. The results of current investigation demonstrate that dandelion root is a valuable source of dietary fibers and natural antioxidants and could be successfully used in foods with the potential to improve digestion and prevent from oxidative stress related diseases.

Key words: *dandelion roots, inulin, total phenolic content, antioxidant activity.*

INTRODUCTION

The interest in medicinal extracts constantly increased because of their improved healthy effect and protective properties against oxidative stress disorders (Aleksieva et al. 2013; Ivanov et al., 2014).

Dandelion *Taraxacum officinale* (L.) Weber ex F.H. Wigg is a medicinal plant member of Compositae family, subfamily Cichorioideae, tribe Lactuceae. It is widely distributed in the warmer temperate zones of the Northern Hemisphere as a perennial weed (Schütz et al., 2006 a). The plant reaches an average length of 15–30 cm and sometimes up to 70 cm (Nnamdi et al., 2012) with roots 60–100 cm in length (Schütz et al., 2006 a).

From century ago infusions and decoctions of the dandelion roots and herbs have been utilized for the treatment of various ailments such as kidney disease, dyspepsia, heartburn, spleen, liver complaints, hepatitis and anorexia (Sweeney et al., 2005; Schütz et al., 2006 a). In Bulgarian traditional herbal medicine, this plant is used for treatment of digestive diseases, prevention of renal gravel and loss of appetite (Pamukov and Ahtardjiev, 1990). The main suppliers of dandelion are Bulgaria, followed

by former Yugoslavia, Romania, Hungary and Poland (Bisset et al., 1994).

Dandelion active ingredients are found in both the roots and leaves (Amin et al., 2013). Its roots contain sesquiterpenes, tetrahydroridentin B, taraxacolide-*O*-β-glucopyranoside, triterpenes, phytosterols (taraxasterols, their acetates and 16-hydroxy derivatives, arnidol, faradiol, α- and β-amyrin, β-sitosterol, β-sitosterol-D-d-glucopyranoside and stigmasterol), several phenolic compounds (chicoric acid, monocaffeoyltartaric, 4-caffeoylquinic, chlorogenic, caffeic, *p*-coumaric, ferulic, *p*-hydroxybenzoic, protocatechuic, vanillic, syringic and *p*-hydroxyphenylacetic acids), as well as three coumarins (umbelliferone, esculetin and scopoletin) (Williams et al., 1996; Schütz et al., 2006 a). Apart from above mentioned secondary metabolites, the dandelion roots are a rich source of polysaccharides, mainly inulin-type fructans and smaller amounts of pectin, resin, and mucilage (Schütz et al., 2006 a; Amin et al., 2013). Inulin and its short chains - fructooligosaccharides are fructans that consists mainly of β-(2→1) fructosyl fructose units (Fm), and usually, but not always, the chain terminates with α-glucopyranosyl unit (1→2) (GFn). Fructan content in dandelion roots ranges from 2% in spring to 40% in autumn (Bisset et al., 1994). Inulin and FOSs are soluble dietary fibers that stimulate growth of *Bifidobacteria*, low glucose blood level, improve mineral absorption and possess immunomodulation effects (Gibson and Roberfroid, 1995; Barcley et al., 2010). Trojanov´a et al., (2004) proved the prebiotic activity of dandelion oligofructans. They stimulate the growth of bifidobacterial cultures. Therefore, the presence of enormous variety of biologically active substances in dandelion roots enhances their nutritional and healthy effects. Leaves of this plant are often consumed as salads, their roots are a coffee substitute, while dandelion extracts have also been used as flavour enhancers in soft drinks and baked goods. In addition, dandelion is often marketed as a health food (Leung et al., 1996). Its roots (Taraxaci radix) are processed into pharmaceutical preparations such as teas, tinctures, capsules, tablets and juices (Schütz et al., 2006b).

Several health-promoting benefits, including diuretic, laxative, cholagogue, anti-rheumatic, antiinflammatory, choleretic, anti-carcinogenic, analgesic, anti-hyperglycemic activities, anti-coagulatory and prebiotic effects have been attributed to the use of dandelion extracts or the plant itself (Schütz et al., 2006 a; Kenny et al., 2014 a). Dandelion root has been reported to possess antioxidant activity that is linked to the presence of phenolic based compounds (Hagymasi et al., 2000, Cho et al, 2002). It was reported that water, ethanol and methanol extracts from the aerial art and root of dandelion possessed antimicrobial activity against *B. cereus, E. coli* and *S. aureus* as this effect was again linked to the phenolic content (Kenny et al., 2014).

Until now, there are uncompleted information about presence of inulin, total phenols, total dihydroxycinnamic derivatives and flavonoids in root of *Taraxacum officinale* L., growth in Bulgaria, especially during the spring season. Not detailed investigations have been reported regarding evaluation of radical-scavanging activities of dandelion roots. Only antioxidant potencial of water infusion from herb *Taraxacum officinale* L. evaluated by ABTS method (Ivanova et al., 2005) and leaves extracts measured by DPPH, FRAP and CuPRAC assays (Ivanov, 2014) were reported.

Therefore, the aim of the current research was to determinate the content of biologically active substances in 95% ethanol and subsequent water extracts from dandelion roots and to evaluate their *in vitro* antioxidant activities.

MATERIALS AND METHODS

All used reagents and solvents were of analytical grade scale. Carbohydrate standards fructose, sucrose, 1-kestose and nystose have been purchased from Sigma-Aldrich (Steinheim, Germany). Fructooligosacchrides Frutafit®CLR, and inulin Frutafit®TEX were supplied by Sensus (Roosendaal, the Netherlands).

The taproots of several randomly chosen dandelion plants were gathered from territory of South Bulgaria – Plovdiv (Plovdiv region), Parvomay (Krushevo village) and Chirpan (Stara Zagora region) during 13-20 April 2013. The underground parts were air-dried, finely

ground and passed through a 0.5 mm sieves. The root powder with approximately 12-14 % moisture content was stored in crew-capped containers for further use.

Extraction procedure

Dandelion roots (20 g) were extracted successively in a Soxhlet apparatus with hexane, $CHCl_3$ and ethyl acetate to remove lipophilic compounds (Olennikov et al., 2009) and the residue was dried. The extraction procedure was performed by previously described method (Petkova & Denev, 2013a). Dandelion dry roots (0.8 g) were placed into a round-bottom flask. A total of 40 mL of 95% ethanol was added and the sample was boiled under reflux for 60 min. The extraction process was repeated twice with 40 mL and 20 mL solvents, respectively. The residue was dried and it was extracted successively with 40 mL, 40 mL and 20 mL boiling distilled water under reflux as the duration for each extraction procedure was 60 min. The obtained dandelion root extracts were analyzed for total fructans, total phenolic content, total dihydroxycinnamic derivative, total flavonoids and radical scavenging activity.

Total fructan content

The fructan content in the obtained extracts expressed as fructose equivalent were analyzed spectrophotometrically at wavelength 480 nm by resorcinol-thiourea reagent (Petkova and Denev, 2012). Hundred microliters extract were place in glass tube of 10 mL, and 100 μL resorcinol (1% ethanol solution), 100 μL thiourea (0.1% ethanol solution), 800 μL 95% ethanol and 900 μL HCl were added to them. The sample was heated 8 min at 80 °C, cooled and filled with water until 10 mL. Then the absorbance was measure against distilled water.

Identification of mono-, di-, fructooligosaccharides (FOS) and inulin by Thin layer chromatography

TLC analysis were used to elucidate the presence of mono-, di-, fructooligosaccharides (FOS) and inulin in the ethanol and water extracts obtained from dandelion roots. Five microliters of each sample were performed on silica gel 60 F_{254} plates (Merck, Germany) with mobile phase *n*-BuOH:*i*-Pro:H_2O:CH_3COOH (7:5:4:2) (v/v/v/v). The TLC plates were dipped in the detecting reagent diphenylamine-aniline-H_3PO_4–acetone (Lingyun et al., 2007), heated and scanned as previously described (Petkova and Denev, 2013a).

Carbohydrate analysis by HPLC-RID method

HPLC-RID methods were used for quantification of sugars (glucose, fructose, sucrose), 1-kestose, nystose and inulin in dandelion root extracts. Chromatographic separation was performed on HPLC Shimadzu, coupled with LC-20AD pump, refractive index detector Shimadzu RID-10A and software program LC solution version 1.24 SP1 (Shimadzu Corporation, Kyoto, Japan). The analysis were performed on an analytical column Shodex® Sugar SP0810 with Pb^{2+} (300 mm × 8.0 mm i.d.) coupled with a guard column (50 × 9.2 mm i.d.) at 85 °C. The mobile phase used for separation was distilled water with flow rate 1.0 ml/min. The injection volume of the samples was 20 μL (Petkova et al., 2014).

Total phenolic content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu reagent according to the described procedure (Stintzing et al., 2005) with some modifications. Basically, 0.2 ml dandelion root extract was mixed with 1 ml Folin–Ciocalteu reagent diluted five times and 0.8 mL 7.5 % Na_2CO_3. The reaction was performed for 20 min at room temperature in darkness. Then the absorbance was measured at 765 nm against blank sample developed the same way but without extract. The results were expressed in mg equivalent of gallic acid (GAE) per g dry weight (dw), according to calibration curve; build in range of 0.02 - 0.10 mg (Ivanov et al., 2014). All determinations were performed in triplicate (n = 3).

Determination of total dihydroxycinnamic derivative

The content of total dihydroxycinnamic acid (including caffeoyl derivatives) was expressed as chlorogenic acid as previously described in the European Pharmacopoeia (6th ed. 2008). The dandelion root extract (1 ml) was added to 2 ml 0.5 M HCl, 2 ml Arnow's reagent (10 g

sodium nitrite and 10 g sodium molybdate made up to 100 ml with distilled water), 2 ml 2.125 M NaOH and 3 ml water. Each sample was compared with the same mixture without Arnow's reagent. Absorbance was read at 525 nm. The results were calculated and expressed as mg chlorogenic acid derivates per g dw (Fraisse et al., 2011).

The total flavonoids content

The total flavonoids content was analysed by $Al(NO_3)_3$ reagents. The absorbance was measured at 415 nm. The results were presented as mg equivalents quercetin (QE) per g dry weight (DW) (Kivrak et al., 2009) according to the calibration curve, linear in range of 10-100 µg/mL quercetin as a standard.

Antioxidant activity (AOA):

The antioxidant activity of ethanol and subsequent water extracts from dandelion roots (*Taraxacum officinale L.* Weber ex F.H. Wigg) was evaluated by two methods: DPPH (1,1-diphenyl-2-picrylhydrazyl radical based on mixed hydrogen atom transfer (HAT) and single electron transfer mechanisms and FRAP (ferric reducing antioxidant power) based only on single electron transfer mechanism.

DPPH radical scavenging activity

Dandelion root extract (150 µl) was added to 2.85 ml freshly prepared DPPH solution (0.1 mM in methanol). The sample was incubated for 15 min at 37 °C in darkness. The reduction of absorbance at 517 nm was measured by spectrophotometer in comparison to the blank containing methanol and % inhibition were calculated (Ivanov et al., 2014). A standard curve was built with Trolox in concentration between 0.005 and 1.0 mM. The results are expressed in mM Trolox® equivalents (TE) per g dry weight (dw).

FRAP assay: 0.1 ml of investigated extracts were added to 3 ml FRAP reagent (0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), 20 mM $FeCl_3 \times 6H_2O$ (10:1:1; v/v/v) and allowed to stand for 10 min at 37 °C in darkness. The absorbance of the formed coloured product was measured at 593 nm (Benzie and Strain, 1996). The results from both antioxidant methods were expressed as mM Trolox® equivalents (TE) per g dry weight (dw).

All determinations were performed in triplicate(n = 3) and the data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using MS Excel 2010. A difference was considered statistically significant, when $P < 0.05$.

RESULTS AND DISCUSSIONS

Dandelion roots were evaluated as a rich source of inulin-type fructans with different chain length. TLC analysis showed that extracts from spring plants were characterized with high levels of fructo-oligosaccharides and sugars (Figure 1). The presence of fructose (R_f = 0.50), sucrose (R_f= 0.44), 1-kestose (R_f= 0.37), nystose (R_f=0.32) and FOSs until 8 monomer units (from GF3 to GF7) equivalent to inulin standard Frutafit CLR was detected in 95 % (v/v) ethanol extracts (Figure 1 a). Water extracts obtain after 95 % ethanol treatment contained small amount of residual sucrose, 1-kestose, nystose and also FOSs up to GF8 dominated together with high molecular inulin with DP 22 similar to the used standard (Figure 1 b).

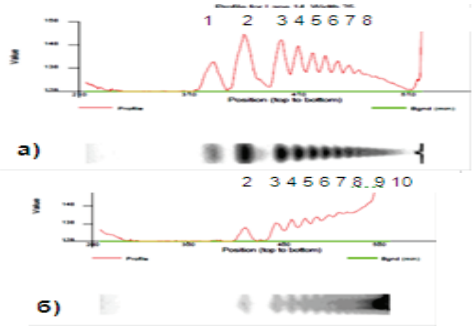


Figure 1. Thin-layer chromatograms of 5 µl a) 95 % ethanol and b) water extracts from root of *Taraxacum officinale L.* Weber ex F.H. Wigg where 1. fructose, 2. sucrose, 3. 1-kestose (GF2), 4. nystose (GF3), 5-8. fructooligosaccharides (respectively GF4, GF5, GF6, GF7, GF8) and 10. inulin

The total fructan content in dandelion roots expressed as fructose equivalents was in range from 17.7 to 19.7 g/100 g dw (Table 1). The high molecular fraction dominated above the ethanol soluble low molecular fraction (sugars and FOSs). Low-molecular fraction (Fru, Suc and FOSs) were around 8 g/100 g dw, while inulin fraction was in range from 8 to12 g/100

g dw. The results obtained were in accordance with previously reported by us data for spring harvested dandelion roots collected from Northern and South Bulgaria locations - 7 and 5 g/100 g dw, respectively (Petkova et al., 2013b). At the same time, the total fructan content in dandelion root harvested in April were lower than the reported content in summer and autumn plants, 21% and 34 %, respectively (Bagaoutdinova et al., 2001; Petkova et al., 2012; Petkova et al., 2013b). Dandelion roots contained more sugars and FOSs in 95 % ethanol extracts than the same extract from the autumn plants (Petkova et al., 2013b).

The detailed analysis of carbohydrate content in 95 % ethanol and the subsequent water extracts was performed by HPLC method (Table 2). The separation of the present compounds was shown on HPLC chromatograms (Figure 2).

Generally, it was considered that glucose levels in dandelion roots seemed to be much lower than fructose and sucrose levels, reaching the maximum level in April and May (Schütz et al., 2006b). Nevertheless, in our case the presence of glucose was not found. Its content could be too small to be detected. The level of fructose reached up to 5 g/100 g dw, while sucrose was in range from 1.8 to 2.2 g/100 g dw (Table 2). In accordance with Wilson et al., (2001) the highest fructose content was defined as dandelion plants initiated new growth. It was found that 1-kestose was the predominant fructooligosaccharide, followed by nystose. The content of 1-kestose and nystose, both considered as best probiotics (Van Loo et al. 1995), reached about 2 % dw. This was consistent with previous reports for common chicory roots and *Helianthus tuberosus* L. tubers (Petkova et al., 2013c; Denev et al., 2014). In contrast, 1-kestose and nystose total amount in dandelion roots exceeded that of artichoke (Schütz et al., 2006b) and elecampane (Petkova et al., 2015), reflecting the short chain characteristic of the inulin. The detected amounts of fructose, sucrose, 1-kestose and nystose expressed on a dry weight basis (Table 2) coincided with those reported previously for dandelion roots harvested in May (Schütz et al., 2006b). The inulin content in the investigated spring dandelion roots was relatively high – 8-11 g/100 g dw (Table 2).

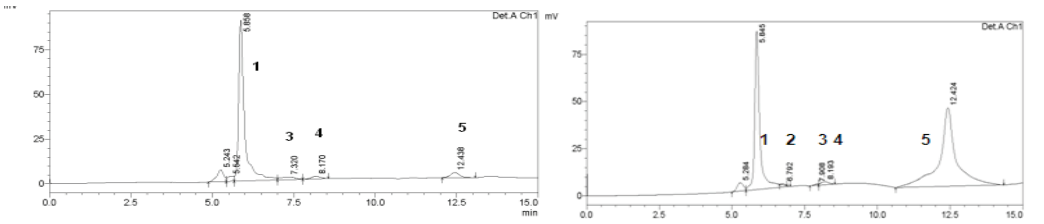
Table 1. Fructan content expressed as fructose equivalents in dandelion roots, g/100 g dw[1] (mean ± SD[2], n=3)

Location	Low-molecular fraction (Fru[3], Suc[4] and FOSs)	High-molecular fraction (inulin)	Total
Chirpan	8.8 ± 0.5	8.8 ± 0.1	17.7 ± 0.6
Plovdiv	7.0 ± 0.6	11.8 ± 0.9	18.8 ± 1.6
Parvomay	7.8 ± 0.1	11.9± 0.3	19.7± 0.3

[1]dry weight, [2]SD – standard deviation, [3]Fru – fructose, 4 - sucrose

Table 2. Carbohydrates content in root extracts of *Taraxacum officinale L. Weber ex F.H. Wigg,* g/100 g dw

Location	Fructose	Sucrose	1-Kestose	Nystose	Inulin
	mean ± SD, n=3				
Chirpan	5.3± 0.1	2.2± 0.2	1.0± 0.1	0.8± 0.1	8.4± 0.1
Plovdiv	4.2± 0.2	1.8± 0.1	0.9± 0.1	0.7± 0.3	10.9± 0.2
Parvomay	4.3± 0.1	2.1± 0.2	1.2± 0.2	1.0± 0.1	11.2± 0.3



a) b)

Figure 2. HPLC chromatograms of extracts obtained from dandelion taproots from different locations: a) Plovdiv and b) Chirpan, where 1. inulin; 2. nystose, 3. 1-kestose 4. sucrose, 5 fructose

Table 3. Total phenolic contents, total dihydroxycinnamic derivatives, total flavonoids contents and antioxidant activity of the extracts obtained from dandelion root extracts

Location	Extracts	TPC, mg GAE[1]/g dw	Total dihydroxycinnamic derivatives, mg CAE[2]/g dw	Total flavonoids, mg EQ[3]/g dw	DPPH	FRAP
		Mean±SD[3]				
Chirpan	95% EtOH	4.5 ± 0.1	1.9 ± 0.1	0.5 ± 0.1	11.4±0.3	17.4±0.2
	water	6.4 ± 0.2	8.4 ± 0.6	1.7± 0.4	37.0±1.6	40.3±0.5
Plovdiv	95% EtOH	6.1 ± 0.4	7.0 ± 0.9	0.7 ± 0.1	20.8±0.9	25.7±2.9
	water	7.1 ± 0.4	13.8 ± 0.1	1.5 ± 0.1	38.7±1.3	52.9±0.3
Parvomay	95% EtOH	7.4± 0.3	3.6± 04	0.5± 0.1	51.2±0.8	26.6±1.8
	water	9.2± 0.3	9.8± 0.9	1.4± 0.1	83.1±3.2	46.9±1.3

[1]Expressed as milligram of gallic acid per gram dry material,[2]cinnamic acid equivalent, [3]Expressed as milligram of quiercetin per gram dry extract, [3]SD – standard deviation (n=6)

During our study the tendency of increasing the concentration of higher polymerized fructan and sucrose was observed similarly to early reported (Van den Ende et al., 2000; Wilson et al., 2001. This fructans served as a starter for selective bifidogenic fermentation in the colon, whereas the long-chain inulin is fermented twice as slowly than the low polymerized inulin, maintained the metabolic activity of the improved flora in more distal parts of the colon (Roberfroid et al., 1998). Therefore, dandelion roots harvested in April can be applied as a rich source of prebiotics in preparation of healthy food and nutrition formula.

The results obtained for total phenolic content, total dihydroxycinnamic derivatives, total flavonoids, anti-radical scavenging activity measured by DPPH and FRAP assays were shown in Table 3. In all cases the subsequent water extracts showed the higher value of antioxidant capacities and phenolic contents in comparison to their 95 % ethanol extract. The amount of total phenolics ranged from 4.5 to 9.2 mg GAE/g dw. The highest total polyphenol content, total dihidrixycinnamic derivatives and total flavonoids were registered by subsequent water extraction (9.2 ± 0.3 mg GAE/ g DW, 13.8 ± 0.1 mg CAE/ g DW and 1.7 mg EQ/g dw, respectively) (Table 3). The highest level of total phenolic content was found in water extracts from dandelion roots gathered from Parvomay location, while the lowest was in ethanol extracts from Chirpan location. In our study the content of total dihidrixycinnamic derivatives in both extracts were higher than reported in teas obtained from the roots 1.2 mg/g cinnamic acids (Williams et al., 1996). The results obtained for total phenolic content and total dihidrixycinnamic derivatives from roots extracted with 95 % ethanol were higher than the reported by Ivanov (2014) for dandelion leaves.

The highest level of total flavonoids was observed in water extracts obtained after ethanol pretreatment. The reported results were lower than their content in roots of common chicory (2.8 ± 0.2, mg EQ/g dw) (Denev et al., 2014).

Taraxacum officinale L. subsequent water roots extracts from Parvomay location demonstrated the highest antioxidant activity (DPPH, 83.1± 3.2 mg TE/g dw; FRAP, 46.9 ± 1.3 mg TE/g dw), while water extracts from Plovdiv location showed high activity defined only by FRAP assay: 52.9±0.3 mg TE/g dw. The reported results for antioxidant activity of dandelion roots were near to previously published data for dandelion leaves (Ivanov, 2014). In the current study the TE values reported for both the DPPH and FRAP assays and phenolic content for roots were higher than those for crude methanol extract (80% v/v) from dandelion described by Wojdylo et al. (2007) - DPPH (53.312 ± 1.191 mg TE/g dw), FRAP (3.979 ± 0.776 mg TE/g dw and the phenolic content 0.126 ± 0.03 mg GAE/g dw, respectively. Our results for radical scavenging activity were near to the data for DPPH and FRAP assays of water extracts from dandelion root reported by Liu et al., (2008), Amin et al., (2013) and higher than the results published by Kenny et al., (2014b) for water extract and dialysates. The reason for the last can be explained with that the results were reported according to the extract, not on the dry weight of the plant, as was in the case of our study. In contrast, the

findings of the present study have shown that water dandelion root extracts had considerably higher total phenolic content and antioxidant activity from the same extract obtained by same extraction procedure from elecampane and common chicory (Denev et al, 2014; Petkova et al., 2015).

According to Schütz et al., (2006b) the healthy-effect of dandelion root were attributed to their high phenolic content, mainly hydroxycinnamic acid derivatives and flavonoids. From the results obtained a strong relationship between antioxidant activity and phenolic content in the extracts for dandelion roots was observed. The presence of inulin and FOS in dandelion roots additionally increase their biological activity and will improve the health benefits for human nutrition.

CONCLUSIONS

The current study represented the first comprehensive report for evaluation of biologically active substances: total fructan, individual sugars, inulin, as well as total phenolic content, total dihydroxycinnamic derivatives, total flavonoids and the radical scavenging activity of dandelion root harvested in spring. The wide distribution of this edible, medicinal plant and its roots reveals the enormous potential for their application as a commercial crop and a cheap natural source of inulin and antioxidants. The carried research has shown the efficacy of extracts from its root to be considered as food additive and natural antioxidant preservatives for functional food production with the potential to delay oxidative stress and to stimulate biffidobacteria growth.

REFERENCES

Alexieva I., Mihaylova D., Popova A., 2013. Evaluation of the antioxidant capacity of aqueous extracts of fresh samardala (*Allium bulgaricum* L.) leaves, Scientific works, vol. LX, Plovdiv, p. 826-831

Amin M, Sawhney S, Jassal M., 2013. Comparative antioxidant power determination of *Taraxacum officinale* by FRAP and DTPH method. Pharmaceut Anal Acta, 4, p. 221.

Bagaoutdinova I., Fedoseyeva P., Okoneshnikova F., 2001. Fructose-containing carbohydrates in plants of different families localization and content, Chemistry and Computational Simulation. But. Com., Vol. 2 (5), p. 13-16

Barclay T., Ginic-Markovic M., Cooper P., Petrovsky N., 2010. Inulin - a versatile polysaccharide with multiple pharmaceutical and food chemical uses. Journal Excipients & Food Chemistry, 1(3), p. 27-50

Benzie F., Srain J., 1996. Ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem., 239, p. 70-76

Bisset G., Phillipson, D., Czygan F.C., Frohne D., Holtze D., Nagell A., Pfander H.J., Willuhn G., Buff W. (Eds.), 1994. Herbal Drugs and Phytopharmaceuticals: A Handbook for Practice on a Scientific Basis. CRC Press, Boca Raton, Ann Arbor, London, Tokyo, p. 486–489.

Cho SY, Park JY, Park EM, Choi MS, Lee MK, Jeon SM et al., 2002. Alteration of hepatic antioxidant enzyme activities and lipid profile in streptozotocin-induced diabetic rats by supplementation of dandelion water extract. Clin Chim Acta, 317, p. 109–117.

Denev P., Petkova N., Ivanov I., Sirakov B., Vrancheva R., Pavlov A., 2014. Determination of biologically active substances in taproot of common chicory (Cichorium intybus L.), Scientific Bulletin. Series F. Biotechnologies, Vol. XVIII, p. 124-129.

European Pharmacopoeia (6th ed. 2008)

Fraisse D., Felgines C., Texier O., Lamaison J-L., 2011. Caffeoyl derivatives: Major antioxidant compounds of some wild herbs of the Asteraceae family, Food and Nutrition Sciences, 2, p. 181-192

Gibson G., Roberfroid M., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr., 125, p. 1401–1412.

Hagymasi K, Blazovics A, Feher J, Lugasi A, Kristo ST, Kery A. 2000. The invitro effect of dandelion antioxidants on microsomal lipid peroxidation, Phytother Res, 14, p. 43–44.

Ivanov I., 2014. Polyphenols Content and Antioxidant Activities of *Taraxacum officinale* F.H. Wigg (Dandelion) Leaves International Journal of Pharmacognosy and Phytochemical Research, 6(4), p. 889-893

Ivanov I., Vrancheva R., Marchev A., Petkova N., Aneva I., Denev P., Georgiev G., Pavlov A., 2014. Antioxidant activities and phenolic compounds in Bulgarian Fumaria species, Int. J. Curr. Microbiol. App.Sci., 3(2), p. 296-306

Ivanova D., Gerova D., Chervenkov T., Yankova T., 2005. Polyphenols and antioxidant capacity of Bulgarian medicinal plants, Journal of Ethnopharmacology, 96, p. 145–150

Kenny O., Smyth T.J., Walsh D., Kelleher C.T., Hewage C.M., Brunton N.P., 2014a. Investigating the potential of under-utilised plants from the Asteraceae family as a source of natural antimicrobial and antioxidant extracts, Food Chemistry, 161, p. 79–86

Kenny, O., Smyth, T., Hewage Ch, Brunton N. 2014b. Antioxidant properties and quantitative UPLC-MS/MS analysis of phenolic compounds in dandelion (*Taraxacum officinale*) root extracts, Free Radicals and Antioxidants, Vol.4 (1), p. 55-61.

Kivrak I., Duru M., Öztürk M., Mercan N., Harmandar M., Topçu G., 2009. Antioxidant, anticholinesterase and antimicrobial constituents from the essential oil and ethanol extract of Salvia potentillifolia. Food Chem., 116, p. 470-479

Leung AY, Foster S., 1996. Dandelion root. In: encyclopedia of common natural ingredients used in food,

drugs and cosmetics, 2nd ed. John Wiley & Sons, New York

Lingyun W., Jianhua W., Xiaodong Zh., Da I., Yalin Y., Chenggang C., Tianhua F., Fan Zh., 2007. Studies of the extraction technical conditions of inulin from Jerusalem artichoke tubers. J. of Food Engineer, 79, p. 1087-1093

Liu H., Qiu N., Ding H., Yao R., 2008. Polyphenols contents and antioxidant capacity of 68 Chinese herbals suitable for medical or food uses, Food Research International, 41, p. 363–370

Nnamdi, Ch., Uwakwe A., Chuku C**.,** 2012. Hypoglycemic effects of aqueous and ethanolic extracts of dandelion (*Taraxacum officinale* f.h. Wigg.) leaves and roots on streptozotocin-induced albino rats, GJRMI, 1(6), p. 211–217

Olennikov D., Tankhaeva L., Rokhin A., 2009. Glucofructans from Taraxacum officinale roots, Chemistry of Natural Compounds, 45 (2), p 143.

Pamukov P. and Ahtardjiev H., 1990. Prirodna apteka, Zemizdat, Sofia (on Bulgarian)

Petkova N., Denev P., 2012. Extraction and determination of fructans (oligofructoses and inulin. Proceeding papers of 9th scientic-practical conference with international participation "Ecology and Health" Academic press of Agricultural university, Plovdiv, 399-404. (in Bulgarian)

Petkova N., Denev P., 2013a. Evaluation of fructan content of the taproots of *Lactuca serriola* L. and *Sonchus oleraceus* L. Scientific Bulletin, Series F "Biotechnologies", Volume XVII, Bucharest, p 117-122.

Petkova N., Ehlimanov Eh., Ivanov I., Denev P., 2013b. Evaluation of Bulgarian medicinal plants as a potential source of inulin-type prebiotics, Proceeding book of International Scientific-Practical Conference, "Food, Technologies & Health", p. 142-146.

Petkova N., Denev P., Ivanova M., Vlaseva R., Todorova M. 2013c. Influence of harvest time on fructan content in the tubers of *Helianthus tuberosus* L., Nutrihort proceeding papers: Nutrient management, innovative techniques and nutrient legislation in intensive horticulture for an improved water quality, p. 284-289.

Petkova N., Vrancheva R., Denev P., Ivanov I., Pavlov A., 2014. HPLC-RID method for determination of inulin and fructooligosacharides, Acta Scientifica Naturalis, 1, p. 99-107.

Petkova N., Vrancheva R., Mihaylova D., Ivanov I., Pavlov A., Denev P., 2015. Antioxidant activity and fructan content in root extracts from elecampane (Inula helenium L.) J. BioSci. Biotechnol., 4 (1), p. 101-107.

Roberfroid M., Van Loo JAE, Gibson G., 1998. The bifidogenic nature of chicory inulin and its hydrolysis products, Journal of Nutrition; 128 (11).

Schütz K., Carle R., Schieber A., 2006a. Taraxacum - A review on its phytochemical and pharmacological profile, Journal of Ethnopharmacology, 107, p. 313–323

Schütz K., Muks Er., Carle R., Schieber A., 2006b. Separation and quantification of inulin in selected artichoke (*Cynara scolymus* L.) cultivars and dandelion (*Taraxacum officinale* Web ex Wigg.) roots by high-performance anion-exchange chromatography with pulsed amperometric detection, Biomedical Chromatography, 20, p. 1295-2006.

Stintzing C., Nerbach M., Mosshammer M., Carle R., Yi W., Sellappan S., Acoh C., Bunch R., Felker P., 2005. Color, betalain pattern, and antioxidant properties of cactus pear (Opuntia spp.) clones, J. Agric Food Chem. 53 (2), p. 442-451

Sweeney B., Vora M., Ulbricht C., Basch E., 2005. Evidence-based systematic review of dandelion (*Taraxacum officinale*) by natural standard research collaboration. Journal of Herbal Pharmacotherapy, 5, p. 79–93.

Trojanov´a, I., Rada, V., Kokoˇska, L., Vlkov´a, E., 2004. The bifidogenic effect of *Taraxacum officinale* root. Fitoterapia, 75, 760–763.

Van den Ende W., Michiels A., Van Wonterghem D., Vergauwen R., Van Laere A., 2000. Cloning, developmental, and tissue-specific expression of sucrose:sucrose 1-fructosyl transferase from *Taraxacum officinale*. Fructan localization in roots. Plant Physiology 123, 71–79.

Van Loo, J., Coussement P., De Leenheer L., Hoebregs H., Smits, G., 1995. On the presence of inulin and oligofructose as natural ingredients in the Western diet. Critical Reviews in Food Science and Nutrition, 35, p. 525–552

Williams C.A., Goldstone F., Greenham J., 1996. Flavonoids, cinnamic acids and coumarins from the different tissues and medicinal preparations of Taraxacum officinale. Phytochemistry 42, p.121–127.

Wilson R, Kachman S., Martin A., 2001. Seasonal changes in glucose, fructose, sucrose, and fructans in the roots of dandelion. Weed Science, 49, p. 150.

Wojdyło A., Oszmianski J., Czemerys R., 2007. Antioxidant activity and phenolic compounds. Food Chemistry, 105, p. 940-949.

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Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

RHEOLOGICAL PROPERTIES OF OIL-IN-WATER EMULSIONS WITH FLAXSEED GUM

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Abstract

There are many hydrocolloids that are used as thickening agents in food emulsions and improve their quality and shelf life. Flaxseed is one of the healthy grains that could be used in functional foods development. The objective of this work was to evaluate the effect of flaxseed gum concentration and temperature on the rheological properties of oil-in-water (O/W) emulsions with flaxseed gum (FG). FG solution and O/W emulsions with different hydrocolloids were prepared. The rheological behavior of the samples was determined, the flow behavior index (n) and a consistency index (k) values were assayed. The emulsions containing the greatest percentage of gums presented higher viscosity than the reference emulsions. This study shows a good potential of flaxseed gum to be used as a thickening agent in O/W emulsions.

Key words*: flaxseed gum, O/W emulsions, rheological properties.*

INTRODUCTION

The interest in flaxseeds renewed in recent years. Flaxseed is a food source of lignans, α-linolenic acid, and flaxseed gum (C. Hall Iii et al., 2006). Soluble dietary fibre has significant benefits for human health. Flaxseed gum has nutritional value as a dietary fibre preventing colon and rectal cancer, coronary heart disease risk and the incidence of obesity. Included in the daily ration, flaxseed gum reduces the levels of blood glucose and cholesterol in type 2 diabetic patients (G.Thakur et al., 2009).

Previous studies revealed that flaxseed gum is a mixture of water-soluble polysaccharides composed mainly of L-galactose, D-xylose, L-rhamnose, and D-galacturonic acid. A recent study showed that flaxseed gum contained about 20% (w/w) of dietary fibres, not reported before. Monosaccharide compositions depend on the type of extraction. (H. D.Huihuang et al., 2014).

Flaxseed gum forms weak gel similar to guar gum. Included as a food hydrocolloid, flaxseed gum can significantly increase the water-holding capacity (WHC) of food (J**.**Sun et al., 2011).The effect of the addition of flaxseed gum on the physicochemical properties of oil-in-water emulsions was investigated. It has been demonstrated that the electrostatic interactions between flaxseed gums and protein-stabilized emulsions need to be controlled in order to prevent bridging flocculation (S. Khalloufiet al., 2009).

The food emulsions are thermodynamically unstable systems and should be stabilized. Stabilizers are used to provide long-term emulsions stability. The most used emulsifiers and thickener agents are proteins, biopolymers and polysaccharides.

Polysaccharide gums are used in food due to their ability to control texture and improve the sensory attributes. Xanthan gum is produced by some bacteria of the genus *Xanthomonas.*This polysaccharide is widely used in various food applications including the stabilization of food emulsions (J. Higiro et al., 2007; S. Desplanques et al., 2014; V. Krstonošicet al., 2015).

In the present study the influence of temperature and pH on rheological properties of flaxseed gum water solutions and oil-in-water emulsions has been studied.

MATERIALS AND METHODS

Aaccording to an adapted methodology, the flaxseed gum (FG) was extracted from flax seeds by an aqueous extraction process at 80ºC, pH 6.5 –7.0 and water: seed ratio 13:1 (W Cui. et al., 1994). Flaxseed gum (FG), Xanthan gum (XG) and Xanthan + Guar Gum (X-GG) aqueous solutions were prepared at room temperature (25ºC) at three different concentrations (0.8, 1, and 1.2 % w/w).

Commercial xanthan and guar gum were used for analysis, with characteristics according to the producer's specification.

Xanthan gum and guar gum were dissolved in water and left at room temperature for 4 h to total dissolution.

Double-distilled water was used to prepare solutions and emulsions. Emulsification was performed on a laboratory homogenizer POLYTRONR KinematicaGmbH PT-G 45/80, (Switzerland) for 30 sec at $523s^{-1}$ and 20°C. The oily phase for oil-in-water (O/W) model emulsions was sunflower oil at volume fraction 30%, v/v.

Commercially available sunflower oil was obtained from local market and incorporated into emulsion preparations without further purification.

Rheological measurements, apparent viscosity and shear stress of model emulsions were measured at various shear rates via digital viscometer FUNGILAB PREMIUM (Spain).

Power law model (Eq. (1)) was used to analyze the flow curves:

$$\tau = K.(\dot{\gamma})^n \quad , \qquad (1)$$

where τ (Pa) is shear stress, $\dot{\gamma}$ (s^{-1}) is shear rate, **n** (no dimensional) is flow behavior index and **K** (Pa. s) is consistency index.

Emulsion stability was evaluated visually by measuring the extent of gravitational phase separation. For this test, 10 ml of the prepared emulsions were transferred in graduated cylinders at ambient temperature (25 ±1 C°).

RESULTS AND DISCUSSIONS

The influence of temperature and gum concentration on the rheological characteristics of the flaxseed solutions is shown in Figure 1. The increase in gum concentration led to the increaing viscosity and non-Newtonian behavior. These results are in accordance with previously reported results by Chen H et al., 2006. The most common reason of the shear thinning behavior in FG solutions is the disruption of particle aggregates caused by the increase in shear rate. The solution viscosity increased with temperature decrease. Flaxseed gum is a hydrocolloid with good water-holding capacity and similar to other gums is influenced by temperature (Dhiaa, 2012).

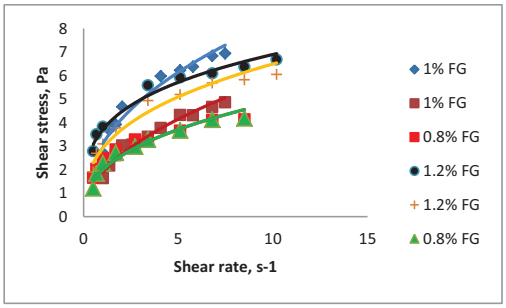

Fig.1 Shear stress versus shear rate for FG solutions with different concentration of gum at 25°C (▲, ♦, ●) and 60°C (■, ■, +)

The influence of temperature and pH on the viscosity of the oil-in-water emulsions with flaxseed gum is illustrated in Figure 2.The emulsions showed shear-thinning flow behavior. The viscosity decreased with increasing the shear rate. The results indicate that temperature did not affect the viscosity of the O/W emulsions but this viscosity increased with decreasing pH. These results are necessary for future use of FG gum in food applications.

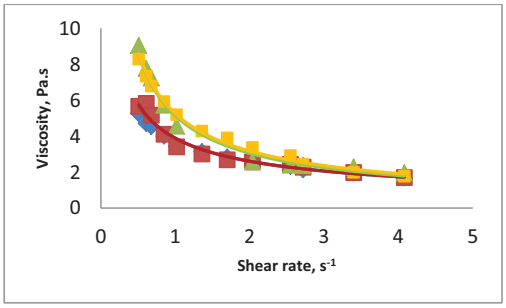

Fig. 2 Viscosity of oil-in-water emulsions with 1 % FG at 25°C, pH- 6.5-7 -♦, at 25°C, pH- 4- ▲; 60°C, pH- 6.5-7 - ■ and 60°C, pH- 4 - ■

The rheological characteristics of O/W emulsions with different concentration of FG were illustrated by the flow curves (shear stress versus shear rate) shown in Fig. 3. They demonstrated the rheological behavior of the samples. The viscosity decreased with increasing shear stress (pseudoplasticity). The differences between the viscosity of emulsions with 1 and 1.2 % FG were not significant. The

emulsions exhibited a shear-thinning behavior that may be caused in part by the presence of aggregated particles.

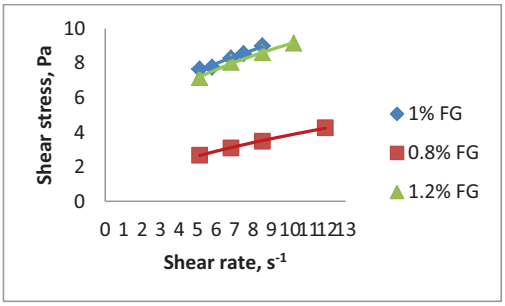

Fig 3 Shear stress versus shear rate for FG emulsions with different concentration of FG

O/W emulsions with different hydrocolloids were analyzed – Flaxseed Gum (FG), Xanthan Gum (XG) and Xanthan + Guar Gum (X-GG). The data of the shear stress versus shear rate for the emulsions fitted well the power law model equation (the values of R^2 were higher than 0.95). Table 1 shows the effect of different hydrocolloids of different concentrations on the consistency index (k) and flow behavior index (n) of oil-in-water emulsions.

According to Table 1, it can be observed that the flow behavior index (n) increased with decreasing the hydrocolloids concentration (HYDC). The increase of (HYDC) led to a higher viscosity (higher k values) and pseudoplasticity (lower n values). Regarding the type of hydrocolloid, the results indicated that the emulsions with X-GG showed the highest viscosity.

The consistency index K of model emulsion with FG were lower than the emulsion with other thickening agents.

The data of emulsion stability (ES) were collected and evaluated. The emulsions with different hydrocolloids thickeners exhibited stability during the entire investigated period.

Table 1 Effect of the type of hydrocolloid at different concentrations on the consistency index (k) and flow behavior index (n) of oil-in-water emulsions

Hydrocolloids	%, w/w	k (Pa.s)	n
FG	0.8	1.1^{a}	0.54^{a}
	1	2.67^{b}	0.48^{b}
	1.2	3.57^{c}	0.43^{b}
XG	0.8	12.17^{d}	0.18^{c}
	1	18.38^{e}	0.18^{c}
	1,2	19.37^{f}	0.11^{d}
X-GG	0,8	10.17^{g}	0.77^{e}
	1	22.95^{h}	0.62^{f}
	1,2	29.24^{i}	0.55^{a}

Different letters mean significant differences ($p<0.05$) in the same column

CONCLUSIONS

Flaxseed gum exhibit good thickness property and can be used as a thickener agent in O/W food emulsions. The results showed that temperature did not affect significantly the viscosity of model emulsions.

The viscosity of all evaluated model emulsions increased with decreasing pH.

The rheological characteristics of the investigated oil in water emulsions showed dependency on the type and concentration of the used thickeners. The viscosity increased with increasing of gum concentration.

The viscosity increasing was most pronounced at the emulsions with X-GG.

The model emulsions with flaxseed gum exhibited non-Newtonian behavior. The analysed samples with higher viscosity presented pseudoplasticity.

Regarding the measured emulsions stability, all samples were stable over the storage period at room temperature.

The results indicated that the viscosity of emulsion with FG was the lowest compared with other thickeners. FG should be used in combinations with other hydrocolloids for food technologies application.

REFERENCES

Chen H.H., Xu S.-Y.& Wang Z. 2006. Gelation Properties of Flaxseed Gum. Journal of Food Engineering, 77(2), 295-303.

Cui W., G. Mazza, B. D. Oomah, C.G. Biliaderis. 1994. Optimization of an Aqueous Extraction Process for Flaxseed Gum by Response Surface Methodology. LWT - Food Science and Technology, Vol. 27:4-, 363–369.

Desplanques S., M.Grisel, C.Malhiac, F.Renou, 2014. Stabilizing effect of acacia gum on the xanthan helical conformation in aqueous solution. Food Hydrocolloids, Vol. 35: 181-188.

Dhiaa, A. 2012.The temperature effect on the viscosity and density of xanthan gum solution. Kufa Journal of Engineering, Vol.3: 17-30.

Hall IiiC., M. C. Tulbek, Y. Xu, 2006. Flaxseed. Advances in food and nutrition research, Vol. 51:1-97.

Higiro J., T. J. Herald, S. Alavi, S. Bean, 2007. Rheological study of xanthan and locust bean gum interaction in dilute solution: Effect of salt.Food Research International, Vol. 40:435–447.

Huihuang H. D., S. W. Cui, H. D. Goff, Q. Wang, J. Chen,N. F. Han, 2014. Soluble polysaccharides from flaxseed kernel as a new source of dietary fibres: Extraction and physicochemical characterization. Food Research International, Vol.56: 166–173. doi: 10.1016/j.foodres.2013.12.005.

Khalloufi S., M. Corredig, H.D. Goff, M. Alexander, 2009. Flaxseed Gums and their Absorption on Whey Protein-Stabilized Oil-in-Water Emulsions. Food hydrocolloids, 23(3), 611-618.

Krstonoŝic V., L. Dokic, I. Nikolic, M. Milanovic. 2015. Influence of xanthan gum on oil-in-water emulsion characteristics stabilized by OSA starch. Food Hydrocolloids, Vol 45: 9-17.

Qian K.Y., S.W. Cui, Y. Wu, H.D. Goff, 2012. Flaxseed gum from flaxseed hulls: Extraction, fractionation, and characterization. Food Hydrocolloids, 28 (2): 275–283.

Sun J., X. Li, X.Xu, G. Zhou, 2011. Influence of Various Levels of Flaxseed Gum Addition on the Water-Holding Capacities of Heat-Induced Porcine Myofibrillar Protein. Journal of Food Science, Vol. 76(3):C472–C478.

Thakur G., A. Mitra, Pal K, Rousseau D., 2009.Effect of flaxseed gum on reduction of blood glucose and cholesterol in type 2 diabetic patients. International. Journal of Food Science and Nutrition, 60 Suppl (6):26-36.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

CHARACTERIZATION OF *Enterococcus* BACTERIA ISOLATED FROM BOVINE COLOSTRUM AS PROBIOTICS

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Abstract

Aims of the research was to characterize probiotic properties of Enterococcus bacteria isolated from bovine colostrum. The research done experimentally with factorial pattern of Completely Randomized Design including two factors such type of bacteria and the characters of probiotics and replicated three times. Parameters of the research was pH tolerance, bile salts, antimicrobial activity, the ability of auto-aggregation. Data analyzed by ANOVA and Duncan's Multiple Range Test (DMRT). Two Enterococcus bacteria isolated from the colostrum which identified with the API 20 Strep were known as E. faecalis and E. faecium. The result showed that E. faecalis and E. faecium were pH 4 tolerant with the population of 1.623 x 10^2 and 1.316 x 10^2 CFU/ml. Availability to survive at high bile salt concentrations (0.3% and 0.5%) which shown by 15.683 x 10^{10} and 23.667 x 10^{10} CFU/ml population. Enterococcus faecalis and E. faecium also had antimicrobial properties which indicated by clear zone diameter towards E. coli (9.7 and 10.3 mm), S. thypimurium (9.7 and 10 mm), and L. monocytogenes (9 and 12 mm). The inherent ability of the isolated Enterococcus was better than the test bacteria (E. coli, S. thypimurium, Listeria) with the auto-aggregation values were 61.783% and 60.425%.

***Key words** : Characterization, Probiotic, Enterococcus, Colostrum.*

INTRODUCTION

Bovine colostrum has known contain nutrients, antimicrobial agents, and antibodies (Kelly, 2003). The components can be used to treat health problems and infections caused by bacteria, viruses, parasites, and fungi. Beside bovine colostrum could be given as a nutritional supplement for diarrhea and sinusitis, it also improve patient immunological factors (Rawal, et al., 2008). The ability was not only caused by immune factors, but also indigenous microorganisms that live inside the bovine colostrum.

Indigenous microorganisms contained in colostrum were diverse and several of them are round-shaped bacteria (cocci). Cocci bacteria in colostrum can act as beneficial bacteria. Catalase-negative cocci bacterial was lactic acid bacteria (LAB) that already known has a role as probiotic.

Probiotics are living microorganisms that gave health benefit for the host when given in adequate amounts (Shinde, 2012). There are not all microorganisms could be called as probiotics, the characterization is needed to determine selected microorganism as a probiotic.

Probiotics has ability to survive in digestive tract conditions such as acidic conditions and can grow in the presence of bile salt exposure. In a normal condition filled stomach has a pH of 4.0-5.0 while the pH of empty stomach was 1.5-2.0 (Shinde, 2012). At pH 1.5, some of probiotics *Lactobacillus* strain showed 55% loss of viability, while at pH of > 2 the strain retained relative constant viability (Vamanu, 2014). In the human intestine, probiotic should survive 0.3% bile salt concentration (Jacobsen, et al., 1999). Meanwhile, bile salt concentration of 0.5% with 4h of exposure gave maximum decrease in probiotics viability (Vamanu, 2014). Both are

main character for the selection of probiotics microorganisms.

Preliminary studies has been done to isolate two cocci bacterial, gram-positive, catalase negative and have been identified by API-50 CHL kit (bioMerieux) as *E. faecalis* and *E. faecium*. *E. faecalis* and *E. faecium* were *Enterococcus* bacteria that have been widely used as probiotic (Fuller, 1989). *Enterococcus* has resistant to gastric acid and bile salts (Moreno, et al., 2006). The study aims to characterized isolated *Enterococcus* ability in surviving low pH and bile salts so that could recomended as probiotic.

MATERIALS AND METHODS

Research design and statistical analysis

Low pH resistance test done experimentally using completely randomized design (CRD) with 2 x 3 factorial pattern and each treatment was performed 3 replications. The first factor is type of probiotic bacteria candidate (*E. faecalis, E. faecium*) and second factor was pH levels (2,4,6). While, bile salts resistance test done with CRD experimental method with 2 x 2 factorial pattern with 3 replications. Probiotic bacteria candidate (*E. faecalis, E. faecium*) as first factor and bile salts (Oxoid) level (0.3%, 0.5%) as second factor. Data analyzed using Analysis of Variance (ANOVA) with level of 95% and treatment effect analyzed by Duncan's Multiple Range Test (DMRT) (Gomez, 1995).

Probiotic Characterization

Parameter measured by Total Plate Count method of grown bacteria on MRS-Agar (Oxoid) with acetic acid addition for low pH resistance test and MRS-Agar (Oxoid) with various bile salts addition for bile salts (Oxoid) resistance test (Hardianingsih, et al., 2006; Gomez, 1995).

RESULTS AND DISCUSSIONS

Probiotic resistance towards low pH

Resistance to acid used as indicators of probiotic bacteria abilitiy to survive in the human stomach (pH of 2-4). Results showed that pH and *Enterococcus* type significantly affected the *Enterococcus* total colonies, so that it proceeds with the DMRT (Table 1). The decreased of pH also decreased the number of bacterial colonies. Highest number of colonies has shown by *E. faecalis* at pH 6 with a number of 40.633 x 10^8 CFU / ml. Both types of bacteria could not grew at pH 2, but still grew at pH 4. The number of colonies of *E. faecalis* at pH 4 was 1.32 x 10^2 CFU / ml and the number of colonies of *E. faecium* was 1.62 x 10^2 CFU / ml. Gastric acidity vary from pH 2-5, filled stomach has pH range of 4-5 and if empty can reach pH of 2, so that both strains could still be categorized as a probiotic (Jacobsen, et al., 1999). Generally, microorganisms grew in pH range of 6.5-7.5 (Pelczar, 2006). *Enterococcus faecalis* and *E. faecium* could grew in a wide range of pH 4.6 to 9.9 and optimally grew at the pH of 7.5 (Fisher and Philips, 2009).

Table 1. Duncan Multiple Range Test (DMRT) Probiotics Tolerance towards Low pH

pH	Bakteri (CFU/ml)	
	E. *faecalis*	E.*faecium*
2	nd C a	nd C a
4	1,32 x 10^2 B B	**1,62 x 10^2 B** **a**
6	**40,633 x 10^8 A** **A**	27,433 x 10^8 A b

Description:

The same uppercase letter read vertically showed no significant difference

The same lowercase letters read vertically to the side showed no significant difference

nd = not determined (number of colonies = 0 CFU / ml)

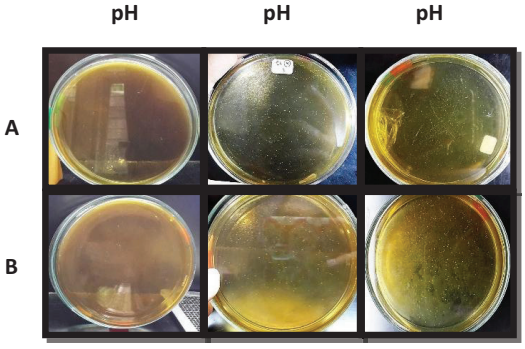

Figure 1. (A) *Enterococcus faecalis* colonies at the pH of 2, 4, 6. (B) *Enterococcus faecium* colonies at the pH of 2, 4, 6.

Enterococcus are less resistant to acid, when external pH <3.0, magnesium ions out of the cell and lead to death less than 4 hours (Bender, et al., 1986). The number of cocci-shaped Lactic Acid (LAB) including *Enterococcus* would declined when the pH of the medium decreased into 5 (Hutkins and Nannen, 1993). Acidity level below 5 will disrupt the structure of the cell membrane, the cell membrane becomes saturated by hydrogen ions thus limiting membrane transport and intracellular component out. Poisoning that occurs at low pH cause by acid substances did not decompose into the cell that resulting in ionization and pH of cells changed then inhibit growth and may even kill microorganisms (Hutkins and Nannen, 1993). Only small number of *E. faecium* could grew at pH of 0.5, 1, 2, and 3 then dead after 4 hours incubation (Chavarin, et al., 2003). None of the LAB could grew at pH of 2.5 more than 4 hours incubation while the incubation of the research was done 6 hours so that the result showed that no colonies found at the media with the pH of 2 (Jacobsen, 1999, Chavarin, et al., 2013).

Tested probiotic candidates showed that have ability to survive in low pH conditions and has tolerance to acid (Jacobsen, 1999). If the bacteria survived and active in gastric acid condition can be regarded as probiotic bacteria (Salminen and von Wright, 2004). Based on the results, *E.faecalis* and *E. faecium* can be said probiotics because survived at the pH of 4. *Enterococus faecalis* and *E. faecium* isolated from bovine colostrum did not resistant to gastric acid if consumed when the stomach empty, but if consumed when the stomach filled they have opportunity to survive.

Probiotic resistance towards bile salts

The results on Table 2 showed that there was no significant effect between the interactions of bile salt concentrations (0.3% and 0.5%) and *Enterococcus* type, while significant effect found at type of probiotic bacteria (*E. faecalis* and *E. faecium*) treatments. *Enterococcus faecalis* and *E. faecium* has ability to grow in different bile salt concentration of 0.3% and 0.5%. *Enterococcus faecalis* has greater ability to grow on both concentration of bile salts than *E. faecium* with total colonies of 23.667 x 10^{10} CFU / ml.

Bile salt tolerance was an indicator of probiotic bacteria's ability to survive in the upper gastrointestinal tract. *Enterococcus faecalis* and *E. faecium* were able to grow on the medium with the addition of 0.3% and 0.5% bile salts that indicated selected bacteria were able to survive and grow in the upper intestinal tract where bile salts secreted. Both strains were able to grew more in the bile salt concentration of 0.3% compared to 0.5%, but did not show significant difference. Higher concentration of bile salts resulting in higher amount of bacterial cell death. Bacteria resistant ability to bile salts caused by peptidoglycan layer and thicker wall owned by gram-positive bacteria so that protected from lysis when exposed to bile salts. In addition, lipid components owned by Gram-positive bacteria kept the membrane structure and decreased cell leakage caused by bile salts (Kimoto, et al., 2002).

Table 2 Average probiotics colonies with different bile salts treatments

Probiotics	Average colonies (CFU/ml)	$p < 0.05$
E. faecium	15,683 x 10^{10}	b
E. faecalis	23,667 x 10^{10}	a

Description: Different letter showed significant difference

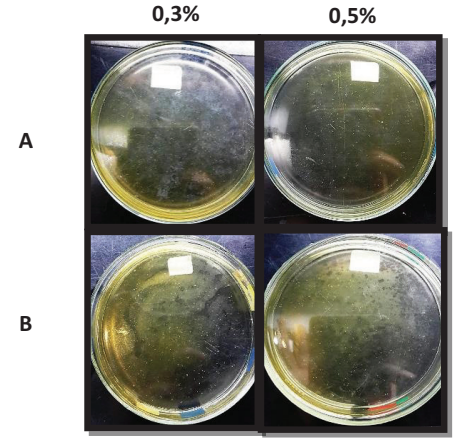

Figure 2. (A) *Enterococcus faecalis* colonies on 0.3% dan 0.5% bile salts added media. (B) *Enterococcus faecium* colonies on 0.3% dan 0.5% bile salts added media.

The concentration of bile salts in the small intestine ranged from 0.2 to 2% (w/v) depend on the organims, type and amount of food ingested, while the equivalent concentration of bile salts in the intestine was 0.5% (Kristoffersen, 2007; Puspawati, 2010). Bile salt concentration of 0.3% was high enough for probotic selection that were resistant to bile salts, so that the bile salt concentration of 0.3% also called as the critical concentration (Jacobsen, 1999).

Bacteria ability to grow in unfavorable environmental conditions, such as the presence of bile salts is one of probiotics bacteria characteristics. *Enterococcus* have been targeted as probiotic because the bacteria have characteristics such as resistance to bile salts (Araujo and Ferreira, et al., 2013). According to the results, concentrations of bile salts until 0.5% did not inhibit bacteria growth. *Enterococcus faecalis* and *E. faecium* can be categorized as probiotics because they could grew on critical concentration of 0.3% or higher of 0.5%.

CONCLUSIONS

Enterococcus faecalis and *E. faecium* isolated from bovine colostrum had resistant to low pH until 4 and bile salt concentration until 0.5%. Both character was main character that must be owned by the probiotics bacteria. Therefore isolates of *E. faecalis* and *E. faecium* have probiotic characteristics. However both bacteria should be tested further to determine other properties of probiotics, such as inhibition ability towards pathogenic bacteria, adhesion to the intestinal epithelial cells, and pathogenicity test.

REFERENCES

Araújo, T. F. and Ferreira, C. L. L.F. 2013. The Genus *Enterococcus* as Probiotic: Safety Concerns. Braz. Arch. Biol. Technol. 56 (3) : 4.

Bender, G. R.. S.V.W. Sutton, and R. E. Marquis. 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral *Streptococci*. Infect. Immun.53:331.

Chavarin, R., Wacher., Campos, E., Chabela, P. 2013. Probiotic Potential of Thermotolerant Lactic Acid Bacteria Strains Isolated From Cooked Meat Products. International Food Research Journal 20(2): 991-1000.

Fisher, K., Philips, C. 2009. The Ecology, Epidemiology and Virulence of Enterococcus. Microbiology. 155 : 1749–1757.

Fuller, R. 1989. A Review Probiotic in Man and Animals. Journal of Applied Bacteriology. 66: 365-378.

Gomez, K.A. dan Gomez A.A. 1995. Prosedur Statistik untuk Penelitian Pertanian. Edisi Kedua. Jakarta : UI – Press.

Hardianingsih, R., Napitulu, R.N.R., Yulinery, T. 2006. Isolasi dan Uji Resistensi Beberapa Isolat Lactobacillus pada pH Rendah. Biodiversitas. 7 (1) : 15-17.

Hutkins, R.W., Nannen, N.L. 1993. pH Homeostasis in Lactic Acid Bacteria. Faculty Publications in Food Science and Technology. Paper 28.

Jacobsen, C.N., Nielsen, V.R., Hayford, A.E., Møller, P.L., Micahelsen, K.F., Pærregaard, A.,Sandstrom, B., Tvede, M., Jakobsen, M. 1999. Screening of Probiotic Activities of Forty-Seven Strains of *Lactobacillus spp.* by In Vitro Techniques and Evaluation of the Colonization Ability of Five Selected Strains in Humans. Appl. Environ. Microbiol. 1999, 65 (11) :4949.

Kelly, G.S. 2003. Bovine Colostrums: A Review of Clinical Uses. Alternative Medicine Review. 8 (4).

Kimoto, H., Ohmomo, S., Okamoto, T. 2002. Enhancement of Bile Tolerance in *Lactococci* by Tween 80. Journal of Applied Microbiology. 92: 41±46

Kristoffersen, S.M., Ravnum, S., Tourasse, N.J., Okstad, O.A., Kolsto, A.B., Davies, W. 2007. Low Concentrations of Bile Salts Induce Stress Responses and Reduce Motility in Bacillus cereus ATCC 14570. J. Bacteriol. 189 (14) : 5302.

Moreno, M.R.F., Sarantinopoulos, P., Tsalkalidou, E., Vuyst, L.De. 2006. The Role and Application Of *Enterococci* in Food and Health. International Journal of Food Microbiology 106 : 1 – 24.

Pelczar, M.J., Chan, E.C.S. 2006. Dasar-Dasar Mikrobiologi. Jakarta: UI-Press

Puspawati, N.N., Nuraida, L., Adawiyah, D.R. 2010. Penggunaan Berbagai Jenis Bahan Pelindung Untuk Mempertahankan viabilitas Bakteri Asam Laktat yang Di Isolasi Dari Air Susu Ibu Pada Proses Pengeringan Beku. J.Teknol. dan Industri Pangan, 21 (1).

Rawal, P., Gupta, V., Thapa, B.R. 2008. Role of Colostrum in Gastrointestinal Infections. Indian Journal of Pediatrics, Volume 75.

Salminen, S. and Atte von Wright. 2004. Lactic Acid Bacteria : Microbiology And Functional. 2nd Edition. Revised and Expanded. Marcel Dekker, inc., New York.

Shinde, P B. 2012. Probiotic: An Overview For Selction And Evaluation. International Journal of Pharmacy and Pharmaceutical Sciences. 4 (2).

Vamanu, E. 2014. Testing in vitro viability of thermophilic probiotic strain in simulated gastrointestinal condition. Ann. Microbiol. 64 : 1439-1442.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE INFLUENCE OF PROCESSING ON ACTIVE - BIOLOGICALLY COMPOUNDS OF SOME BERRIES – A REVIEW

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Abstract

Berries are forest fruit characterized by rich content on active - biologically compounds (phyto-hormones, plant pigments - carotenoids, flavonoids and indole quinone pigments etc., metabolic products - derivatives of carbohydrates, lipids, proteins, glycosides, lignin, tannins, essential oils, resins, balsams, alkaloids, antibiotics and other fitoncide substances). This article aims to present the results of several studies regarding the determination of the influence of processing on active - biologically substances of eco-berries. In preparing this article, a review of the state of the art literature has been done and based on it a synthesis of the research results obtained by specialists will be presented. In general, the transformation of fresh berries in food products (juice, natural syrups etc.) is done through a specific technology which consists of the concentration of active - biologically substances from fruit and removing a significant amount of water. Active - biologically substances existing in raw fruit, consisting of vitamins, minerals, enzymes, hormones etc. remain in a big proportion in juices, after cold processing. Absence of thermal processing of fruit protects many nutrients and catalytic compounds which should be finding also in final juices. Berries processed at 100^0C, for up to 20 minutes, retains important amount of active - biological compounds, then their bioactivity decreased significantly with the time. Berry juices with pulp contain important components of the raw fruit and maintain good flavour and aroma of raw material.

Key words*: berries, active - biologically compounds, cold processing, juice, natural syrups.*

INTRODUCTION

Natural products made from berries, cold pressed, supplements are complex in terms of active - biologically compounds (phyto-hormones, plantpigments - carotenoids, flavonoids and indole quinine pigments etc., metabolic products - derivatives carbohydrates, lipids, proteins, glycosides, lignin, tannins, essential oils, resins, balsams, alkaloids, antibiotics and other substances).

Transforming fresh berries in various products, in this case the juice, natural syrups that is through a specific technology which consists of the concentration of biologically - active fruit and removing a significant amount of water.

The main goal in the production of berries juices is extracting as many potentially biologically - active components of the feed stock, such as: vitamin C, total phenolics, anthocyanins, carotenoids and other antioxidants. The main source of antioxidants is fresh fruit, especially berries and vegetables, but their consumption does not provide even half of the body's biological needs. Thus it is possible to compensate for this deficiency by consuming biological fruit juices.

MATERIALS AND METHODS

In preparing this literature review, the merit of the work is to synthesize and assemble of research results obtained in this field by researches. This paper is a review of research regarding the influence of processing on active - biologically compounds of some berries.

Juices which have a very high level of active - biologically compounds that are: sea buckthorn *(Hippophae rhamnoides)*, black berries *(Ribes nigrum)* and blue berry (*Vaccinium myrtillus)*, which contains, first, a large amount of vitamin C (Казаков А. В., 2009).

Preserving specific natural flavour of fruit in hand is another important aspect that gives

sensory qualities of juices and enhances their quality. Therefore, it attaches importance to fresh fruit hand ling from harvesting to processing.

Harvesting the fruit is the first step to obtain juices and syrups technological nature, but paramount importance. Fruit juices or syrups for harvested fully ripe but not too late to prevent degradation of quality and quantity. This period is influenced by the area and the local weather conditions. After harvesting, fresh fruits are stored for not more than 12 hours, as it can begin the process of natural fermentation, which degrades the physic-chemical and sensory qualities of the products.

In practice, appropriate methods should be selected for fruit harvesting and cold processing, so as to maintain a favourable ratio between the price of fruit and final price for products from berries on the open market.

RESULTS AND DISCUSSIONS

1. The influence of processing on the antioxidant activity of berries

A relevant study on antioxidant activity of bioactive compounds from berries was made by a team of researchers led by Ana Slatner, Slovakia (Slatner Ana, Jerneja J., Franci S., Robert V. and Polona J., 2012). In this research were determined the antioxidant activity in vitro and in vivo fruit juices containing large amounts of phenols. The juice was extracted from 5 species of berries in several locations in Slovakia: mountain cranberry, blue berry crop, black berry, elderberry and aronia. Types of phenols (anthocyanins, flavonols, flavones) were determined by HPLC method.

The antioxidant activity in vitro was determined by the method of DPPH free radical scavenging and in vivo using Saccharomyces cerevisiae. The largest number of phenols was found in cranberry juice.

The greatest power in vitro antioxidant found in black berry (Slatner A., Jerneja J., Franci S., Robert V. and Polona J., 2012).

The results show significant differences in antioxidant activity in vivo and in vitro berry juices. The key factor determining the antioxidant activity was the ratio of compounds. It was found a high content of anthocyanins and flavonols, low concentration of the hydroxyl acids, which resulted in a lower intracellular oxidation. Intracellular oxidation increased with higher consumption hidroxicinamic acids and a lower intake of anthocyanins in cells (Slatner A., et. al., 2012).

Their concentration is usually higher in the epidermis and sub-dermal tissue and lower central part of the fruit (Prior R.L.,et. al., 1998).

In the group of the hydroxyl acids were identified three compounds: chlorogenic acid - determined in all analysed juices, crypto-clorogenic acid detected only in blue berry juice and caffeic acid in black berry juice (Milivojevic J., et. al.2012).

A group of flavonoids is characterized by phenolic diversity; were identified 20 different compounds. The greatest diversity of flavonols was found in black berry juice, but the concentration of individual compounds was low. A high concentration of anthocyanins was found in aronia, then blue berries, black berry and then the shock (Benvenuti S., et. al., 2004).

The anthocyanins are between 25 and 92% of analysed phenolsin berries juice (Moyer R. A., et. al., 2002). Due to the huge antioxidant activity, black berry ensuring of human balance by effective action of numerous compounds found in the product (Suryakumar G., Gupta A., 2011).

Sea buckthorn fruits were harvested from a higher altitude presents a more intense colour and are rich in biologically - active compounds, showing a stronger antioxidant activity. (Korekar G., et al, 2014).

Another study confirmed a smaller loss of vitamin C during pasteurization and concentration of black berry juice against sea buckthorn juice (Lydia P., 2012).

The antioxidant activity of anthocyanin is associated with the biological properties of berries (blue berries and black berries).

The anthocyanins helps neutralize free radicals which are unstable molecules and are responsible for the development of many degenerative diseases (Deividas B., et. al., 2009).

The antioxidant activity of phenols and anthocyanins time is from 4.18 to 5.27 mmol/100g of product. Between phenols and anthocyanins content on the one hand and on the other antioxidant activity are closely connected (Giovanelli G., Buratti S., 2009).

2. Influence of processing in preserving biological properties of berry juices.

In a series of studies (Avila A. P., 2012) were tested different techniques of processing berries cold and hot. Also, were made comparisons between the antioxidant activities of blue berry juice, obtained in different thermal conditions. Juice after pressing warming caused an increase in antioxidant activity. The study was done on three samples of berries pressed at: 22 ^{0}C, 75 ^{0}C and 43 ^{0}C. *The results* confirmed the most powerful antioxidant in blue berries pressed at 75 ^{0}C.

Purpose of the studies was carried out by means of high hydrostatic pressure to 400 MP for 10 minutes, 20 minutes and 30 minutes. The results were within the juice obtained by hot pressing with the highest antioxidant power, followed by juice pressed at 43 ^{0}C, then finally pressed juice at 22 ^{0}C. Juice samples pressed at 75 ^{0}C gave the highest values of anthocyanin, total phenolics and antioxidant capacity.

There was a substantial difference in the level of anthocyanin and phenols in samples processed using high pressure equipment. The most effective from the point of view of the pressing antioxidant activity was pressed for 30 minutes. Juice pressed at 75 ^{0}C for 30 minutes was the most accepted by the consumer for that retained the best sensory qualities, then juice pressed at 43 ^{0}C.

Study on keeping sensory qualities of hot - pressed juices (75 ^{0}C) was made by another team of researchers, aiming to preserve the colour purple - blue very attractive to the consumer (Ju Z.Y., Howard L.R., 2003).

The team led by Buchert experienced pectolytic enzyme processing, adjusting the pressure and temperature in order to increase the extraction efficiency and enhancing the quality of the juice. Have the following steps: crushing fruit, partial maceration with pectolytic enzymes, adjustment of temperature and pressure during processing (Buchert J., 2005).

Gorinstein team has experimented influence of thermal processing times at 100 °C on the quality of berries. The juice extracted in three different solvents: water, acetone and hexane. The correlation between total phenols, flavonols and antioxidant activity was found to be higher in water, medium in acetone and low in hexane. By processing reduces biological substances - active. In this experiment, the fruit were thermally processed for 10, 20, 40 and 60 minutes at a temperature of 100 ^{0}C.

Only thermal processing for 10 and 20 minutes keep the content of bioactive compounds.

Thermal processing for 40 and 60 minutes show significant decreases in bioactivity fruit.

After 20 minutes of thermal processing content of bioactive products in berries juice was reduced at: polyphenols - 97.5%; flavonoids - 95.7%; flavonols - 98.7%; tannins - 91.1%; anthocyanins - 95.2%; ascorbic acid - 97.4%.

After 60 minutes, the loss of anthocyanin was: 60% polyphenols, 57.7% flavons, 49.5% flavonoids, 58,6% anthocyanins, 59% tannins.

Conclusion: thermally processed fruits less than 20 minutes demonstrate bioactivity (Gorinstein S., 2010).

3. The influence of technological process on biological - active substances

The technological process of obtaining berry juice begins with the selection of fresh fruit, fruits departing it, sick, other parts of the plant (leaves, stems). Then wash the fruit, which is important in removing dust and dirt. Wash the air tank (pressurized water). The best washing temperature is 400 ^{0}C (Zhang W., 1989).

Sea buckthorn juice can be prepared by conventional extraction techniques or by supercritical CO_2 extraction (Beveridge T., 1999)

Juice extraction by cold pressing technology is based on pressing for removal of the sheath fruits juice. The presses are made of canvas, trapped in the screw or strip. The best are the press fabric in the form of grid which gives a yield of 67%. Then the juice is allowed to settle and it is centrifuged at high speed.

The removal of oil leads to a product called soda pulp with the aqueous phase. If allowed to settle 1-2 days will be separated into 3 phases: a phase that floats a clear liquid in the middle and at the bottom a residue. Then go to pulp removal by filtration and centrifugation. The product obtained is the juice itself. Sea buckthorn juice may contain particles in suspension between 0.5 and 800 μm (micrometers) (Knekt P. T., et. al., 1996).

An alternative method has been experimented by Heilschet team using frozen sea buckthorn. The process starts with flushing, thawing and grinding in a mill of the fruit. Cold fruits are

passed through a system of sieve from 2 mm to 0.8 mm. Last sieve retains the seed and skin from which is extract oil. The remaining sheaths are heated to 50 - 55 ^{0}C and mixed with crystalline sugar. Allow1-3 hours and then are centrifuged. The slurry is treated with a proteolytic enzyme for 46 hours at 55 ^{0}C and then heated to 95 ^{0}C, followed by centrifugation. The obtained juice can be used for the preparation of several beverages.

CONCLUSIONS

The largest amount of phenols of berries is mountain blue berries (*Vaccinium myrtillus*), followed by fruits of blackberry (*Ribes nigri*).
The largest amount of ascorbic acid is in black berries and then followed by blue berry and sea buckthorn. There is a relationship between the content of anthocyanin and other phenolic constituents, on the one hand, and antioxidant power, on the other hand. The high content of anthocyanin and low of flavanols and hidroxicinamic acids caused an intracellular oxidation lower than the high level of hidroxicinamic acids and low levels of anthocyanins (Slatner A., et. al., 2012). Anthocyanin content is higher in fruit peel and lower in the central part of the fruit (Prior R. L., et. al., 1998). The anthocyanin are present in the plant cell vacuole. Buckthorn berries harvested from a higher altitude presents a more intense colour and richer content in bioactive substances, including total phenols were found to have a stronger antioxidant activity (Korekar G., et al, 2014). Heating juice, cold or hot pressed, increase antioxidant power. Research has shown that the most powerful antioxidant had cranberry juice obtained by hot pressing at 75 ^{0}C, compared with 43 ^{0}C and 22^{0}C. The samples pressed at this temperature had the highest values of anthocyanin, total phenolic and antioxidant activity. With regard to the effective time of the pressing was carried out in 30 minutes (Avila P., 2012). Processed fruits heat to100 ^{0}C, and the time up to 20 minutes retains bioactive compounds (Gorinstein S.,2010).

REFERENCES

Avila P. A., Namiesnik J., Toledo F., Werner E., Martinez-Ayala A. L., Rocha-Guzmán N. E., Gallegos-Infante J. A., Gorinstein S., 2012, The influence of different time durations of thermal processing on berries quality, Food Control 26, p.587-593

Benvenuti S., Pellati F., Melegari M., Bertelli D., 2004, Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of Rubus, Ribes, and Aronia. J Food Sci 69: C164–C169.

Beveridge T., Thomas S. C. Li, B., Oomah D., Smit A., 1999, Sea Buckthorn Products: Manufacture and Composition, J. Agric. Food Chem. 47, p. 3480−3488

De Souza V. R.., Pereira P.A., Da Silva T.L., De Oliveira Lima L. C., Pio R., Queiroz F., 2014, Determination of the bioactive compounds, antioxidant activity and chemical composition of Brazilian blackberry, red raspberry, strawberry, blueberry and sweet cherry fruits, Food Chemistry. 125, p. 362–368

Gorinstein S., Haruenkit R., Poovarodom S., Vearasilp S., Ruamsuke P., Namiesnik J., et al., 2010, Some analytical assays for the determination of bioactivity of exotic fruits, Phytochemical Analysis, 21, p. 355-362

Ju Z.Y., Howard L.R., 2003, Effects of Solvent and Temperature on Pressurized Liquid Extraction of Anthocyanins and Total Phenolics from Dried Red Grape Skin, Journal of Agricultural and Food Chemistry 51: 5207-5213.

Казаков А В.,Фиофилактов О.В., 2009. Symbiotic complexes and their functional properties, Пищеваяпромышленность, 12, p. 70-71

Knekt P., Reunanen A., Jarvinen R., Maatela J., 1996, Flavonoid intake and coronary mortality - Reply Br Med J. 312: 1480–1480.

Korekar G., Dolkar P., Singh H., Srivastava B. R., Stobdan T., 2014 - Variability and the genotypic effect on antioxidant activity, total phenolics, carotenoids and ascorbic acid content in seventeen natural population of Seabuckthorn (Hippophae rhamnoides L.) from trans-Himalaya, LWT - Food Science and Technology 55, p. 157-162

Milivojevic J., Slatnar A., Mikulic-Petkovsek M., Stampar F., Nikolic M., et. al., 2012, The Influence of Early Yield on the Accumulation of Major Taste and Health-Related Compounds in Black and Red Currant Cultivars (Ribes spp.). J Agric Food Chem 60: 2682–2691.

Moyer R.A., Hummer K.E., Finn C.E., Frei B., Wrolstad R.E., 2002, Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. J Agric Food Chem 50: 519–525.

Prior R.L., Cao G.H., Martin A., Sofic E., McEwen J., et. al., 1998, Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of Vaccinium species, J Agric Food Chem46: 2686–2693

Slatner Ana., Jerneja J.,Franci S.,Robert V. and Polona J., 2012, The effect of Bioactive Compounds on In Vitro and In Vivo Antioxidant activity of different berry juices, Plos One Journal, 7(10), e47880

Zhang W., Yan J., Duo J., Ren B., Guo J., 1989, Preliminary study of biochemical constitutions of berry of sea buckthorn growing in Shanxi province and their changing trend. Proceedings of International Symposium on Sea Buckthorn (H. rhamnoides L.), Xian, China, Oct 19 - 23, p. 96 - 105

INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BIOCHEMICAL FEATURES OF ACIDOPHILIC BACTERIA INVOLVED IN THE BIODEGRADATION PROCESS OF ORGANIC AND INORGANIC COMPOUNDS

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Abstract

Successful biodegradation process of different complexes required attention to biological parameters (such as choice of microorganisms), control of the chemical and physical environment (pH, redox potential, temperature and metallic ions) and consideration of the metal mines condition. In this context, the present paper presents the study regarding the main interactions between acidophilic bacteria and toxic metal ions can occur through active processes, involving the metabolic sequences of living microorganisms or passive processes, independent of cellular metabolism. The acidophilic bacterial cultures used in the degradation experiments of organic compounds were selected on the basis of their capacity to hydrolyse the starch in the presence of different inorganic forms of heavy metal ions The heterotrophic bacterial capacity by producing organic acid was very high, which confirm the adaptation of these populations to higher concentrations of heavy metallic ions. Comparative results bring an improved bacterial growth and sensitivity to specific environmental conditions and bacterial populations although the effect is not maintained after longer times. Our analysis regarding the efficiency of extracellular enzymes from Acidiphilium populations shows continuous agitation conditions (21 days) in the presence of 2-3g/l starch induce an increased extracellular hydrolytic activity (50-60%) when sulphates is present in the environment.

Key words**:** *enzymatic activity, heavy metal ions, acidity, bioremediation technology.*

INTRODUCTION

In the last years, environmental pollution with heavy metallic ions to the aquatic environment in particular, has attracted attention especially in order to the toxic metals leave, deposits to build up in sediments and tailings. The vast majority of heavy metallic ions are not found in the form of water soluble or, if it exists, the respective chemical species are complexed to inorganic or organic ligands which radically affect their toxicity. The main criteria for selection of chemicals in terms of the environmental impact, prevailing in the world are: (1) toxicity, (2) resistance; (3) ability for bioaccumulation; (4) carcinogen and teratogenic effects [7, 16, 23, 34].

The research activities on the microbial ecology of acidic extreme environments provide a basis for the uncontrolled discharge of industrial waste waters into the river systems containing metallic ions induce profound changes of water quality with inhibiting mineralization, accumulating of heavy metals in elevated concentrations in certain the aquatic organisms and are ultimately to humans [6, 24, 27].

Due to the serious changes of environmental factors in the certain areas close to major industrial platforms are found disappearance of local species of the flora and fauna. In other areas there was a change in the structure of ecological communities due to the mainly eutrophication of the water and soil degradation [3, 15, 26].

On the whole, the accumulation of products with inhibitory activity and the antagonism phenomena contribute, together with the quality and quantity changes of the nutrients, to the appearance of changes in the microorganism communities' structure underlying the succession of populations in an ecosystem. In addition, in the pilot centers of coal desulphurization it has been found that the number of acidophilic bacteria that oxidize

minerals decreases rapidly in the presence of flagellates acidophilic species [18, 29, 35].
Therefore, these eukaryotes are able to bring control in the situations mentioned above (for example, mine waste). At the same time, indigenous microorganisms act on the lack of balance produced by the temporary ecological disorders (eg. discharges of wastewater into the soil or natural waters) [4, 8, 11, 19].
New Gram-positive and Gram-negative bacteria were isolated from Poeni oily sludge, using enrichment procedures. The Gram-positive strains belong to *Bacillus*, *Lysinibacillus* and *Rhodococcus* genera. The Gram-negative strains belong to *Shewanella*, *Aeromonas*, *Pseudomonas* and *Klebsiella* genera. Isolated bacterial strains were tolerant to saturated, monoaromatic and polyaromatic hydrocarbons, and also resistant to different antimicrobial agents (i.e., antibiotics, dyes, synthetic surfactants) [31, 32].
However, many bacterial strains have already been identified as oil-degrading bacteria, many of them belonging to other genera as those isolated by Stancu and Grifoll (2011), which could be used for remediation of oily sludge contaminated environments.
Bioremediation is a complex process of treating a contaminated area by using microorganisms able to metabolize certain harmful substances with structural complexity, by reducing them to biodegradable simpler compounds, with a low degree of toxicity [2, 7, 11, 21].
The study conducted for the first time in Romania on the influence of some intercalation graphitic compounds on the morphogenetic processes over *Sequoia sempervineus*. Endel in vitro culture has demonstrated positive effects on their existence, especially for extended periods of time (60 days or 90 days). For two of the three graphitic compounds it has been noticed that they are non-toxic and do not inhibit the normal processes of the growth and the development in the species studied in vitro cultures, probably due to the existence of specific mechanisms involved in the development of these processes [28, 37].
Recent research has revealed that certain groups of acidophilic microorganisms are able to concentrate metals in solution due to their different qualities: reduction/oxidation, absorption and precipitation. Increased resistance of these microorganisms to the Cu^{2+} ion could result from their adaptation to contaminated environments because they were isolated mainly from mining effluents and tailings, containing increased concentrations of Cu^{2+} [10, 12, 38].
In this context, due to the effect of inorganic compounds on the bacterial degradation of organic compounds, our results revealed the efficiency of biodegradation processes of organic compounds using acidophilic heterotrophic populations in the presence of different inorganic substances.

MATERIALS AND METHODS

Starting from the identification of the environmental pollution source, the pollutants characterization and the degree of the contamination that cause extreme and hostile conditions that influence the zonal microbiota, due to changes in physic-chemical characteristics of the affected area, the presence of the physiological groups of bacteria with biotechnological potential is microbiologically analysed [1, 5, 33, 36].
The research regarding the microbiological analysis of samples from processing plant revealed a big variety of acidophilic bacteria from different physiological groups. Acidophilic bacteria, like as *Acidithiobacillus ferrooxidans* and *Acidiphilium* sp., isolated from metal mines in Romania [14, 19, 39].
In order to obtain isolates of the *Acidiphilium* genus were used selective liquid culture medium GYE (organic medium with a pH of 3.0), in which the energetic substratum was represented by the starch in the optimum concentration of 3g/l.
In the biodegradation experiments of organic substrate by the cultures of *Acidiphilium* sp. were followed the growth of acidophilic heterotrophic bacteria and extracellular enzymatic activity by Wohlgemuth method in six experimental variants with two types of toxic metallic ions sub forms of chloride, such as: (1) GYE medium + 3g/l starch + 0.1% $NiCl_2$ + 0.1% $MgCl_2$; (2) GYE medium + 3g/l starch + 0.1% $NiCl_2$ + 0.1% $MgCl_2$ + 0.1%

$CaCl_2$; (3) GYE medium + 3g/l starch + 0.1% $NiCl_2$ + 0.1% $MgCl_2$ + 0.1% NaCl; (4) GYE medium + 3g/l starch + 0.1% $ZnCl_2$ + 0.1% $MgCl_2$; (5) GYE medium + 3g/l starch + 0.1% $ZnCl_2$ + 0.1% $MgCl_2$ + 0.1% $CaCl_2$; (6) GYE medium + 3g/l starch + 0.1% $ZnCl_2$ + 0.1% $MgCl_2$ + 0.1% NaCl.

RESULTS AND DISCUSSIONS

The study on enzymatic hydrolytic activity of acidophilic bacteria, such as heterotrophic cultures belonging to the *Acidiphilium* genus, present in extreme acidic environments has strong influences on the mining technologies.
In this context, these biodegradation experiments of organic compounds using acidophilic heterotrophic populations mentioned above improved the mineralization mechanisms of complex inorganic forms from polluted industrial environments subject to organic substances contamination.
The test of microbial populations for their capacity to degradation complex organic forms in the presence of different metallic ions composition was made by comparative experiments of the study that metallic ions compounds present in industrial effluents have on the extracellular hydrolytic activity of acidophilic isolates belonging to the *Acidiphilium* genus. Growth experiments were carried out at different species type, heavy metal ion type and contact times between cell and these ions. The comparative studies regarding the effects of toxic metallic ion concentrations on the acidophilic heterotrophic bacteria revealed a increase of growth in GYE medium with 0.1% $NiCl_2$, respectively 0.1% $MgCl_2$ and 0,1% $CaCl_2$, at a concentration value of the organic substratum is established at 3g/l starch.
The biodegradation experiments have revealed the high capacity of the bacterial isolates belonging to the *Acidiphilium* genus to reduce the organic compounds in the presence of Ni^{2+} in the experimental variant V_2 and in the presence of Zn^{2+} in the experimental variant V_6. They have degraded the starch as nutritive substratum in percentages between 35-65% depending on the type of metal ion in solution, pH and bacterial cultures used (fig. 1-4).

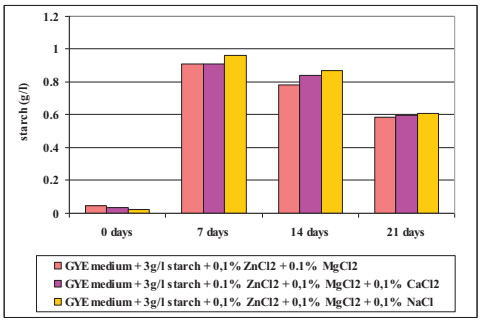

Fig.1. The growth of the P_4 population in GYE medium with 0.1% $NiCl_2$ in the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+}) sub form of chloride at 3g/l starch

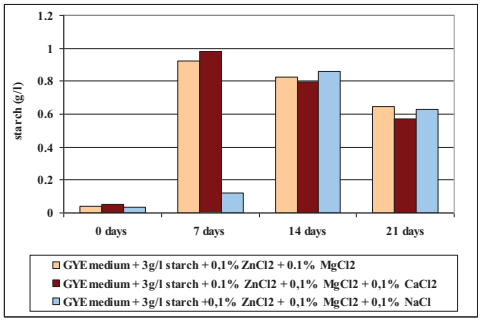

Fig.2. The growth of the P_7 population in GYE medium with 0.1% $NiCl_2$ in the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+}) sub form of chloride at 3g/l starch

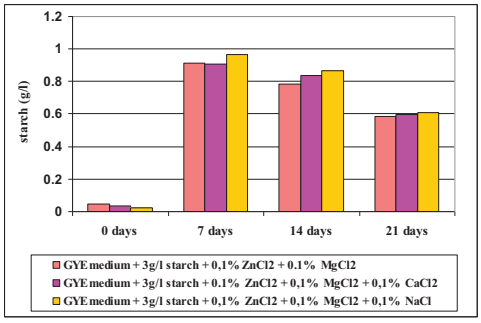

Fig.3. The growth of the P_4 population in GYE medium with 0.1% $ZnCl_2$ in the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+}) sub form of chloride at 3g/l starch

Appreciable percentage of the starch degradation in the experimental variants demonstrated the efficiency of the microbiological process of the environments remediation contaminated with toxic metal ions.

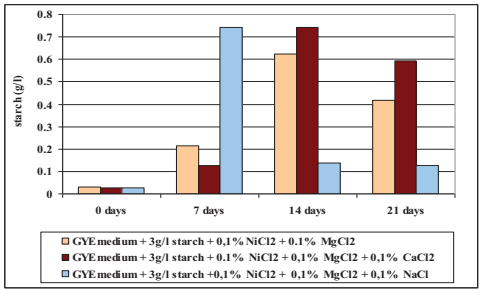

Fig.4. The growth of the P_7 population in GYE medium with 0.1% $ZnCl_2$ in the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+}) sub form of chloride at 3g/l starch

Also, depending on the variation of their habitat essential parameters (temperature, pH, Eh, concentration of O_2 and CO_2, type of metal ions) are produced enzymatic activity changes of the *Acidiphilium* population (fig. 5-8).

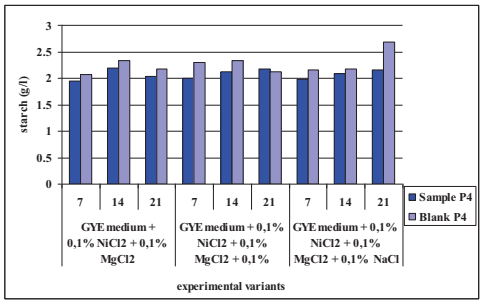

Fig.5. The extracellular degradation activity of the P_4 population in GYE medium with 0.1% $NiCl_2$, in the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+})

A slight influence of sodium sulphates on the extracellular organic degradation activity was obtained under the action of the acidophilic bacterial population with slower tolerance at higher concentrations of toxic metal ions. High percentages degradation of organic compounds were obtained using populations of acidophilic heterotrophic bacteria, which confirms the adaptation of the extracellular hydrolytic activity to the concentration of toxic metallic ions used. The biodegradation experiments of the organic substrate with cultures of *Acidiphilium* sp. at different salts composition showed the fact that at 0,1% $ZnCl_2$ in the presence of Mg^{2+} and Na^{+} it was obtained a higher efficiency of extracellular hydrolytic activity from organic compounds, getting to percentages of 40-75% (fig. 6-8).

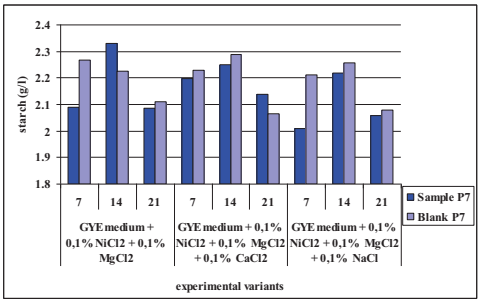

Fig.6. The extracellular degradation activity of the P_7 population in GYE medium with 0.1% $NiCl_2$, the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+})

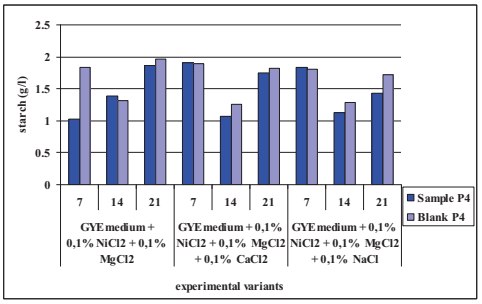

Fig.7. The extracellular degradation activity of the P_4 population in GYE medium with 0.1% $ZnCl_2$ in the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+})

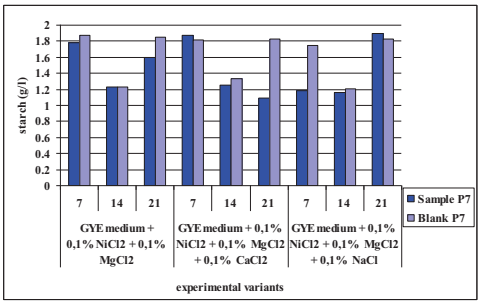

Fig.8. The extracellular degradation activity of the P_7 population in GYE medium with 0.1% $ZnCl_2$, the presence of different cations (Mg^{2+}, Ca^{2+} and Na^{+})

Also, studies of the influence of the metal ions on the metabolic activity of acidophilic heterotrophic bacteria has proved the effectiveness of using starch as an energy underlayer in the stimulation and intensification of bioaccumulation or biosorbtion of heavy metal ions.

The use of *Acidiphilium* populations in the biodegradation experiments of organic compounds has shown a higher correlation

between the growth and the extracellular hydrolytic activity of these bacteria (fig. 1-8).
To understanding the extracellular activity of acidophilic heterotrophic bacteria constitute a landmark in establishing the high influence of chemical conditions on the biodegradation process of organic complexes in the presence of inorganic salts by their action on the heavy metals sorption in the polluted environments with these ions [13, 17, 25, 30].

CONCLUSIONS

The comparative studies regarding the influence of the metallic ions solution on the growth and the starch degradation activity is developed with a maximum intensity up to 7 days of incubation periods at the same toxic metal ion concentrations.
The biodegradation experiments of organic complexes revealed a selective sensitivity to actions in the presence of chloride compounds in environments with different toxic metal ions (Zn^{2+} and Ni^{2+}), which reflects the correlation between specific culture conditions and corresponding bacterial populations.
An advantage of the biodegradation process of organic and inorganic compounds under the action of *Acidiphilium* populations is that the optimum pH and temperature value coincides with their optimum growth values. The experiments of organic compounds degradation under the action of acidophilic heterotrophic bacteria belonging to the *Acidiphilium* genus have revealed the possibility of using starch as a nutritive substratum in processes of ecological restoration of the habitats contaminated with heavy metal ions.

ACKNOWLEDGEMENTS

The study was funded by project no. RO1567-IBB05/2015 from the Institute of Biology Bucharest of the Romanian Academy.

REFERENCES

[1] Asoodeh, A., Chamani, J., Lauzian, M., 2010, A novel thermostable, acidophilic alpha-amylase from a new thermophilic "*Bacillus* sp. Ferdowsicons" isolated from Ferdows hot mineral spring in Iran: Purification and biochemical characterization. International Journal of Biological Macromolecules. 46(3): 289-97.
[2] Asoodeh, A., Alerni, A., Heydari, A., Akbari, J., 2013, Purification and biochemical characterization of an acidophilic amylase from a newly isolated Bacillus sp. DR 90. Extremophiles. 17(2): 339-48.
[3] Baker-Austin, C., Dopson, M., 2007, Life in acid: pH homeostasis in acidophiles. Review, TRENDS in Microbiology, Elsevier, 15(4):165–171.
[4] Cismasiu, C.M., Teodosiu, G., Cojoc, L.R., Ciobanu, L., 2007, Desulphurization coal microbiological procedures. Capacity Building on the Ecomining Principle: Proceedings of the 2nd International Seminar ECOMINING-Europe in 21st Century, Sovata & Praid Salt Mine, 403-413.
[5] Cismasiu, C.M., 2010, The acidophilic chemolithotrophic bacteria involved in the desulphurization process of lignite and pit coal from Halânga, Mintia and Petrila mines, Romanian Biotechnological Letters, 15(5): 5602-5610.
[6] Colleran, E., 1997, Uses of Bacteria in Bioremediaton. Bioremediation Protocols Methods in Biotechnology, 2: 3-22.
[7] Giller, K.E., Witter, E., McGrath, S.P, 1998, Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils; a review. Soil Biology and Biochemistry, 30(10-11)**:** 1389–1414.
[8] Gomez, J., Steiner, W., 2004, The Biocatalytic Potential of Extremophiles and Extremozymes (review), Food Technology Biotechnology. 42(4): 223-235.
[9] Ibrahim, S.A., Eltorn A.M., Abdul-Rahman, A.M., Saeed, B.O., 1994, Correlation of some biochemical parameters with clinical features of protein energy malnutrition, East African medial journal, 71(2):77-83.
[10] Jordan, M.A., McGinness , S., Philips, C.V., 1996, Acidophilic bacteria-their potential mining and environmental applications. Minerals Engineering. 9(2): 169-181
[11] Johnson, B.D., Hallberg, K.B., 2003, The microbiology of acidic mine waters. Research in microbiology. 154(7): 466-73.
[12] Johnson, B.D., 2012, Geomicrobiology of extremely acidic subsurface environments. FEMS Microbiology ecology, 81(1): 1-12.
[13] Karigar, C.S, Rao, S.S., 2011, Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. Enzyme Research Volume. Article ID 805187, 11 pag.
[14] Kay, C.M, Rowe, O.F., Rocchetti, I., Coupland, K., Hallberg, K.B., Johnson, B.D., 2013, Evolution of microbial "streamer" growths in an acidic, metal-contaminated stream draining an abandoned underground copper mine. Life (Basel Swizerland) 3(1): 189-210.
[15] Kimura, S., Bryan, C.G., Hallberg, K.B., Johnson, D.B., 2011, Biodiversity and geochemistry of an extremely acidic, low-temperature subterranean environment sustained by chemolithotrophy. Environmental Microbiology, 13(8): 2092-104.
[16] Korehi, H., Blöthe, M., Schippers, A., 2014, Microbial diversity at the moderate acidic stage in three

different sulfidic mine tailings dumps generating acid mine drainage. Research in Microbiology, 165(9): 713-718.
[17] Mostafa, Y.S., Alamri, S.A., 2014, Biosynthesis of thermostable α-amylase by immobilized *Bacillus subtilis* in batch and repeat batch cultures using fortified date syrup medium. African Journal of Microbiology Research, 8(12): 1292-1301.
[18] Nancucheo, I., Johnson, D.B., 2012, Acidophilic algae isolated from mine-impacted environments and their roles in sustaining heterotrophic acidophiles. Frontiers in Microbiology, 3: 325.
[19] Okibe, N., Gericke, M., Hallberg, K.B., Johnson, D.B., 2003, Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred-tank bioleaching operation. Applied Environmental Microbiology, 69(4): 1936-43.
[20] Oliveira, A., Pampulha, M.E., 2006, Effects of long-term heavy metal contamination on soil microbial characteristics. Journal of Bioscience and Bioengineering, 102(3): 157-161.
[21] Pacesila, I., 2012, Biotic and abiotic factors controlling organic matter decomposition in aquatic ecosystems of Sfântu Gheorghe, the Danube Delta. Muzeul Olteniei Craiova, Oltenia, Studii şi comunicări, Ştiintele Naturii., 28(2): 137-143.
[22] Popescu, G., Dumitru, L., 2009, Biosorption of some heavy metals from media with high salt concentrations by halophilic archaea. Biotechnology & Biotechnolology EQ 23: 791-795.
[23] Petrisor, I.G., Komnitsas, K., Lazar I., Voicu, A., Dobrota, S., Stefanescu, M., 2003, Biosorption of heavy metals from leachates generated at mine waste disposal sites, The European Journal of Mineral Processing and Environmental Protection (EJMP&EP) - Biotreatment & Biosorption 1 - Special Issue, 2(3): 158-167.
[24] Rampelotto, P.H., 2010, Resistance of Microorganisms to Extreme Environmental Conditions and Its Contribution to Astrobiology, Sustainability, 2: 1602-1623.
[25] Reddy, N.S., Nimmagadda, A., Sambasina Rao, K.R.S., 2003, An overview of the microbial α-amylase family. African Journal of Biotechnology, 2(12): 645-648.
[26] Rowe, O.F., Sánchez-Espana, J., Hallberg, K.B., Johnson, D.B., 2007, Microbial communities and geochemical dynamics in an extremely acidic metal-rich stream at an abandoned sulphide mine (Huelva, Spain) underpinned by two functional primary production systems. Research in microbiology, 154(7): 466-473.
[27] Sampson, M.I., Philips, C.V., Blake, R.C., 2000, Influence of the attachement of acidophilic bacteria during the oxidation of mineral sulfides. Minerals Engineering. Elsevier. 13(4): 373-389.
[28] Sarbu, A., Brezeanu, A., Smarandache, D., Cogălniceanu, G., Pascale, G., Stoiculescu, R., 2008, Morpho-anatomical studies on vegetative organs of *Sequoia sempervirens,* in *in vitro* culture on carbon microstructured substrates. Acta Horti Botanic Bucarest, 35: 51-59.
[29] Shankar, V., Nehete, N.P., Kothari, R.M., 1993, Immobilization of amyloglucosidase. Indian Journal of Biochemistry & Biophysics, 30(1): 62-70.
[30] Shrivastava, A., Singh, V., Jadon, S., Bhadauria, S., 2013, Heavy metal tolerance of three different bacteria isolated from industrial effluent. International Journal of Pharmaceutical Reseach and Bio-Science. India. 2(2):137-147..
[31] Stancu, M.M., Grifoll, M., 2011. Multidrug resistance in hydrocarbon-tolerant Gram-positive and Gram-negative bacteria. Journal of General and Applied Microbiology, 57:1-18.
[32] Stancu, M.M., 2014, Physiological cellular responses and adaptations of *Rhodococcus erythropolis* IBB_{Po1} to toxic organic solvents. Journal of Environmental Sciences, 26: 2065-2075.
[33] Stefanescu, M., Voicu,, A., Cirstea, D.,2008 State of the art concerning the possible biotechnological applications for environmental protection, International Conference on New Research in Food and Tourism, Biofoods and public health, FH.27, 10pg., www.unitbv.ro/at/bioatlas.
[34] Šmejkalová1, M., Mikanová, O., Borůvka, L., 2003, Effects of heavy metal concentrations on biological activity of soil micro-organisms. Plant Soil and Environment, Agricultural Journals, 49(7): 321-326.
[35] Singh, R., Singh, P., Sharma, R., 2014, Microorganism as a tool of bioremediation technology for cleaning environment: A review, Proceedings of the International Academy of Ecology and Environmental Sciences, 4(1): 1-6.
[36] Spasova, I., Nicolava, M., Georgiev, P., Groudev, S., 2014, Mine waters cleaning connected with electricity generation. Annual of the University of Mining and Geology „St. Ivan Rilski". 57(2): 137-139.
[37] Udubasa, S.S., Constantinescu, S., Popescu-Pogrion, N., Sârbu, A., Stihi, C., Udubasa, G., 2010, Nanominerals and their bearing on metals uptake by plants. Review Roumain Géologie, 53-54: 47-60.
[38] Voicu,, A., Cismasiu, C.M., Dobrota, S., Petrisor, I.G., Stefanescu, M., Lazar, I., 2000, The resistance of seversl microorganisms to Cu^{2+} ions and their role in biohydrometallurgical applications. Proceedings Institute of Biology, 3, 295-304.
[39] Voicu,, A., Stefanescu, M., Cornea, C.,P., Gheorghe, A., 2009, Microorganisms with biotechnological potential isolated from natural environments. Biotechnology & Biotechnological Equipment 23(2): 747-750 www.diagnosisp.com.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

OPTIMIZATION STRATEGIES AND SCALE-UP THE PRODUCTION OF A RECOMBINANT PROTEIN IN A METHYLOTROPIC YEAST *PICHIA PASTORIS* FROM EGGSHELLS

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Abstract

The goal of achieving a high quality final product of Collagen type 1 suitable for both cosmetical and pharmaceutical industry is a constant battle between the production cost and maintaining a superior improved standard working protocol.
The aim of this study is to optimize the production of Collagen type 1 from different waste materials such as eggshells and methanol by using a fermentation protocol with genetic modified Pichia pastoris yeast.
In our first experiment we used as carbon sources a mixture of eggshells and a glycerol and methanol feeding solution and a fermentation procedure with oxygen limited strategy. The intermediary product was lyophilized and then capped in sterile bottles.
The production method that we tried to optimize regarding the profitability and also the obtaining of high quality final product of Collagen type 1 was successfully achieved. Taking into considerations this result, we believe that the productions costs could be further lowered by adding as a carbon source from marine Black sea algae such as Ulva familae, Porphyra leucostica or Punctaria latifolia.

Key words: *Collagen type 1, Pichia pastoris.*

INTRODUCTION

Collagen is a fibrous protein that strengthens and supports many tissues in the human body In this direction collagen has a number of biomedical uses in plastic surgery like prosthetic implants, tissue replacement, angioplasty sleeves even in cornea repair surgery. Collagen is a fibrous protein found in 20 - 30% of the human body and contains 20 amino acids. The most important amino acid is Gly - glycine about 33% of the collagen molecule, prolyne - Pro and hydroxiprolyne about 22%, alanine 11% and aspargic and glutamic acid 12%. As an important observation specific only for collagen molecule is hydroxiproline which is about 10%. (Traub 1969, Kuhn 1987, Buehler 2006, Fratzl 2008) The goal of our project is to obtain undenaturated *collagen type 1* with a fermentation method and from such waste materials as eggshell and Black Sea marine algae. Marine algae do have an unusual metabolism breath which results in nutritional qualities, including protein and peptides, minerals and vitamins (Kalpa S. and You-Jin J. 2012). They are useful research tools and their natural products are practicable in the treatment of the human disease. Marine algae might be good candidates for harvesting bioactive peptides against cancers. Recently (Chen et.al 2011) it was reported that the protein waste from C. Vulgaris derived peptides has been shown to inhibit UVB-ultraviolet solar B. This can be used for the cosmetic industry as well. In another study has been proved the antitumor activities of C. Vulgaris. For the food industry, in red and green seaweeds - macroalgae that for example Prphyra tenera there have been found about 21 up to 47 grams of protein per 100 grams of dry weight and Ulva perusa form 20-26 grams of protein per 100 grams of dry weight . In the pharmaceutical industry there is a permanent research of the utilization of the marine algae with respect to their bioactive peptides. With new isolation techniques under optimized conditions this could become a time and cost effective process. The therapeutic proprieties of the eggshell and marine algae together with the fact that are toxically free and present a high bioactivity and also bio specificity for

the research and clinical trials are also the reason of the present project.

The basic principle is that we use those waste materials each proven to be useful for the collagen production and with available biotechnology techniques. We also consider optimization of the process in the future post research studies.

For the fermentation we use oxygen limited method and also *Pichia pastoris* yeast for the intracellular collagen production.

Pichia pastoris as a methilotropic yeast was used in many research projects, also in some industrial projects. The efficiency of *Pichia* is also due to it being easily manipulated. (Lin C.Y. and Cregg J.M. 2000)

We use the *Pichia pastoris* strain expressing *Collagen type I* (Project Innovas - Bioingenium S.L). The development of the strain expressing 4-hydroxylase has subunits alpha and beta *Collagen type I* subunits A1 and A2. The process was conducted for the intracellular expression so until a specific technological stage we will use the pellet resulted from the centrifugation process.

In a first stage encoding genes prolyl 4-hydroxylasse subunits alpha and beta were cloned into 2 independent *P. pastoris* shuttle expression vectors and later were transformed into wild-type of *Pichia pastoris* strain. This dual integration was confirmed for isolated clones by genomic PCR and was screened by expression analysis of both prolyl 4-hydroxylasse subunits by western blot. After selecting a prolyl 4-hydroxylase producing clone, an expression vector encoding both subunits of *collagen type 1* (alpha1 alpha2) was transformed into it. Resistant clones were isolated and screened by expression analysis of both *collagen type 1* subunits by western blot. We separate by size and transfer them to solid membrane support with a specific antibody for a better visualization.

We use as carbon sources a mixture between of eggshells, methanol and glycerol feeding solutions in a fermentation process using a 5 L Braun bioreactor. For fermentation we used an oxygen limited procedure and for methanol addition we used a method with feed-back control. (Shiova 1992, Carrondo 2005, Inan 2000).For that reason we monitories the DO - dissolved oxygen (in percent feeding), pH, capacitance (in microfarad) - in relation with turbidity, temperature and the foam level of course. For optimization of the entire process during the cultivation phase we use as carbon first source glycerol and second phase methanol. We supplement the mixture with eggshell initially treated in base solution for 24 hours. In the future-summer season - we will use also specific Black Sea algae, because of the rich glycine and hydroxi-proline composition. As we know from literature hydroxiproline is characteristic only for collagen and it confers stability because of the hydrogen linkages. The initial alkaline treatment of the mixture wills action through molecular linkage without destroying them.

Maximization of the protein production is one of the objectives of the research and post research studies as well. We determine a specific fermentation moment, when viable cell density was measured by capacitance and optical density by spectrophotometer. At an optical density of 300 (OD=300) and a measured capacitance of 32 microfarad we start methanol addition. This is the proper moment for methanol feeding program. From now on we measure the methanol concen-tration by Raven Biotech Me OH sensor online and with our own software made by Innovas Waste Technologies. The calibration methanol sensor was made by initial fermen-tation volume and the methanol feeding percentage as per manufacturer indications.

The methanol addition was performed in 1% shots from culture volume. Special attention must be taken because any exceed of that concentration could become toxic for the culture.(Heisey 1997)

Our specific work is that we use as a multiple carbon and nitrogen source a mixture of eggshell and Black Sea marine algae together with the glycerol and methanol feeding solutions. Our study is about the collagen production technology with waste material like eggshells and marine algae as a carbon and nitrogen source and we believe that after our completed research studies our type 1 collagen will be with basic triple helical structure (tropocolagen) suitable for the pharmaceutical and cosmetic industry.

MATERIALS AND METHODS

Pichia pastoris strain expressing *Collagen type 1* (project Innovas - Bioingenium S.L)
5 litre Braun bioreactor, type 880
Raven Biotech online methanol sensor and control
Cole Parmer peristaltic pumps SDS electrophoresis unit
Lyophiliser unit
In that study we used a series of laboratory equipment and materials provided by Innovas Waste Technologies

Culture maintenance

Pichia pastoris was grown on YPD agar medium plates containing 1% yeast extract, 2% peptone, 2% dextrose and 2% agar. *Pichia* yeast master plate must be stored at 4°C for a one two months use. For long term the cultivated plates should be stored at -80° C, according to the Invitrogen recommendations for medium store.Source of the strain was the project Innovas-Bioingenium S.L

Inoculum Preparation

With 24 hours before fermentation we will use a pre-inoculum in shake flasks from a fresh agar plate containing the strain. We achieve an optical density of 1 and we use 150 ml shake flask incubated at 30°C at 250 rpm with BMGY complex medium (7 g yeast extract, 14 g peptone, potassium phosphate buffer, YNB stock and glycerol) from Sigma Aldrich.

Medium preparation

We prepare trace salts and biotin solutions and store at 4°C. We prepare also fermentation basal salts medium for our bioreactor.
For each litre of fermentation basal salts we will use 4.35 mL of trace salts. We start from 3 (three litre) according to our bioreactor volume.
We prepare the mixtures from eggshell and marine Black Sea algae with alkaline water.

Bioreactor setup and sterilization

For this operation we use a vertical autoclave so that we can completely sterilize the whole bioreactor. The same procedure was applied to all air filters and pipes that were mounted to be sterilized.
We protect the filters and sensors with aluminium foil. We introduce the assembled bioreactor with the medium inside the vessel into the autoclave for 20 min to 121°C.

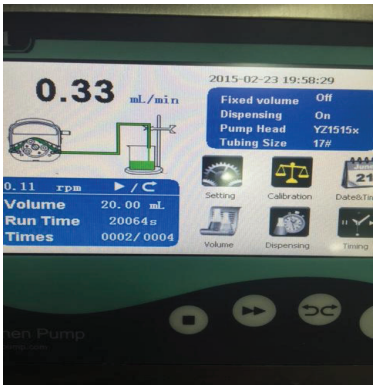
Fermentation procedures

We use an oxygen limited strategy fermentation for improving production. The oxygen is controlled and consumed in connection with the liquid gas transfer and with the methanol additions. (Picture 1, Picture 2)
After autoclaving we connect the cooling system and we add the prepared mixture and we set all the parameters as following. The cultivation conditions were stirring rate 1000 rpm, temperature 30°C and dissolved oxygen in a limit of 20%. Initial working volume was 3L. All medium salts were autoclaved or filter sterilized by 0.2 micrometre Millipore filter. Air outlet was secured also by 0.22 micrometre PTFE filter. We adjust the pH to 5-6 with a base and with an acid. We used NaOH, ammonium hydroxide as a base pH adjustment and HCl or acetic acid for the acid pH. For the level controlling pH we use peristaltic pumps from Master Flex and Tygoon rubber connections. We add trace salts through a sterile septum.
All sensors must be calibrated so we can start polarization of the dissolved oxygen sensor. For that sensor we use one or two point calibration. All prepared so the entire controllers could be started. Special attention must be taken about the feeding with oxygen or air to be sterile as well. Special filters with pharmaceutical quality standards must be used in gas line connection.
We measure the optical density from the fermenter and we calculate the necessary volume of inoculum to assure an OD of 1. By a sterile septum or an inoculum bottle we inoculate the bioreactor fermentation medium. When we check the dissolved oxygen and capacitance and we detect a rapid increase of those values we start with glycerol fed-batch phase. Glycerol feed pump must be connected and a special addition program will be implemented to add flow rate from 20 up to 100 mL per hour. We measure optical density

for each 30 minutes and when we will have a value of 350 we stop the glycerol addition.
After that phase we start methanol feeding stage in shots of maximum 1% of the bioreactor volume. We observe the online methanol measurement and the dissolved oxygen values also. A sudden peak is observed when methanol is depleted. We measure the desired protein concentration and when we realize it is steady we decide to end the fermentation. In all the methanol addition phase, because our process is exothermal we must strictly control the temperature. (Picture 3)
Once the process is finished the fermentation culture is stopped by the aid of controllers, sensors and stirring. This could be observed by no increasing of the desired protein concentration observed.
We collect the bioreactor by draining valve in a sterile bottle by a sterile connection.

Picture 1 - Computer control and software for on line monitoring from Innovas Waste Technologies

Picture 2 - Obtained centrifugation pellets



Picture 3 - Glycerol and methanol feeding

Picture 4 - Innovas fermentation software

Early downstream

We initiate early downstream to recover and purify the fermentation product.

The obtained product from the fermentation process was centrifuged at 6000-8000 g for about 20-30 min.

The supernatant will be used for extracellular production or cell pellet for intracellular production mode. We add pepsin solution 0.2 mg/ml according to Nokelainen et al., 2001 and we gently stir. A washing step was done for cell pellet in Phosphate Buffer Solution from Sigma Aldrich at 4^0C and we centrifuge again at 6000-8000 g for 25-30 min. The obtained pellet we re-suspend in a suitable binding buffer for the purification step. The intracellular product must to be disrupted by one of the available known techniques such as chemical treatment, enzymatic digestion treatment or French press, sonication, glass beds etc. We used French Press because *Pichia pastoris* has a strong cellular membrane.

In that stage some protease inhibitors were used to prevent the protein to be proteolysis. After the disruption the lysed solution was centrifuged at 10000g for 15 min. With the obtained supernatant we start filtration procedures. As a filtration steps we first used is a macro filtration by a 30 micrometre filter, then a filtration with 5-10 micrometre filter and a clarification or sterilization by 0.45 - 0.2 micrometre filter. The concentrate the product by ultrafiltration treatment Sartocom cassettes of 100 kDa was performed. The intermediary product was lyophilized and then capped in sterile bottles.

Late downstream stages

For characterization we use SDS-page electrophoresis and UV-VIS analysis for concentrate protein.

RESULTS AND DISCUSSIONS

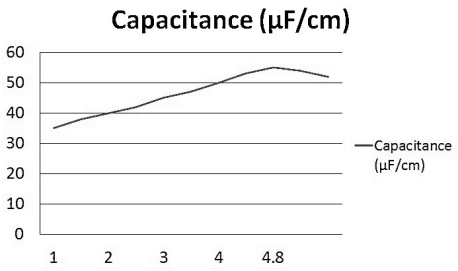


Figure 2. Capacitance variations by the number of fermentation days

Capacitance online measurements were very useful for our project, taking into consideration that the methanol addition should be started when a few parameters like optical density OD or turbidity reach the values of 300 respectively 35. The proper moment for the methanol feeding is very important to be accurately determined.

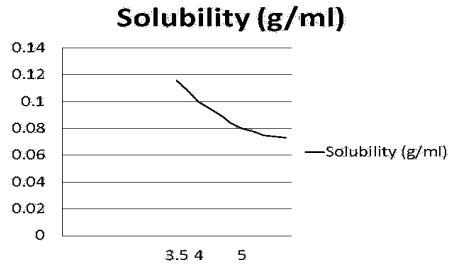


Figure 3. Variation of solubility at different pH values

The pellet obtained after the second centrifugation must be treated with acid for lowering the pH. We have found that in alkaline pH collagen solution incline to precipitate. So for any pepsin treatment or another filtering operation we must be sure that the solution is as liquid as it is possible.

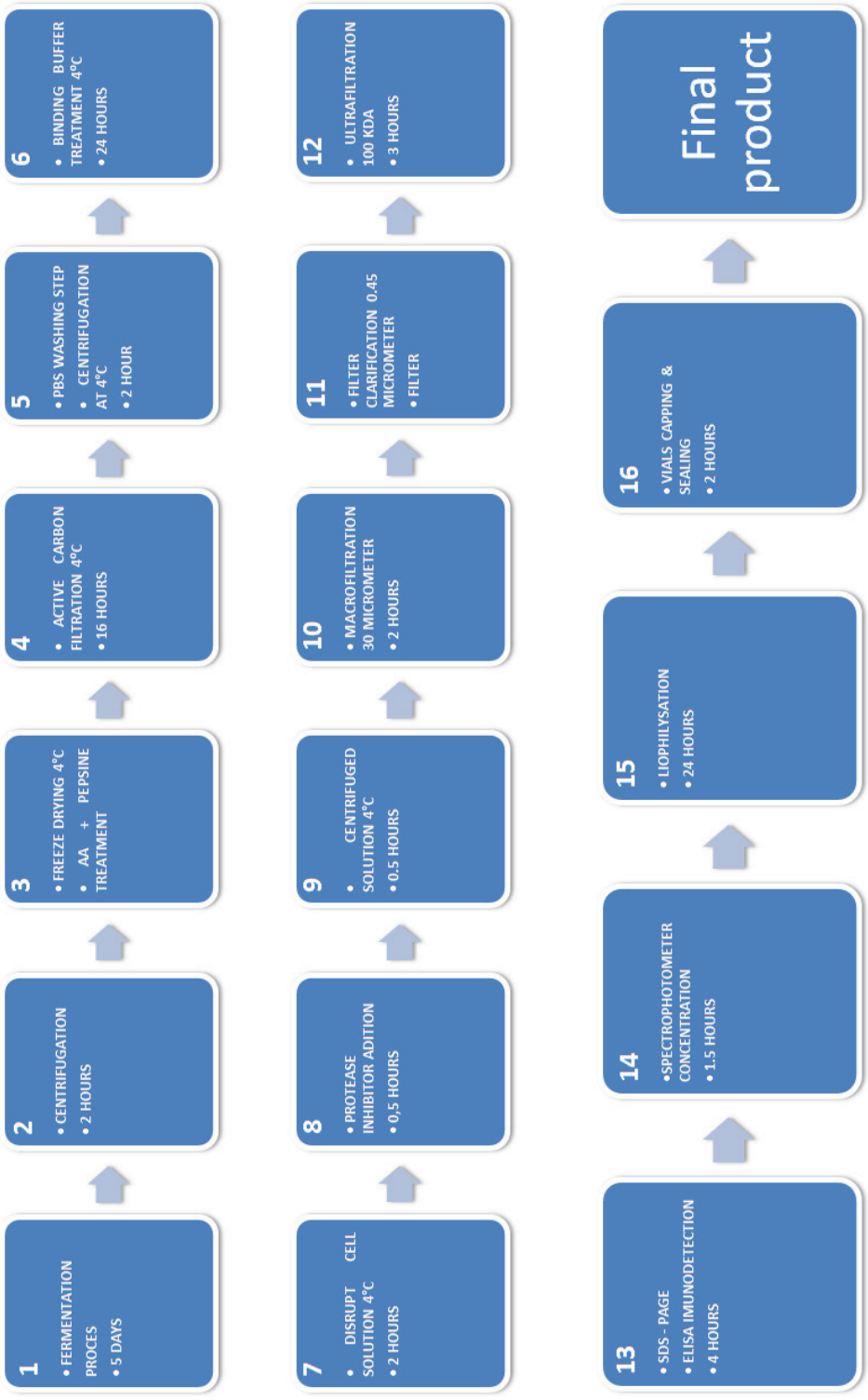


Figure 1. Methods plan with operations time calculation

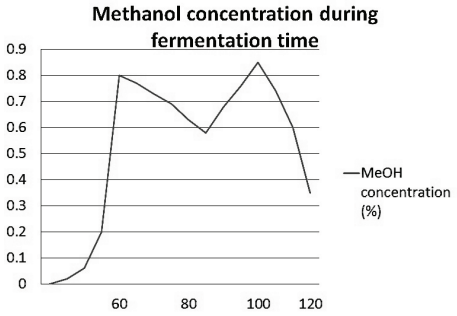


Figure 4. Methanol concentration during fermentation time (min)

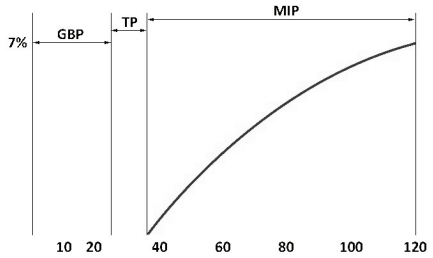


Figure 5. Fermentation phases yield during fermentation time. GBP=Glycerol base phase, TP=Transition phase, MIP=Methanol induction phase

Methanol concentration is very important to be checked online and controlled if possible because of the potential toxic concentration value which can affect the *Pichia pastoris*. During the fermentation phases in GBP (glycerol base phase) we have no methanol addition. In TP (transition phase) we must wait until the glycerol is completely depleted and in approximately 40 hours from the fermentation we started MIP (methanol induction phase). The feeding rate should be less than 1% from the existing bioreactor volume in that time.

CONCLUSIONS

During the present project there have been a lot of modifications and variations of the results. These could be from the eggshell composition, from algae composition or from different fermentation condition despite our computer monitoring. For a small industrial scale we are looking in the future to scale up the production in order to create a production platform that is time and cost efficient. We also have the intention to optimize the screening, purification, filtration and product transfer and finally to make comprehensive analysis accepted by the pharmaceutical industry and also by the cosmetically and food industry as well.

The proposed collagen production was obtained in small quantities based on the production strategy and from the proposed mixture. The online methanol control in concordance with the capacitance sensor was very useful to observe the most important moments of the fermentation process and on the other hand to avoid any negative effects for the protein production.

We strongly believe that such waste materials have the potential for the mentioned industries taking into account that there are clean and prolific sources from the biochemical and ecological point of view.

However the results need to be improved for any future commercial use, taking into consideration the productivity and rentability.

ACKNOWLEDGEMENTS

Special thanks to Francisco Valero from UAB Department of Chemical Engineering, David Resina and Antonio Barreiro from Bioingenium S.L and to Innovas Waste Technologies for the equipment and laboratories use.

REFERENCES

Buehler, M. J. 2006. Nature designs tough collagen: Explaining the nanostructure of collagen fibrils. (33): 12285–12290.

Carrondo MJT., Clemente JJ., Cunha A.E., Oliveira R., 2005. Adaptive dissolved oxygen control throught the glycerol feeding in a recombinant Pichia Pastoris cultivation in conditions of oxygen transfer limitation. J Biotechnol 116:35-50

Chen C.L., Lion S.F., Chen S.J., Shih M.F.,2011. Protective effects of Chlorella derived peptide on UVB-induced production of MMP-1 and degradation of procollagen genes in human skin fibroblasts-Regulatory Toxcology and Pharmacology 60: 112-119

Cregg J. M., Tolstorukov I., Kusari A., Jay Sunga A., Madden K., Chappel T. 2010 Pichia Protocols (second edition). Humana Press, Totowa, New Jersey

Deyl Z., Miksi'k I.,1995. Separation of collagen type I chain polymers by electrophoresis in non-cross-linked polyacryl – amide - filled capillaries, J Chromatogr,

Fratzl, P. 2008. Collagen: Structure and Mechanics. New York: Springer

Heisey RF., Matheson NH., Schneider K., Wagner LW., 1997. Use of a silicone tubing sensor to control methanol concentration during fed batch fermentation of Pichia Pastoris. Biotechnol Tech 11: 791-795

Inan M., Meagher MM., Zhang W., 2000. Fermentation strategies for recombinant protein expression in the methilotrophic yeast Pichia Pastoris. Biotechnol Bioprocess Eng 5: 275-287

Kalpa S. and You-Jin J. 2012. Bio-functionalities of proteins derived from marine algae - A review. 948–960

Kuhn K.,1987 The classical collagens: types I, II, and III In: Mayne, Rand Burgenson, R.E., (Eds.) Structure and Function of Collagen Types. Orlando, FL: Academic Press, Inc.,

Lin Cereghino J., Cregg, J. M., 2000. Heterelogous protein expression in the methylotropic yeast Pichia Pastoris. FEMS Microbiol. Rev. 24: 45-65

Se-Kwon K., 2011 Handbook of Marine Macroalgae - Biotechnology and applied phycology. Markono Print Media Pte Ltd., Singapore

Se-Kwon K.,2013 Marine Proteins and Peptides - Biological activities and applications. Markono Print Media Pte Ltd., Singapore

Shioya S. 1992. Optimization and control in fed batch bioreactors. Advances in biochemical engineering/ biotechnology. . 46. p111-142

Traub, W.; Yonath, A. & Segal, D. M.,1969, On the molecular structure of collagen". Nature 221 (5184): 914–917.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

CHARACTERISATION OF BACTERIAL ENZYMATIC COMPLEX USED IN LEATHER WASTES DEGRADATION

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Abstract

The leather waste generated by the leather industry contains a large amount of hard to degrade proteins. Those squanders are destroyed by incineration and it represents a threat for the environment. Through biotechnological methods, this waste can be used as substrate for enzyme production, the leather serving as the unique source of carbon and nitrogen.
This paper presents the isolation and characterization of leather degrading bacterium. The hydrolytic bacteria where isolated from compost of fur and skin .The isolated colonies show their ability to synthetize various hydrolytic enzyme as proteinase (0.709 – 0.868 U/ml), lipases (20-80 U/ml), collagenases (0.344 – 0.373 U/ml), amylases (0.104 – 0.198 U/ml), keratinases (0.4-1.8U/ml) and it can be use in different biotechnological processes. The proteolytic enzymes have an important value in the biotechnological sector and the obtaining process presented in this paper is a nonpolluting alternative to the current disposal system.
The results show that the isolated bacteria have the capacity to produce hydrolytic enzymes able to degrade different types of leathers.

Key words*: degradation, leather wastes, enzymatic product.*

INTRODUCTION

Proteolytic enzymes are groups of enzymes that break the long chainlike molecules of proteins into shorter fragments (peptides) and eventually into their components, amino acids (Anson, 1938). Proteolytic enzymes are present in bacteria and plants but are most abundant in animals. Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. The isolated bacteria have the capacity to produce proteolytic enzymes using skin and fur as unique source of carbon.

MATERIALS AND METHODS

Materials

Minimal media, Luria Bertani, PCA (plate count agar), CMC (carboximetilcelulose) and starch media was used.

Microorganisms

Three isolated strains from skin and fur compostation were used. The bacterial cultures were growing at 35^{o}C and maintained by periodic transfer on agar tubes with the following composition: peptone 1g%, yeast extract 1g%, NaCl 0.5g% and agar 2g%.

Inoculum growth medium and cultivation conditions

40 mL of minimal media (medium (g/l) 1.0g $NaCl_2$, 0.05g $CaCl_2$, 0.7g KH_2PO_4, 0.9g $MgSO_4$, 2.38g K_2HPO_4, 3,0g sucrose, 0.6g skin and fur) was previously sterilized at 120^{o}C, for 20 minutes and incubated with one loop of isolated bacteria. The inoculum was prepared in 100 mL Erlenmeyer flask by incubation at 35^{o}C, for 120 hours on a laboratory shaker (Heidolph – Germany) at 135 rpm.

Biomass separation
At the end of fermentation process the cultivation medium was centrifuged during 20 minutes at 9000 rpm.

Determination of proteolytic activity
Proteolytic activity was spectrophotometric measured at 578 nm, following the method of Anson (1938). The reaction mix contained 0.5 mL enzymatic solution and 1 mL casein 1% in phosphate buffer 0.2M (pH 7), incubated at 37^{o}C for 10 min. Enzymatic reaction was stopped with 2 mL of tricloracetic acid 5%. The reaction mix was kept 30 min at room's temperature and then it was filtrated. For every 0.5mL filtrate was added 0.5mL HCl 0.2N, 2mL NaOH 0.5N and 0.6 mL Folin-Ciocalteu 1:2. After 30 min at room's temperature the extinction was measured.

Determination of lipolytic activity
For the lipolytic activity was used 10 mL emulsion substrate, 2 mL $CaCO_2$, 5mL citrate buffer, 1 mL enzymatic solution. The samples were incubated 60 min at 37^{o}C, 170 rpm. After incubation the reaction was stopped by adding 20 mL acetone: ethanol 1:1 (v/v).
Lipase activity was measured by titrimetric assay with NaOH 0,1N (Anson, 1938). One unit of lipase activity was defined as the amount of enzyme that release 1µmol equivalent of carboxylic groups of fatty acid under analysis conditions (temperature 37^{o}C, pH 7, reaction time 60 min.) (Lupescu et al. 2007).

Determination of keratinolitic activity
Enzyme activity was determined with keratin azure as substrate. The reaction mixture contained 2 mL suspension (1.5 mL Tris Buffer and 0.5 mL culture supernatant) and 80 mg keratin azure (Sigma).
The mix was incubated at 50^{o}C for 60 min and then centrifuged at 10.000 rpm, 20 min. Supernatants were measured at 595 nm. One unit of keratinase activity was define as that amount of keratinase producing an increase of 0.1 absorbent units 595 nm/h (Korkmaz et al., 2004).

Determination of collagenase activity
Collagenase activity was measured by using ninhidrin as substrate following the method of Moore and Stain (1948). The reaction mix contained 2 mL ninhidrin, monoetil-glicol and eter. After boiling for 30 minutes the samples are diluted with 10 mL n-propanol 50% concentration.
The absorbance was determined at 600nm after 15 minutes.

Determination of amylase activity
The amylase activity was determined. An assay mixture containing 0.5 mL phosphate buffer 0,2M (pH 6.9), starch 1g% and 0.5 mL supernatant. The mixture was incubated 10 min at 30^{o}C. The reaction was stopped using 2 mL DNS. The samples were incubated 5 min at 100^{o}C. After cooling for 12mL distilled water was added. The amylase activity was measured at 546 nm. One unit of amylase activity was defined as the number of µ moles of maltose liberate by 1 mL enzyme solution per minute (Tapai M., 2009).

Determination of organic carbon
For the organic carbon determination 0.5g of skin and fur were introduced in filtered enzymatic solution. It was incubated 48 h at 35^{o}C, 135 rpm. After the incubation it was washed and dried.
The reaction mixture used 10mL $K_2Cr_2O_7$ and 20 mL H_2SO_4. The samples were incubated for 20 min at 95^{o}C. 150 mL distillated water was added and ortofenantroline was used as reaction indicator. The titration was made with Mohr salt.

RESULTS AND DISCUSSION

Three bacteria strains producing variable proteolytic zone on PCA agar plates where isolated from decomposition of fur and skins: DA7, DA10 and DA13. The clear zone of hydrolysis around each bacterial colony reflects their extent of extracellular proteolytic activity (Habib et al., 2012).

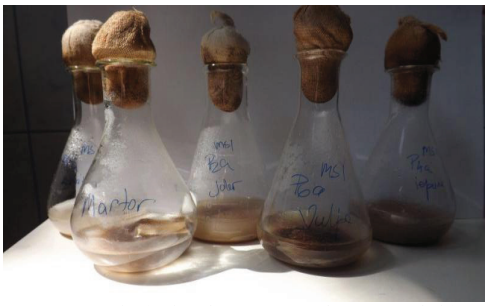

Figure 1. The isolated strains tested on fur and skin into minimal media, after 48 h of incubation

The bacteria were incubated 120 hours in suspension on minimal media, at 35°C, 135 rpm, pH 7.2.

The parameter was performed in fermentation experiments carried out in 100 mL flasks using 40 mL of minimal media.

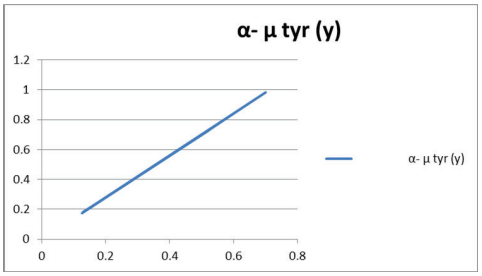

Figure 2. Growth behavior of isolated bacterial strain and rate of extracellular protease production during 120h, after 1:25 dilution

An increase in the cell mass and lipase activity was observed (Figure 3) as a result of a strong aeration (lipase activity = 80 U/mL), pointed out by the lipolytic activity in the above mentioned cultivation conditions.

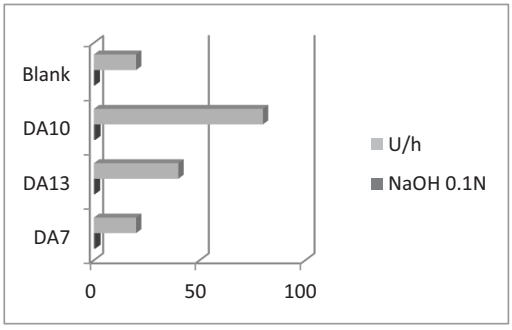

Figure 3. Lipase activity in fermentation media (U/h)

The highest keratinolytic enzyme production by isolated strains was 0.223 U/mL, produced by DA10 strain, in the above mentioned cultivation conditions (Figure 4).

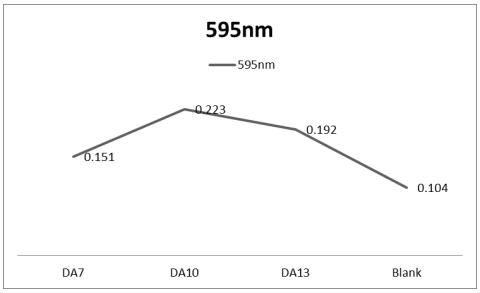

Figure 4. The keratinolitic activity determination at 595 nm

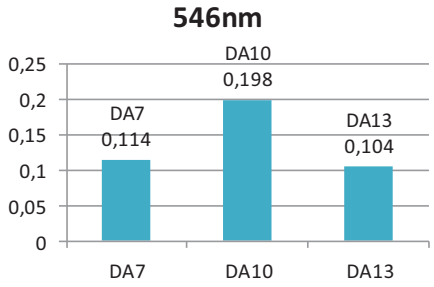

Figure 5. Amylolitic activity of isolates after 120 h of incubation

In submerged fermentation the amylase production has reached maximum of 0.198 U/ml by DA10 strain, at 120 hours of incubation, where it showed a significant increase in enzyme production rate (Figure 5).

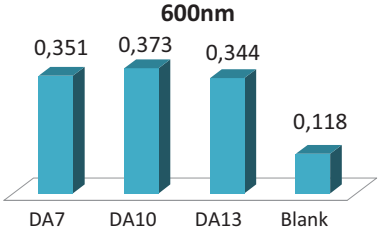

Figure 6. Determination of collagenase activity at 600 nm, after 120 hours of incubation on MM

The collagenazic activity maximum production was 0.373 U/mL, obtained with the DA10 strain in the fermentation medium with

0.6 g of skin and fur as unique source of carbon (Figure 6).

Table 1. Organic carbon determined, after 24 h of incubation of fur and skins in enzymatic solution.

Nr. Crt.	Sample	%
1	Blank	51.68
2	DA7	24.63
3	DA10	24.82
4	DA13	25.75

The organic carbon source has the potential to introduce synthesis of proteases at an optimal level, which was determined through testing of organic compounds, those being utilized as individual resources.

CONCLUSIONS

The isolated strains produced extracellular enzymes which have the capacity to use fur as unique source of carbon. The best results were obtained using DA10 strain.
The strains have high proteolytic activity and are very affective in skins and fur degradation, suggesting its potential use in biotechnological processing involving protein hydrolysis.

BIBLIOGRAPHY

Anson, M.L., 1938, J. Gen. Physiol. 22, 79-89

Habib S.M.A., Fakhruddin A.N.M., Begum S., Ahmed M.M., 2012, Isolation and screening of thermostable extracellular alkaline protease producing bacteria from tannery effluents. J. Sci. Res. 4: 515-522

Korkmaz H., Hür H., Dinçer S., 2004, Characterization of alkaline keratinase of *B. licheniformis* strain HK1 from poultry waste, Annals of Microbiology 54 (2), 201-211.

Lupescu I., Groposila-Constantinescu D., Jurcoane S., Diguta C., Cozea A., Tcacenco L., 2007, Production of lipases by strain of the non-conventional yeast *Yarrowia lipolytica* and isolation of crude enzyme, Roumanian Biotechnology letter, Vol 12, No3, pp 3261-3268.

Moore S., Stein W.H., 1948, Photometric ninhydrin method for use in the chromatography of amino acids, J. Biol. Chem. 176: 367-388.

Tapai (Stoica) Mihaela, 2009, Biosinteza unor amilaze utilizate în hidroliza unor materii prime agricole, Teza de Doctorat, p.65-66, Bucuresti.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

SCREENING OF OLEAGINOUS MICROORGANISMS FOR LIPID PRODUCTION

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Abstract

Among hundreds species of yeasts and molds, only few are able to accumulate more than 25% lipids. The aim of our experiments was to select microorganisms with high potential of lipids producing ("oleaginous" microorganisms). Several strains of yeasts and molds were tested in order to determine their ability to produce and accumulate lipids. The strains were cultivated in Petri dishes on specific media. Evaluation of lipid accumulation was achieved by microscopic observations, through Sudan Black B dyeing technique. Preliminary tests in order to determine the capacity of lipid production were made by Soxhlet extraction, after batch fermentations on liquid media. The results showed that five strains of yeasts and molds, Yarrowia lipolytica, Hansenula anomala, Sacharomyces cerevisiae, Aspergillus niger and Trichoderma viridae, were the best lipid producers, which accumulated up to 40% of lipids in dry biomass. One of the key factors in achieving an economically attractive bioprocess is the conversion yield of the substrate into lipids. In this case, yeasts are superior to fungi with a conversion yield over 20%, in comparison with less than 18% for molds. Given these considerations, additional experiments will be conducted to optimize the conditions of lipid production with Yarrowia lipolytica.

Key words*: biomass, fermentation, lipids, oleaginous microorganism.*

INTRODUCTION

Like all living cells, microorganisms contain lipids for the essential functioning of membranes and membranous structures, but not all microorganisms can be considered as abundant sources of fats and oils. Those microorganisms that do produce a high content of lipids may be termed "oleaginous" (Ratledge, 1994). From 600 different species of yeast, only 25 or so are able to accumulate more than 20% lipid; of the 60,000 fungal species fewer than 50 accumulate more than 25% lipid. The lipids that are accumulated by the oleaginous microorganisms are mainly triacylglycerols (Ratledge, 1993). Generally, oleaginous microorganisms are eukaryotes and thus representative species include algae, yeasts, and molds. Bacteria do not usually accumulate significant amounts of triacylglycerols, but many do accumulate waxes and polyesters (Ratledge, Cohen, 2008).
Oleaginous microorganisms, such as microalgae, yeasts, molds and bacteria can accumulate high levels of lipids and do not require arable land, so that they do not compete with food production (Gouda et al., 2008).
Microbial lipophilic compounds, called single cell oils (SCO), are of potential industrial interest due to their specific characteristics (Rattray et al., 1974). The aim of our experiments was to select microorganisms with high potential of producing lipids.

MATERIALS AND METHODS

Microorganisms and media

Ten strains including molds and yeasts from the Collection of Microorganisms of the Faculty of Biotechnology were tested in order to determine their ability to produce and accumulate lipids: *Trichoderma viridae, Aspergillus niger, Aspergillus oryzae, Rhizopus nigricans, Yarrowia lipolytica, Candida utilis, Saccharomyces cerevisiae, Saccharomyces ellipsoideus, Hansenula anomala, Saccharomyces carlsbergensis.*

The strains were cultivated in Petri dishes on specific media. The yeasts were cultivated on YPG medium (yeast extract, peptone and glucose), meanwhile the molds were cultivated on PDA (potato infusion, glucose) medium (Ma et al., 2009).

Maintenance of strains and shake flask cultivation conditions

The yeasts were maintained on YPG-agar medium at 28^0C for 48-72 hours and then stored in a freezer at 4^0C.
The molds were cultivated on PDA-agar medium at 35^0C for 5 days and then stored at 4^0C (Ratledge et al., 2008).
From the culture stored at 4^0C, a loop of cell mass was transferred aseptically to 20 ml liquid media (YPG/PDB) and then incubated in a rotary shaker incubator at 240 rpm, 28^0C for 24 hours for yeasts, and 220 rpm, 35^0C for 48 hours for molds. Next, this culture was used to inoculate 200 ml of liquid media (Erlenmeyer flasks of 500 ml) using a 10% (v/v) inoculum (Qier, 2013).

Biomass assessment

Samples were collected at 8 hour intervals for the determination of wet cell weight, dry cell weight, cell concentration and microscopy examination. Biomass was assessed by absorbance for yeasts and by weighing wet biomass for molds.
For absorbance measurement of yeast media, 0,5 ml of culture medium were taken at 8 hour intervals during fermentation, properly diluted in water (1:25) and measured with a spectrophotometer, at 570 nm (Subramaniam et al., 2010).
For wet biomass preparation, 5 ml of culture medium were taken during fermentation, transferred to pre-weighted tubes and centrifuged. The supernatants were removed and the tubes containing the cellular sediments were weighted (wet cell weight).
Calibration curves were performed using absorbance measurements for yeasts and wet cell biomass for molds (Liu et al., 2010).

Evaluation of lipid biosynthesis

Preliminary evaluation of lipid accumulation was achieved through Sudan Black B staining method, monitoring lipid production by microscopic observation of the cells cultivated on solid media (Liu et al., 2010; Thakur et al., 1988).
The strains were screened for their capacity of producing lipids in shake flask cultures.
The strain that showed the greatest potential ability to produce lipids was selected for further investigation.

Lipid extraction and determination

Wet biomass was transferred in a thermo-balance were it was dried at 115^0C, to constant weight.
The lipids were extracted processing the dried biomass with a Soxhlet extractor, using chloroform:methanol 2:1 (Ratledge et al., 2008).
Quantitative assay of lipids was done by solvent evaporation and weighing the remaining product (Ratledge, 1993).

RESULTS AND DISCUSSIONS

Strain screening

Ten strains of molds and yeasts were screened through Sudan Black B staining method, in order to highlight the capacity to accumulate lipids.
The results showed that three strains of molds exhibit lipid bodies in their hyphae, when examined with optical microscope.
The analysis of yeasts showed that all of them have the potential to accumulate lipids.
Therefore, these nine strains were further tested for their ability to produce lipids in shake flask culture.

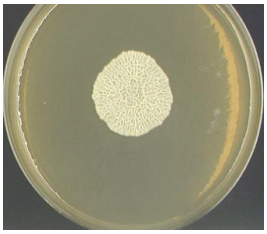

Figure 1. Culture of *Yarrowia lipolytica*

Figure 2. Culture of *Saccharomyces cerevisiae*

Shake flask cultivation and biomass assessment

The nine strains were studied in liquid cultures in order to evaluate the cell growth and lipid accumulation.
Table 1 shows biomass yield of the tested nine strains.

Table 1. Dry biomass yield of tested strains

Strain	Dry biomass (g/l)	Biomass productivity (g/l·h)
Trichoderma viridae	13,3	0,111
Aspergillus niger	14,9	0,124
Aspergillus oryzae	11,8	0,098
Yarrowia lipolytica	15,3	0,213
Candida utilis	11,1	0,154
Saccharomyces cerevisiae	9,1	0,190
Saccharomyces ellipsoideus	10,1	0,210
Hansenula anomala	11,4	0,238
Saccharomyces carlsbergensis	11,6	0,161

Growth curve determination

All yeasts and molds were cultivated in 500 ml shake flasks with 200 ml medium. Optical density/wet biomass were measured every 8 hours. The measurements were used for drawing a growth curve to identify which time points are suitable for harvesting the microorganisms culture.
According to the test results, the best harvesting times were determined to be around 72 hours for *Yarrowia lipolytica,* 48 hours for *Candida utilis*, 48 hours for *Saccharomyces cerevisiae,* 48 hours for *Saccharomyces ellipsoideus,* 48 hours for *Hansenula anomala,* 72 hours for *Saccharomyces carlsbergensis,* 120 hours for *Trichoderma viridae,* 112 hours for *Aspergillus niger,* 120 hours for *Aspergillus oryzae.*

Lipid production

Lipids were extracted from the dry biomass in chloroform:methanol 2:1, with a Soxhlet extractor.
Yarrowia lipolytica displayed the greatest potential ability in accumulating microbial lipids, the lipid content reaching 39.9% of dry cell weights.

The tested molds showed a much lower potential of accumulating lipids then yeasts, of 18.1-21.8% (g lipids /g dry biomass). Table 2 shows lipid yield of the tested yeasts and molds.

Table 2. Lipid yield of tested strains

Strain	Lipid fraction (%)	Lipid productivity (g/l·h)
Trichoderma viridae	21,8	0,024
Aspergillus niger	18,1	0,022
Aspergillus oryzae	18,9	0,019
Yarrowia lipolytica	39,9	0,085
Candida utilis	28,5	0,044
Saccharomyces cerevisiae	38,4	0,073
Saccharomyces ellipsoideus	30,2	0,064
Hansenula anomala	22,6	0,054
Saccharomyces carlsbergensis	20,5	0,033

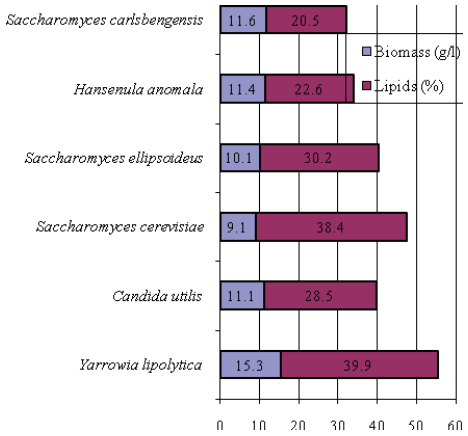

Figure 3. Biomass and lipid content of yeasts

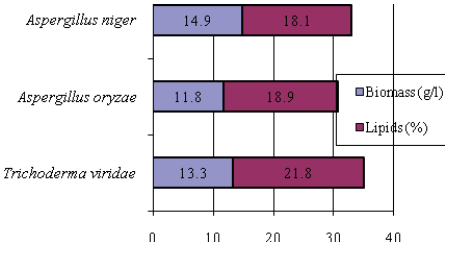

Figure 4. Lipid content of molds

CONCLUSIONS

- Screening of the ten strains of yeasts and molds pointed out a varied potential of lipid biosynthesis.
- The strains characterized by a higher potential of lipid biosynthesis were yeasts, with lipid productivities of: 0,085 g/l·h *Yarrowia lipolytica*, 0,073g/l·h *Saccharomyces cerevisiae* and 0,064g/l·h *Saccharomyces ellipsoideus*.
- In the case of molds, the results were lower than those mentioned in the literature. The best results were obtained from *Trichoderma viridae*, with a lipid productivity of 0,024 g/l·h.
- The strain *Yarrowia lipolytica* showed the greatest potential ability in producing lipids and it was selected for further investigations.

ACKNOWLEDGEMENTS TNR 12

This research was carried out with the support of the program "Invest in people!", project co-financed by the European Social Fund POSDRU2007-2013,
POSDRU/159/1.5/S/132765

REFERENCES

Liu G.Q., Lin Q.L., Jin X.C., Wang X.L., Zhao Y., 2010, Screening and fermentation optimization of microbial lipid-producing molds from forest soils, African Journal of Microbiology Research, 4(14), 1462-1468.

Ma L., Xing D., Wang H., Wang X., Xue D., 2009, Effect of culture conditions on cell growth and lipid accumulation of oleaginous microorganism, CHIN. J. Biotechnol., 25, 55-59.

Thakur S., Prapulla S.G., Karanth N.G., 1988, Microscopic observation of Sudan Black B staining to monitor lipid production by microbes, J. Chem. Technol. Biotechnol., 42, 129-134.

Qier S.,A comparative study on four oleaginous yeasts on their lipid accumulating capacity, 2013, Master´s thesis, Volkmar Passoth, Swedish University of Agricultural Sciences, Department of Microbiology, Uppsala.

Rattray J.B., Schibeci A., Kidby D.K., 1975, Lipids of yeasts, Bacteriol. Rev., 39(3), 197.

Ratledge C., Cohen Z., 2008, Microbial and algal oils, Lipid Technology, 20, 7, 155-160.

Ratledge C., Single cell oils – have they a biotechnological future?, Trends in biotechnology, 1993, 11(7), 278-284.

Ratledge C., 1994, Yeasts, moulds, algae and bacteria as sources of lipids. In: Kamel BS, Kakuda Y (eds) Technological advances in improved and alternative sources of lipids. Blackie, London, 235-291.

Subramaniam R., Dufreche S., Zappi M., Bajpai R., 2010, Microbial lipids from renewable resources: production and characterization, J. Ind. Microbiol. Biotechnol., 37, 1271-1287.

Gouda M.K., Omar S.H., Aouad L.M.,2008, Single cell oil production by Gordonia sp DG using agro-industrial wastes, World J. Microbiol.Biotechnol., 24, 1703-1711.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

FORMATION OF AEROBIC GRANULES IN SEQUENCING BATCH REACTOR SBR TREATING DAIRY INDUSTRY WASTEWATER

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Abstract

Many recent studies in the field of wastewater treatment and environmental protection have focused their attention on the possibility of obtaining aerobic granular sludge in order to develop new innovative wastewater treatment technologies. Compared to conventional activated sludge wastewater treatment plants, aerobic granular technology represent a novel alternative offering numerous advantages such as high biomass retention, good settling ability and simultaneous removal of organic load and nutrients. The main focus of research was to evaluate granules formation and evolution of treatment performances. Two lab scale sequencing batch bioreactors were used in the experiment. The first bioreactor (D) was inoculated with conventional activated sludge while the other one (GM) was inoculated with crushed aerobic granular sludge. Both bioreactors were fed with dairy industry wastewater with high organic and nutrients load (CODCr=1723 – 3550 mg O_2/L, BOD_5 = 492 – 1806 mgO_2/L; NH_4^+= 64,6 - 114 mg/L, P tot = 5,04 – 21,5 mg/L). The first granular structures were observed after 5 days (10 treatment cycles) with a diameter of 67 to 556 µm in D bioreactor and with 392 to 1200 µm in GM bioreactor. By the end of the experiment the granules in D bioreactor reached 2 mm in diameter while the granules in GM bioreactor reached up to 4 mm in diameter. Treatment performances increased along with the growth of granules size.

Key words: *aerobic granular sludge, dairy wastewater, SBR.*

INTRODUCTION

Granular sludge technology is one of the great achievements in environmental biotechnology of the twentieth century, and was first observed in a anaerobic upflow sludge blanket (UASB) reactor designed to treat industrial wastewater at the end of the 1970's (Lettinga et al., 1980). However, the concept of aerobic granular sludge appeared later on - in the 1990's when Mishima et al. (1991) reported the first aerobic granular sludge in an aerobic upflow sludge blanket reactor treating municipal wastewater. Later researches on biofilm structure and on the role of storage polymers (extracellular polymeric substances - EPS) on biofilm formation lead to the idea of growing aerobic granules without carrier material on readily biodegradable substrates in Sequencing Batch Reactor (SBR) (van Loosdrecht, 1997). The light and dispersed flocs are washed out gradually, while the denser sludge particles are retained and accumulated through a repetitive selection in SRB operations, leading to the formation of compact granules. In these aerobic reactors, it was proven to be possible to grow stable granular sludge with integrated simultaneous COD and nitrogen removal capacity. Since that time, SBR has been intensively used by researchers worldwide to develop and understand the concept and mechanism of aerobic granulation (Liu et al., 2004) and to evaluate the performances and practical potential application of this technology. Microbial granules can be considered as dense microbial aggregates. According to Liu et al. (2004), aerobic granular sludge can be defined as an enormous metropolis of microbes containing millions of

individual bacteria due to microbial granulation. Almost all aerobic granular sludge has been obtained and cultivated using sequencing batch reactors (SBRs) (Li et al., 2008, Jang et al., 2003) and has been used to treat high-strength wastewaters containing organics, nitrogen, phosphorous and toxic substances (Adav et al., 2008, Jiang et al., 2004) The granulation process can be affected by a number of parameters, such as seed sludge, substrate composition, organic loading rate, feeding strategy, reactor design, settling time, exchange ratio, and aeration intensity (hydrodynamic shear force).

MATERIALS AND METHODS

The experiments were conducted in two identical column type SBR reactors with a height to diameter ratio of 10 and a total working volume of 8 L in order to evaluate the possibility of forming aerobic granules starting from different inoculum and to evaluate the evolution of treatment performances during startup and steady state conditions. Each of the SBR reactors, as it can be seen in the shematic representation of the AGSBR (figure 1) consisted of: influent vessel (60 L), feeding pump (Heidolph, PUMPDRIVE 5001, peristaltic pump), effluent vessel (60 L) and effluent withdrawal pump (Heidolph, PUMPDRIVE 5001, peristaltic pump). The cyclic operation of the SBR systems was ensured by a Programable Logic Controller (PLC) which controlled the feeding pumps and air inlet and effluent outlet electrovalves. Both bioreactors had identical operational time sequence: anaerobic feeding (45 min.), aerobic reaction (11 h), settling (5min.) and effluent withdrawal (10 min.). During aerobic reaction stage, an air compressor supplied each column at an airflow of 4 L/min. As settling time is an important hydrodynamic selection pressure operational parameter on the microbial community in the bioreactor, a short settling time was preferred and used to allow the selection and growth of fast settling bacteria and the wash out of the sludge with poor settleability. At startup, the two bioreactors used in the experiment were inoculated as follows: 1st bioreactor (D) was inoculated with 5 g/L of conventional activated sludge sampled from a municipal wastewater treatment plant while the 2nd bioreactor (GM) was inoculated with crushed and sieved (0.5 mm) aerobic sludge granules. The granules used were sampled from another lab scale working AGSBR. The idea was to evaluate how fast they recover the granular structure and treatment performances. Both bioreactors were fed with dairy industry wastewater characterized by high organic and nutrients load as shown in table 1.

Table 1. Main quality parameters of the influent

Parameter	Concentration range
CODCr mg O_2/L	1723 – 3550
BOD_5 mg O_2/L	492 – 1806
NH_4^+ mg/L	64.6 – 114
Ntot mg/L	64 – 162
P tot mg/L	5.04 – 21.5

Treatment performances were evaluated based on COD, NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}. COD was analyzed volumetrically based on potassium dichromate method according to the ISO standard (SR ISO 6060:1996) and using heating mantle (Model KI16, Gerhardt, Germany). NH_4^+, NO_2^- and NO_3^- were determined according to the SR EN ISO 14911:2003 and SR EN ISO 10304/1:2009 standards (for the last two indicators), respectively, using ion chromatography system ICS-3000 (Dionex, USA). The granules formation and growth evolution were monitored by particle size analyses carried out using Malvern, Mastersizer S2600 and by microscopic investigation (trinocular Optech microscope and trinocular Motic stereomicroscope with built-in cameras).

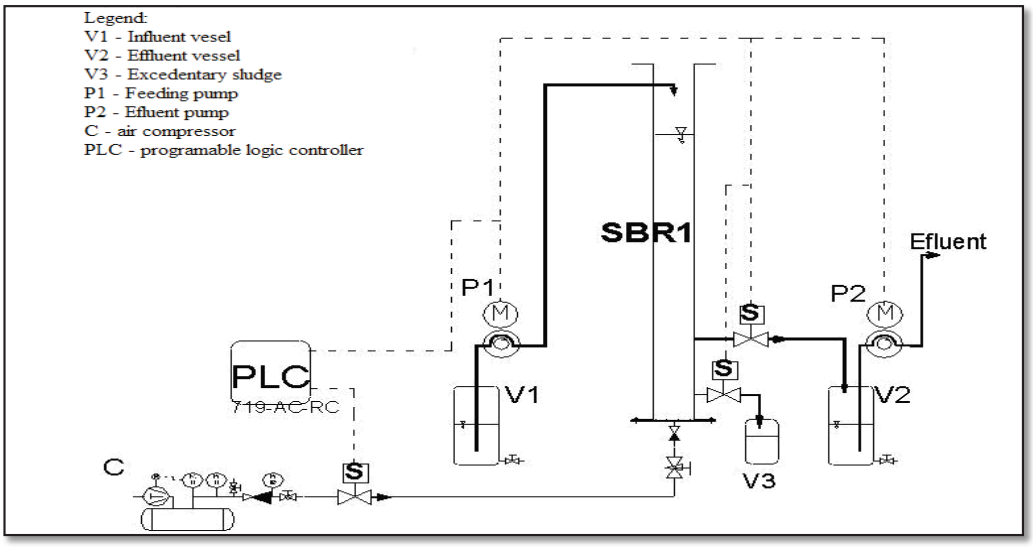

Figure 1. Schematic representation of the aerobic granular sludge SBR

RESULTS AND DISCUSSIONS

In case of the 1st bioreactor (D) inoculated with conventional activated sludge, the first granular structures were observed after 5 days (10 treatment cycles) and continuously increased up to 2 mm in diameter after 22 days (figure 2, and 3).

Microscopic investigations emphasized the tendency of flocs to adhere to each other to form granules and implicitly to grow in diameter. Thus, in figure 2 (a) representing the inoculum we can observe dispersed activated sludge flocs while in 2 (b) and 2 (c) we can observe that the flocs are more compacted and granules are being formed so that after 26 days, the sludge in the bioreactor is under the form of granules with diameter of up to 2 mm (figure 3).

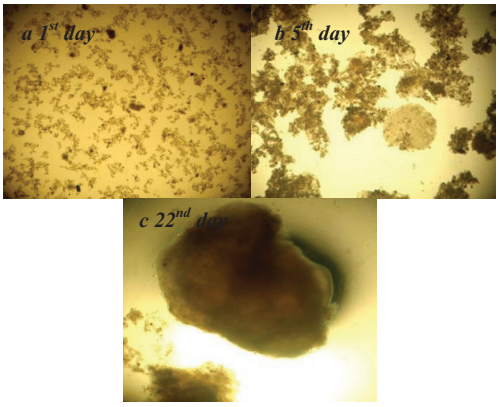

Figure 2. The evolution in time of aerobic sludge granules (microscopic images 4X)

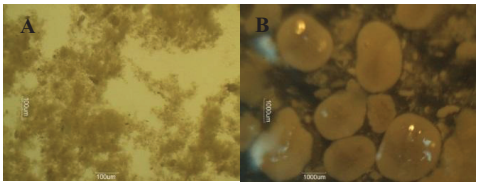

Figure 3. Stereomicroscopic images (10X): A – inoculum –conventional sludge; B – granules 0,1-2 mm (after 26 days)

In case of the second bioreactor (GM) which was inoculated with crushed and sieved granules, compared to the granules obtained in bioreactor D (round shaped and smooth surface) within the same period of time and under the same operational conditions, the granules formed in bioreactor GM had irregular shape variable size but compact structure. (figure 4)

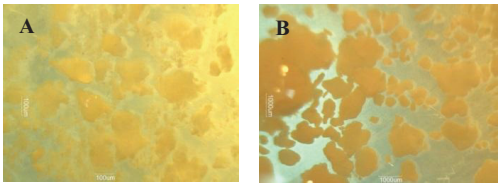

Figure 4. Stereomicroscopic images: A – inoculum – crushed aerobic granular sludge (40X); B – sludge granules (10X) ~0,5-1,6 mm (after 26 days)

Once with the increase of size granules the sludge settling speed and biomass concentration in the bioreactors increased leading to good treatment performances considering the nutrient and organic load of the influent, the total hidraulic retention time of 12 hours and the fact that it is only a one step process (aerobic). The treatment performances obtained in the two experimental SRB reactors, D and GM, are presented comparatively in table 2.

Table 2. Comparative presentation of the treatment performances in both reactors

Parameter	Bioreactor D	Bioreactor GM
CODCr	91 – 95 %	70 - 91 %
BOD_5	93 – 97 %	75 - 93 %
NH_4^+	94 – 99 %	82 - 94 %
P tot	65 – 93 %	65 – 90 %
Ntotal	48 – 81 %	50 – 80 %

Treatment efficiency was higher in D bioreactor than in GM bioreactor. This can be explained by lower specific surface area of the granules and lower diffusion gradients of nutrients within the granules.

CONCLUSIONS

The focus of the research was to evaluate the granules formation and performances evolution during startup and steady state conditions. In both cases the first granules formation were observed after 5 days of inoculation. Even though the two bioreactors used in the experiment underwent the same operational conditions GM bioreactor, inoculated with crushed granular sludge, has shown slightly lower treatment performances during start-up compared to D bioreactor, inoculated with conventional activated sludge. The aerobic granular sludge proved to be stable and adaptable to high nutrients concentrations succeding to efficiently remove the organic load and nutrients from the influent wastewater.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

REFERENCES

Adav SS, Lee DJ, Lai JY., 2008, Intergeneric coaggregation of strains isolated from phenol degrading aerobic granules. Appl Microbiol Biotechnol, 79, 657–661.

Jiang HL, Tay JH, Liu Y., 2003, Ca2+ augmentation for enhancement of aerobically grown microbial granules in sludge blanket reactor. Biotechnol Lett, 25, 95–103.

Jiang HL, Tay JH, Tay STL., 2004, Changes in structure, activity and metabolism of aerobic granules as a microbial response to high phenol loading. Appl Microbiol Biotechnol, 63, 602–608.

Lettinga G., van Velsen AFM, Hosma SW, de Zeeuw W, Klapwijk A., 1980, Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol Bioeng, 22, 699-734.

Li Y, Liu Y, Hai LX., 2008, Is sludge retention time a decisive factor for aerobic granulation in SBR? Bioresour Technol, 99, 7672–7677.

Liu Y, Tay JH., 2004, State of the art of biogranulation technology for wastewater treatment. Biotechnol Adv, 22, 533-563

van Loosdrecht, M.C.M., Pot, M.A. and Heijnen, J.J., 1997, Importance of bacterial storage polymers in bioprocesses. Wat. Sci. Technol., 35(1), 41–47.

Mishima K, Nakamura M., 1991, Self immobilization of aerobic activated sludge – a pilot study of the process in municipal sewage treatment, Water Sci Technol, 23, 981-990.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

DISCOLOURING AND BIOREMEDIATION OF SYNTHETIC TEXTILE DYES BY WASTEWATER MICROBIAL ISOLATES

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Abstract

Fungal biomass has the ability to decolorize textile industry wastewaters by a series of bio-accumulation/bio-sorption mechanisms, posing an economically promising, eco-friendly and feasible alternative to conventional methods. The present study explored the qualitative potential of previously isolated fungal strains from samples of post-finishing textile wastewater in treatment of synthetic dyes solutions based on Bemacid azo-dyes (red, yellow and blue). The analyses were carried out on solid nutritive media with 200g/L dye concentration, for 9 microbial strains. Previously isolated fungal strains were identified by ITS-RFLP method. Qualitative assessments revealed the increased tolerance of microbial isolates to textile azo-dyes, and their capacity to degrade into non-colored intermediates.

Key words: *textile dyes, wastewater, fungi, bioremediation.*

INTRODUCTION

There is a real actual concern regarding dyestuff effluents entering ecosystems, with both national and international regulations for industrial wastewater requiring significant elimination of dyestuff content from the effluents. None of actual effluent treatment methods are sufficiently effective in disposing of typical dyestuff, modern techniques including chemical methods (coagulation / flocculation, activated carbon, adsorption and bio-treatment, ozonation, sodium hypochlorite treatment, photochemical decolourization).

Textile dyes are usually designed to withstand microbiological fading. Usually, dyes concentrations range from 10 to 200 mg/L, for intense coloration. Textile dyes have the tendency to bio-accumulate in living organisms, sustained by their slow degradation. Wastewater recycling represents a viable solution to increase textile industry technologies sustainability, reducing pollutants impact on the environment (X. Lu al., 2010). Textile industry is one of the largest water consumers, due to numerous operations involved in textile processing and finishing, which involve high volumes of water, resulting in dynamic polluting vectors (C. Allegre al., 2006). More than 25% of wastewater resulted from textile and paper industry activities are being released into main water effluents (Shaul al., 1991).Industrial effluents contamination leads to inevitable alteration of pH value, increases biochemical oxygen demand (BOD) and chemical oxygen demand (COD), and leads to intense colorations (Dutta al., 2002; Fang al., 2004; Asad al., 2007), leading to water sources that pose toxic, mutagenic and/or carcinogenic threat for water microbial populations and animals (Gunasekaran al., 2006).

Azo-dyes represent a large class of synthetic dyes used in textile industry, with more that 50% of commercial dyes used in industry belonging to this class. Azo-dyes contain nitrogen as the azo group −N=N− as part of their molecular structures. Most azo dyes contain only one azo group, but some contain two (disazo), three (trisazo) or more. In some conditions, azo-dyes can change structure, resulting in carcinogenic and allergenic aromatic amines. Conventional wastewater

treatment methods, such as coagulation, filtration and chemical flocculation (Gogate al., 2004), based on physical-chemical principles are expensive methods which lead to additional disposable compounds, compared to microbial degradation and bioremediation, with lower costs and environmental impact (Verma al., 2003; Mendez-Paz al., 2005; Pandey al., 2007). Bioremediation of azo-dyes contaminated wastewater can be possible with the use of bacterial strains, such as: *Bacillus cereus, Pseudomonas putida, Pseudomonas fluorescence* (Khehra al., 2005), *Pseudomonas desmolyticum* and *Bacillus sp.*(Kalme al., 2009).

MATERIALS AND METHODS

Fungal isolates

Fungal strains used in this study were previously isolated from a source of wastewater resulted from textile treating and finishing stages. The isolates were plated on 4 synthetic medias: Potato-Dextrose-Agar (PDA), Sabouraud-Agar, Malt-Agar (MA), Czapek-Dox. A total of 9 strains were used in the experiments, from which 8 strains were unknown, and subjected for identification, and 1 collection strain of *Aspergillus niger* IMI 45551.

DNA extraction and PCR fingerprinting

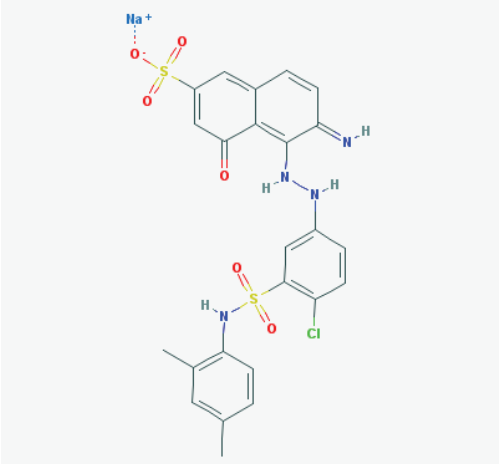
DNA isolation from microbioal strains (new isolates and collection strains) was performed using the method of Raeder and Broda (1985) with slight modification (the cells lysis was optimized by using glass beads and a Mini-Beadbeater BioSpec). For DNA amplification primers for the ITS region of the nuclear small-subunit rRNA gene were used: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC)(White et al., 1990). The PCR reactions were performed in a total volume of 25 μL, containing 1x standard PCR incubation buffer, 0.5 μM of each primer, 0.2 mM of each of the four dNTPs, 0.025 Uμl^{-1} of DreamTaq polymerase and 30 ng genomic DNA. The PCR conditions are: 94°C for 45 sec, annealing at 55.5°C for 45 sec, 60 sec elongation at 72°C and final extension of 10 min at 72°C in 34 cycles. A negative control with all the reaction mixtures except the DNA template was included with each set of the PCR amplification Reactions (Kamala et al., 2015). For restriction fragment analysis, PCR products were cleaved with five restriction enzymes, namely HaeIII, HinfI, HhaI, MseI and SduI., in 16 μL reaction mixtures consisting of: 10x buffer (2.0 μL), enzyme (1 μL), PCR product (10 μL), and pure water (3 μL). Reaction mixtures of HaeIII, SduI, HinfI and HhaI enzymes were incubated for 2 h at 37°C, and 65°C for MseI. The restriction DNA fragments were separated in 2.0% (w/v) agarose gel electrophoresis, and the visualized in UV light after staining with ethidium bromide.

Nutritive substrate

Synthetic nutritive media was used for qualitative assessment of fungal isolates efficiency, with the following composition: 1g/L K_2HPO_4, 0.01g/L $ZnSO_4$, 0.05g/L $CuSO_4$, 0.5g/L $MgSO_4$, 0.01g/L $FeSO_4$, 0.5g/L KCl, 3g/L $NaNO_3$, 10g/L glucose and 20g/L of agar. The media was sterilized for 15' at 121^{o}C, with post sterilization pH value of 5.5.

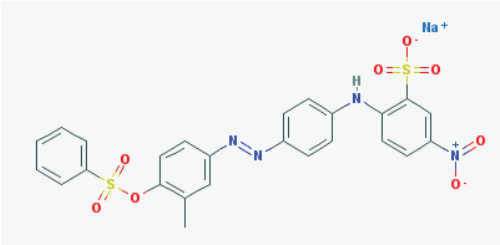
Textile dyes

3 industrial textile Bemacid acid dyes from Bezema, for polyamide and wool were tested: Bemacid ROT N-TF (Figure 1), Bemacid GELB N-TF (Figure 2) and BEMACID BLAU N-TF (Figure 3). Used dye concentration was of 200mg/mL for each dye.



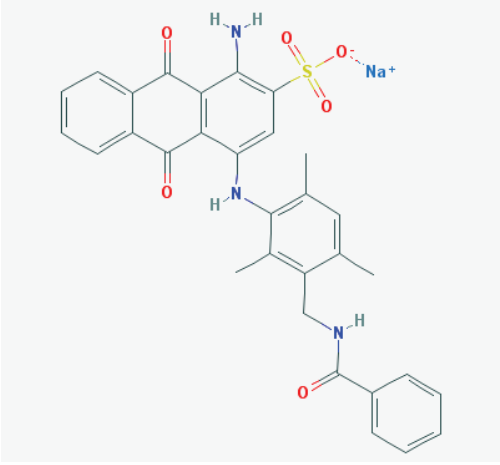
sodium 6-amino-5-((4-chloro-3-(((2,4-dimethylphenyl)amino)sulphonyl)phenyl)azo)-4-hydroxynaphthalene-2-sulphonate

Figure 1. Bemacid Rot N-TF structure



sodium 6-amino-5-((4-chloro-3-(((2,4-dimethylphenyl)amino)sulphonyl)phenyl)azo)-4-hydroxynaphthalene-2-sulphonate

Figure 2. Bemacid Gelb N-TF structure



sodium 1-amino-4-({3-[(benzoylamino)methyl]-2,4,6-trimethylphenyl}amino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate

Figure 3. Bemacid BLAU N-TF structure

RESULTS AND DISCUSSIONS

In this study, it was evaluated the discoloring potential of 9 fungal isolates when used against Bemacid dyes, immobilized in solid nutritive media. Each dye was used in concentration of 200mg/L, and the fungal strains were inoculated in the middle of the plates, and incubated for 16 days, in darkness, room temperature. After the incubation period, the Petri plates were visualized for signs of decolorization and/or dye bioaccumulation by the microbial biomass (Figure 6, 7 and 8)

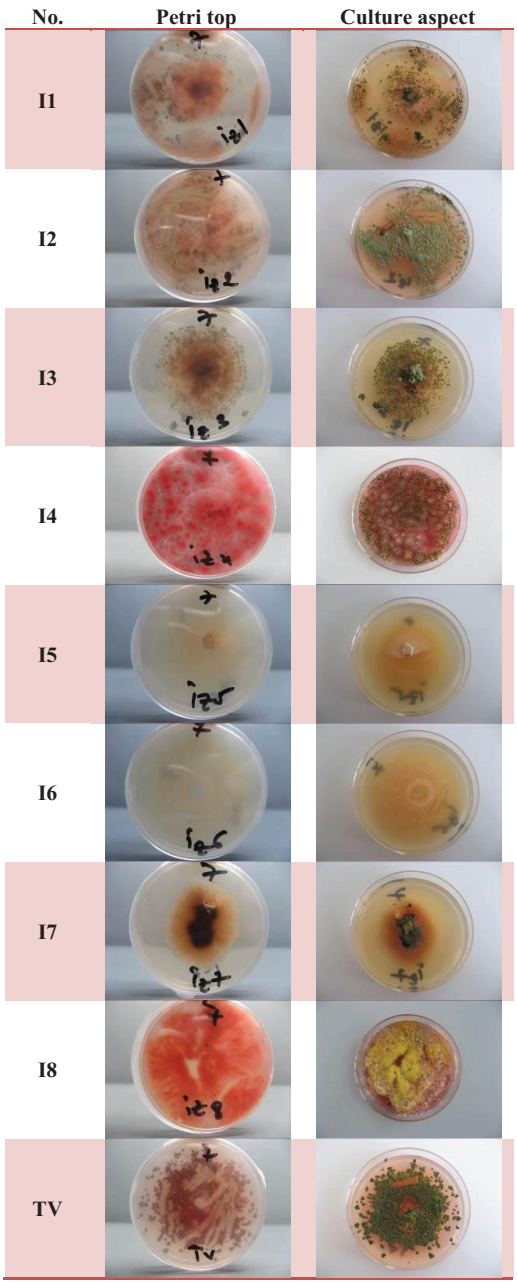


Figure 6. Bemacid ROT vizual discoloration

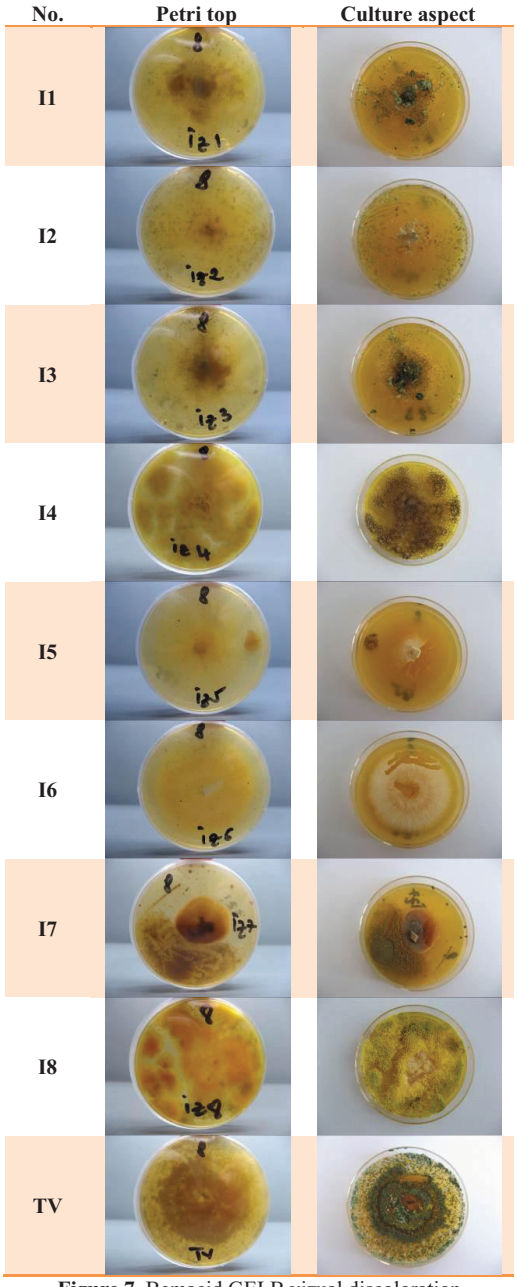


Figure 7. Bemacid GELB vizual discoloration

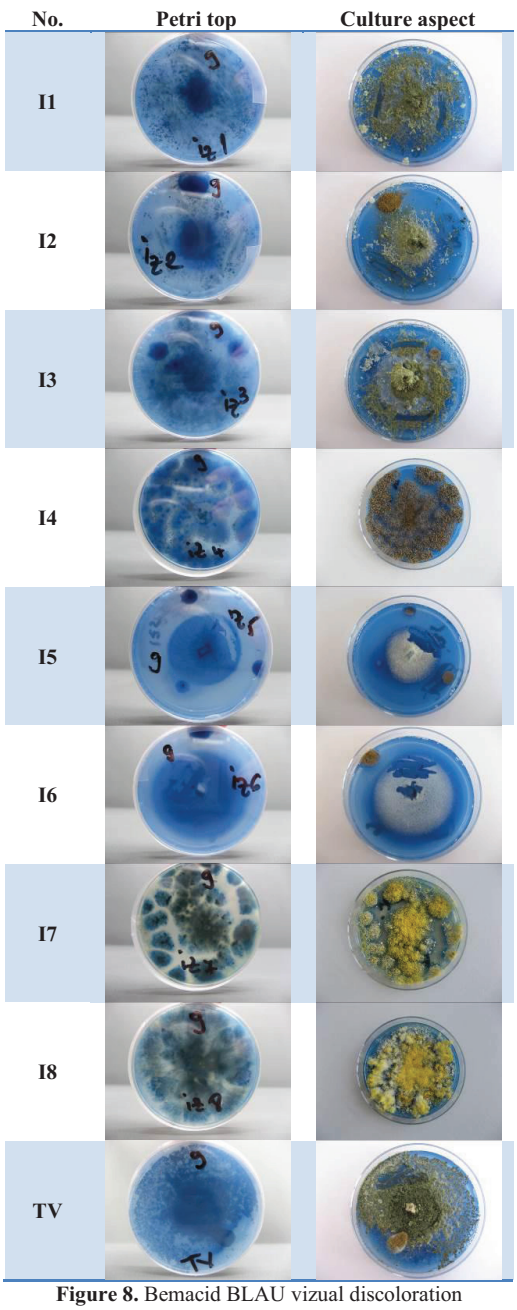


Figure 8. Bemacid BLAU vizual discoloration

Molecular identification

A total of 8 microbial strains, new isolates and collection strains were used in experiments for species identification by ITS-RFLP method. Differences in the length of the amplicons obtained after PCR with primers for ITS1/ITS4 regions were detected after agarose gel electrophoresis (Figure 4).

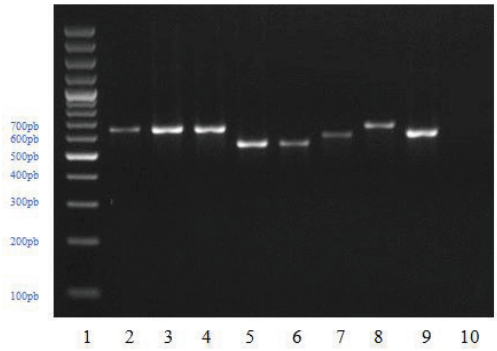


Figure 4. Amplicons obtained with ITS1/ITS4 primers. 1= ladder DNA; 2 – fungal strain 1; 3 = fungal strain 2; 4 = fungal strain 3; 5 = *Polyporus squamosus*; 6 = *Fusarium oxysporum*; 7 = yeast strain 7; 8 = yeast strain 8; 9 = *Trichoderma spp*.; 10 = no DNA

In order to differentiate the strains, restriction analysis of the PCR fragments was conducted, comparing with the data from literature. Comparing the isolates 7 and 8, the restriction profiles (Table 1) were different comparing with several yeast strains used as reference (*Sacharomyces cerevisiae, Yarrowia lipolytica, Metschnikowia pulcherima, Rhodotorula spp.*) (data not shown) and didn't allowed the identification of the species.

Table 1. Restriction profile of the isolates 7 and 8

Strains	Restriction fragments length (bp)		
	*Hha*I	*Hae*III	*Hinf*I
7	100+230+300	210+400	80+210+340
8	70+100+160+200+220	50+190+320	70+140+190+340

When the comparison was realized between the strains 1, 2, 3 and TV (*Trichoderma spp*), the restriction profiles with the enzymes HhaI, SduI and Hae III were similar for the strains 1-3 and with small differences for TV strain (Figure 5).

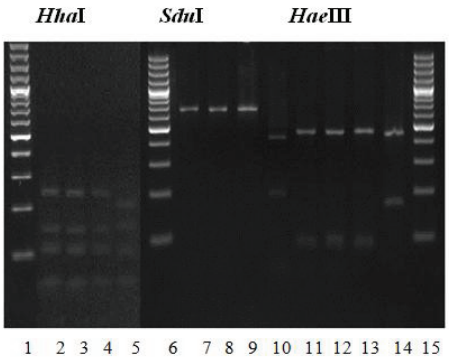


Figure 5. Electrophoretic profile of the restriction fragments obtained with HhaI, SduI and Hae III restriction enzymes. 1, 6, 15 – ladder DNA; 2, 7, 11 – fungal strain 1; 3, 8, 12 – fungal strains 2; 4, 9, 13 – fungal strain 3; 5, 10, 14 - TV

Based on data from literature it is possible that the new isolates could belong to *T. parceramosum / T. reesei / T. longi* group, and TV strain to *Trichoderma atroviride* (Table 2) but further analysis, including the sequencing of PCR products are necessary for confirmation.

Table 2. Restriction profile of the isolates 1, 2, 3 and TV

Strains	Restriction fragments length (bp)		
	*Hha*I	*Sdu*I	*Hae*III
1	110+150+250	630	70+90+480
2	110+150+250	630	70+90+480
3	110+150+250	630	70+90+480
TV	110+160+210	190+430	170+450

Clear identification was obtained for the strains *Fusarium oxysporum* and *Polyporus squamosus* using ITS-RFLP analysis with *Sdu*I, *Hae*III, and *Mse*I restriction enzymes (Table 3).

Table 3. Restriction profile of the strains of *F.oxysporum* (6) and *Polyporus squamosum* (5)

Strain	Restriction fragments length (bp)		
	*Sdu*I	*Hae*III	*Mse*I
5	540	90+110+340	540
6	540	90+110+340	50+90+370

CONCLUSIONS

The results of the present study indicate that the tested strains can yield well to great discolouring potential. Either by enzymatic cleavage of textile azo-dyes into non-coloured intermediates, or by bioaccumulation, the microbial

strains show effective decolourization of solid media immobilized dyes.

The molecular techniques are valuable for genetic analysis of microbial strains and could allow the identification of the species. However, standard strains are necessary in order to perform a precise analysis.

Future work will be concentrated on quantitative assessment of degradation of azo-dyes with the use of fungal isolates, in liquid media, and spectrophotometric dosage of reduction rates.

ACKNOWLEDGEMENTS

This work was financed by Operational Program Human Resources Development 2007-2013, project POSDRU/159/1.5/S/ 132765 using European Social Fund.

REFERENCES

Allegre, C., P. Moulin, M. Maisseu and F. Charbit, J. Membrane Sci., 269, 15 (2006).

Asad, S., Amoozegar, M.A., Pourbabaee, A.A., Sarbolouki, M.N., Dastgheib, S.M., 2007. Decolorization of textile dyes by newly isolated halophilic and halotolerant bacteria. Bioresource Technol. 98, 2082–2088.

Dutta, K., Bhattacharjee, S., Chaudhuri, B., Mukopadhyay, S., 2002. Chemical oxidation of C.I. Reactive red 2 using Fenton like reactions. J. Environ. Monit. 4, 754–760.

Fang, H., Wenrong, H., Yuezhong, L., 2004. Biodegradation mechanisms and kinetics of azo dye 4BS by a microbial consortium. Chemosphere 57 (4), 293–301.

Gogate, P.R., Pandit, A.B., 2004. A review of imperative technologies for wastewater treatment II: Hybrid Methods. Adv. Environ. Res. 8,553–597.

Gunasekaran, P., Puvaneswari, N., Muthukrishnan, J., 2006. Toxicity Assessment and microbial degradation of azo dyes. Indian J. Exp. Biol. 44, 618–626.

Kalme, S., Jadhav, S., Jadhav, M., Govindwar, S., 2009. Textile dye degrading laccase from Pseudomonas desmolyticumNCIM 2112. Enzyme Microb. Tech. 44, 65–71.

Kamala,T., Indira Devi, S., Chandradev Sharma, K., Kennedy, K., 2015, Phylogeny and Taxonomical Investigation of Trichoderma spp. from Indian Region of Indo-Burma Biodiversity Hot Spot Region with Special Reference to Manipur, BioMed Research International Article ID 285261.

Khehra, M.S., Saini, H.S., Sharma, D.K., Chadha, B.S., Chimni, S.S., 2005. Decolorization of various azo dyes by bacterial consortium. Dyes Pigments. 67 (1), 55–61.

Lu, X., L. Liu, R. Liu, J. Chen, 2010, Desalination, 258, 229.

Mendez-Paz, D., Omil, F., Lema, J.M., 2005. Anaerobic treatment of azo dye Acid Orange 7 under fed-batch and continuous conditions.Water Res. 39 (5), 771–778.

Pandey, A., Singh, P., Iyengar, L., 2007. Bacterial decolorization and degradation of azo dyes. Int. Biodeter. Biodegr. 59, 73–84.

Raeder U., Broda P., 1985, Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1: 17-20

Shaul GM, Holdsworth TJ, Dempsey CR, Dostal KA., 1991. Fate of water soluble azo dyes in the activated sludge process. Chemosphere, 22: 107-119.

Verma, P., Madamwar, D., 2003. Decolorization of synthetic dyes by a newly isolated strain of Serratia maerascens. World J. Microb. Biot. 19, 615–618.

White, T.J. T. Bruns, S. Lee, J. Taylor, 1990, "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics: PCR protocols," in A Guide to Methods and Applications, pp. 315–322.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BIOLOGICAL WASTE WATER TREATMENT: 1. MONITORING METABOLIC ACTIVITY OF ACTIVATED SLUDGE AND THE CHEMICAL PARAMETERS OF WASTE WATER TREATMENT.

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Abstract

In this paper there are present the results concerning chemical parameters and the rate of metabolic activity of activated sludge in Constanta Nord waste water plant. Special emphasis is focused on time evolution of nitrates, ammonium, phosphorus concentrations and chemical oxygen demand in inlet, waste water and outlet, purified water. These results are discussed in correlation with metabolic activity of active sludge microbiota measured as the rate of resazurine reduction, and microscopic images obtained both in bright field and in epifluorescence microscopy. The results open *inter alia* the possibility to use the rapid method of resazurine reduction (15-20 minutes) to assess the activity of activated sludge microbiota and to correlate it with standardized, but time-consuming (hours to 5 days) methods, thus increasing the possibility to take good operational decisions in shorter time.

Key words: *waste water, chemical oxygen demand, activated sludge, resazurine reduction, Gram and Neisser stains, SYTOX Green.*

INTRODUCTION

There is an increase in water shortage around the world so great attention is paid to the reuse of wastewater; spent municipal and industrial wastewaters being treated in plants often by an activated sludge process. (Ardern and Lockett,1923; Grady and Lim, 1980; Vaicum, 1981;Negulescu, 1985; Arceivala, 1988; Bitton, 1999; Cheremisino, 2002; Burton and Stensel, 2003).Wastewater treatment is a complex biotechnological activity comprising mechanical processes (screening, sedimentation, filtration, or flotation), chemical processes (disinfection, adsorption, or precipitation) and biological processes involving microbial activity which are responsible for organic matter degradation and removal of nutrients. (Hanel, 1988; Metcvaff and Eddy, 1997;Vaicum, 1981; Templeton and Butler, 2001). Waste water treatment plants are very complex ecological systems built up by mankind, based on the knowledge concerning degradation processes occurring both in natural ecosystems and in natural ecosystems with strong anthropic impact (Godeanu 1973; Curds and Hawkes, 1983; Guterstam, 1996; Mitsch, 1997; Todd, 1997; Gray, 2002; Godeanu, 2013). The aim of this paper is to monitor sludge with respect to both its rate of metabolic activity (rapidly measured as resazurine reduction) and to the outcome of this activity, the decrease in chemical oxygen demand and removal of nutrients during waste water treatment at Constanța North waste water treatment plant.

MATERIALS AND METHODS

Samples were taken form Constanta Nord waste water plant at the following dates: the 13th of June 2014 (I), the 16th of June 2014 (II), the 21st of June 2014 (III), the 28th of June 2014 (IV), the 7th of July 2014, the 11th of July 2014 (VI), the 25th of July 2014 (VII), the 1st of August 2014 (VIII), the 29th of

January 2015 (IX), the 5th of February 2015 (X), and the 14th of February 2015 (XI). Constanța North waste water treatment plant was built in 1959 and further developed, the new plant complying with the EU directives and the Romanian standards (Presură et al., 2014).

Chemical analysis were performed according to standard procedures: chemical oxygen demand (STASS SR ISO 6060), ammonium (STASS SR ISO 7150-1), phosphorus (STASS SR EN 1189) nitrate (STASS SR ISO 7890-1) and dry solids in activated sludge (STASS SR EN 12880).

Quantitative determination of resazurin reduction

Resazurin (10 -oxide7-hydroxy-3H-phenoxazin-3-one) is a blue non-fluorescent dye; it can be reduced to resorfine (pink and highly fluorescent), which is further reduced to hydroresorufina (colorless and nonfluorescent). Resazurine was used primarily as an indicator of oxidation-reduction reactions in cell viability tests. The test is used from many years for monitoring bacterial contamination of milk (Ramsdell et al,1935; Noyer and Cambell, 1963). Recently resazurine became very popular as a simple and versatile method for measuring cell proliferation and cytotoxicity both in prokaryotes and eukaryotes, in different types of samples (Perony and Rossi,1986; Larson, 1997; Al-Nasiry et al., 2007;Duarte et al., 2009; Guerin et al., 2001). Conversion of resazurin to fluorescent resorufin is proportional to the number of metabolically active and viable cells present in a population. Calculation of reduced resazurin was done following standard protocol as previously shown (Ghiță et al., 2013).

Microscopic investigation were done with Zeiss microscope, Gram and Neisser staining being done as shown in Spellman (1999).

RESULTS AND DISCUSSIONS

In figure 1 there are presented some chemical parameters (phosphorus, ammonium, nitrates concentrations and chemical oxygen demand) in inlet, waste water and in outlet, purified water.

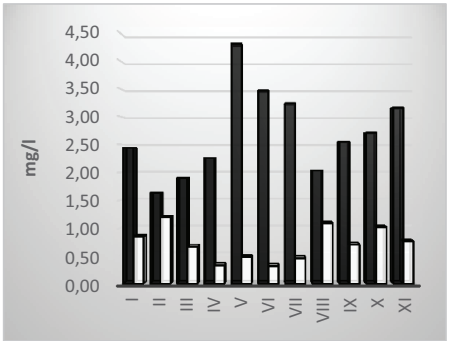


Figure 1A Time evolution of phosphorus concentration in waste water (dark columns) and in purified water (light columns). Roman numbers indicate different times of sampling (see material and methods)

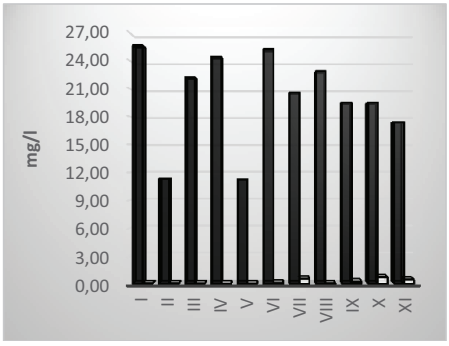


Figure 1 B Time evolution of ammonium concentration in input waste water (dark columns) and in effluent, purified water (light columns). Roman numbers indicate different times of sampling (see material and methods)

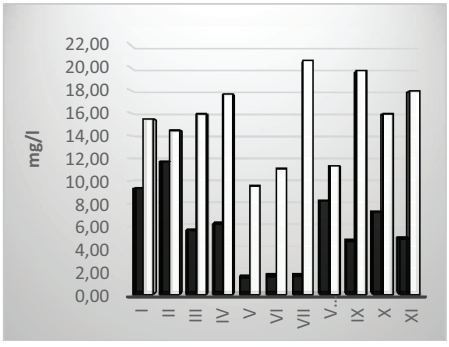


Figure 1 C Time evolution of nitrate concentration in input waste water (dark columns) and in effluent, purified water (light columns)

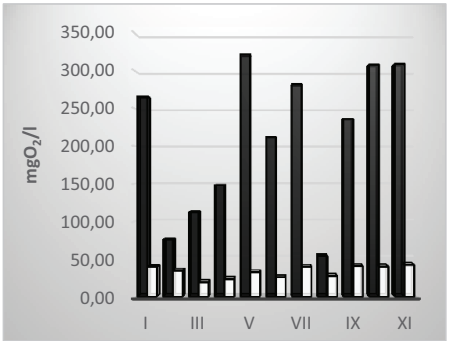
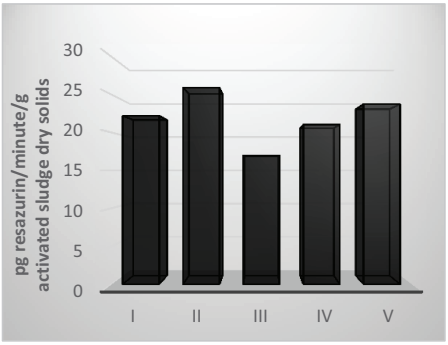


Figure 1 D Time evolution of COD in input waste water (dark columns) and in purified water (white columns).

As one can see in figure 1 A, B and D there is a strong decrease in concentrations of pollutants in effluent as compared with input, waste water; the only exception is the concentration of nitrate (Fig. 1 C) which is under increase, as a consequence of biological oxidations during waste water treatment.

In figure 2 there are presented the rate of resazurine reduction within the experimental period. When resazurine is reduced it is transformed in resurfine which has a strong red fluorescence this fluorescence can be used as a label to see metabolically active bacteria in microscopic preparations (Fig. 2 B). These microscopic pictures of activated sludge could be further correlated with image analysis as already shown for other experimental systems (Armaselu et al., 2011; Sarchizian et al., 2011). Image analysis opens the great opportunity to quantify the metabolic activity at the level of each individual microorganism, either unicelular or filamentous, which can be further correlated with photometric measurements of resazurine reduction by suspension of cells.



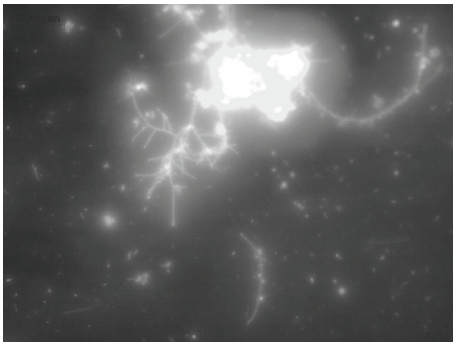


Figure 2 Resazurine reduction by activated sludge. A. The rate of resazurine reduction expressed as pg reduced resazurine/min/mg active sludge (dry weight).B microscopic images (epifluorescence) of cells and filaments containing resourfine produced by reduction of resazurine.

As one can see in figure 2 there are relatively large differences in the ability of active sludge to reduce resazurine when expressed on dry biomass, showing that the metabolic rate of activated sludge is not constant from one sampling time to another, besides its concentrations are maintained relatively constant in time (figure 3).

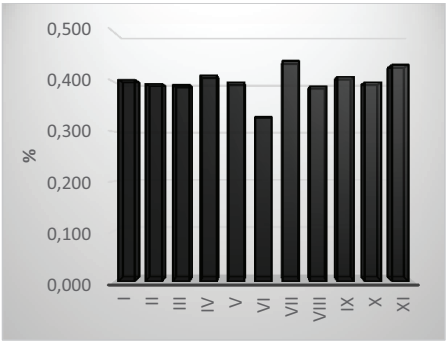


Figure 3 Time evolution of activated sludge dry weight concentration.

These experiments were initiated because the study of the ability of activated sludge to reduce artificial electron acceptors, including resazurine, is a method to a measure metabolic activity (Liu D. 1983; Bensaid et al 2000; McCluskey, et al., 2005; McNicholl et al., 2007). Liu (1983) showed that, the use of resazurine to measure dehydrogenase activity of activated sludge gave good correlations with important parameters such as oxygen consumption and heterotrophic bacterial count, both in the pilot plant and in the laboratory simulations.

Furthermore, the sensitivity of resazurine reduction against changes in nutrient levels or toxic substances recommed it as a tool both for routine plant monitoring and for research in activated sludge activity, as well (Liu, 1983). Bensaid et al (2000) showed that XTT, a tetrazolium salt can be used as a tool for estimating the activity of the electron transport system in activated sludge They found that the production of formazan by reduction of XTT is depended on the initial concentration of the XTT and is proportional to live cell biomass; furthermore, oxygen uptake rate and XTT reduction rate were highly correlated and indicated significant variations depending on the growth conditions (Bensaid et al., 2000). McNicholl and colab (2007) developed a rapid, robust and cost-effective method of assaying the metabolic activity of the biomass of activated sludge plants based on the redox dye resazurin, in which levels of reduction of the dye are proportional to cell biomass and respiration rate.

In figure 4 there are presented microscopic investigations of activated sludge in aerobic and anaerobic bioreactor concerning Gram reaction and Neisser reaction , which are the most used staining procedures for activated sludge Grady and Lim, 1980; Bitton, 1999; Cheremisino, 2002; Burton and Stensel, 2003). For the sick of simplicity, in this paper there are presented microscopic images concerning only one sampling time; qualitatively speaking, there are no microscopic differences between the samples collected at different times.

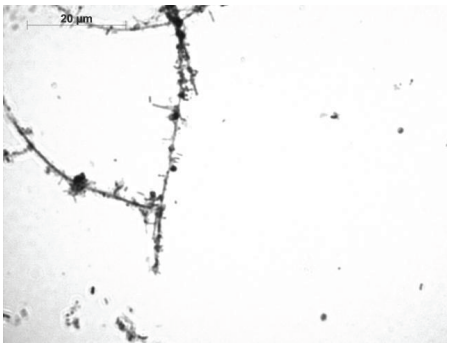


Figure 4 A Gram staining of activated sludge from aerobic tank.

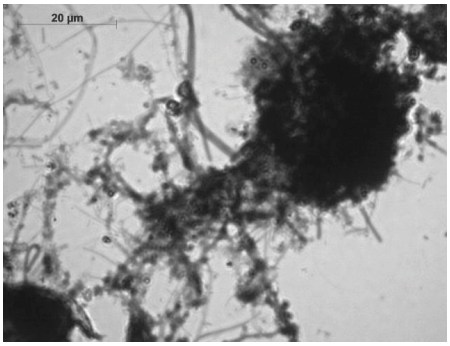


Figure 4 B Neisser staining of activated sludge from aerobic tank

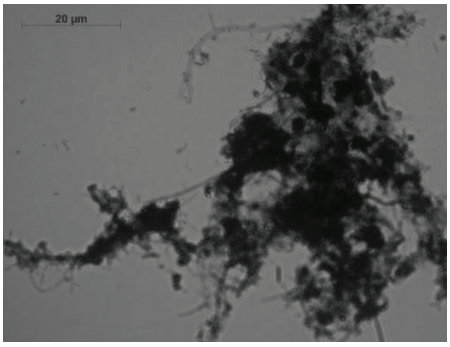


Figure 4 C Gram staining of activated sludge from anaerobic tank

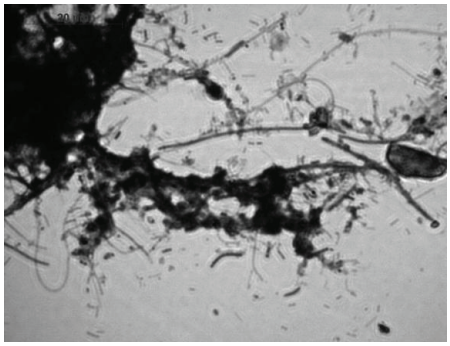


Figure 4 D Neisser staining of activated sludge from anaerobic tank

As one can see in figure 4, qualitatively speacking, there is a dominance of Gram-negative filamentous bacteria both in the aerobic tank and in the anaerobic one (A and C, respectively) and a dominance of Neisser pozitive filamentous bacteria (B and D, respectively). These qualitative results are in agreement with reports in the literature concerning the microbiota of activated sludge under normal operational parameters (Grady and Lim, 1980; Eikelboom and van Buijsen,1981; Arceivala, 1988; Droste, 1997; Bitton, 1999; Cheremisino, 2002; Burton and Stensel, 2003).

The visualization of living cells is an important task in biotechnology. When it comes to activated sludge, the use of already classical cocktail of SYBR Green and propidium iodide is not useful because the activated sludge has a strong red fluorescence (results not shown) that interfere with the fluorescence of propidium iodide, thus making impossible the specific label of dead cells by this fluorochrome. However, dead cells can be labeled by SYTOX green (Roth et al., 1997) which has a green fluorescence. In figure 5 and 6 there are presented microscopic images in bright field (A) and in epifluorescence (B) showing the same microscopic field for aerobic and anaerobic activated sludge, respectively

As one can see in figure 5 and 6 there are some microorganisms (either isolated cells and filamentous bacteria) which are permeable to SYTOX Green, thus considered as dead cells.

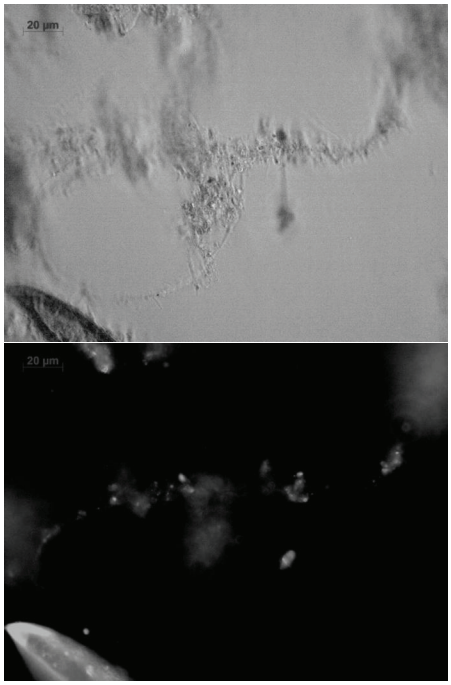


Figure 5 Microscopic images of aerobic activated sludge: A) bright field and B) fluorescence signal of dead cells labeled by SYTOX green.

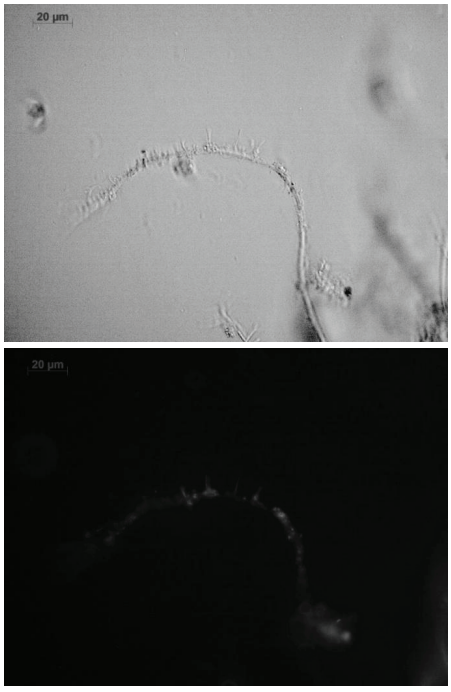


Figure 6 Microscopic images of anaerobic activated sludge: a) bright field and b) fluorescence signal of dead cells labeled by SYTOX green.

CONCLUSIONS

Chemical parameters of the waste water plant are in agreement with National and EU legislation. Qualitative microscope inspection (Gram character and Neisser reaction) show a healthy activated sludge and quantitative determination of the metabolic activity show different intensities of resazurine reduction, suggesting that the activity of activated sludge is variable in time. The qualitative presence of dead cells is seen using the fluorochrome SYTOX Green.

FUTURE PROSPECTS

The use of quantitative image analysis a powerful tool in microbiology to quantify mainly the occurrence of Gram-positive and Gram–negative bacteria, the presence of volutine-containing bacteria and the characterization of flocs (Eikelboom and van Buijsen,1981; Droste, 1997).

A better understanding of the correlation bewteen metabolic activity of activated sludge measured with resazurine or other artificial electron acceptor and/or by other methods e.g respirometry (Walker and Davis,1977) and both operational chemical parameters and microscopic parameters of the activated sludge from aerobic tank, anaerobic tank and re-circulating tank.

A deeper understanding of the overall process would open the possibility for sludge population optimization : i) selecting the most desirable species for a specific function; ii) controlling the growth of unwanted or undesirable microorganisms in the system and iii) optimizing microbial properties, as originally proposed by Yuan and Blackall (2002).

Furthermore, understanding waste water treatment plant not only as an (bio)industrial unit but also as a very complex ecological system in which microbiota strongly interacts with multicellular macroorgansims (Godeanu 1973; Grady and Lim, 1980; Vaicum, 1981; Curds and Hawkes,1983; Arceivala, 1988; Guterstam, 1996; Mitsch, 1997; Todd , 1997; Bitton, 1999; Cheremisino, 2002; Gray, 2002; Burton and Stensel, 2003; Godeanu, 2013) could help the biotechnological professionals to increase the performances of the overal process, friendly for the environment.

ACKNOWLEDGEMENTS

Thanks are due to Mr. Felix STROE (General Manager at S.C. RAJA S.A.), Mr. Aurel PRESURA (Deputy General Manager at S.C. RAJA S.A.), Mr. Nicolae JIANU (Head of Wastewater Laboratory at S.C. RAJA S.A.) for kind professional support to MCI.

REFERENCES

Al-Nasiry S., N.Geusens, M.Hanssens, C.Luyten and R.Pijnenborg 2007. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. Human Reproduction, 22(5):1304–1309.

Arceivala J.S., 1988. Wastewater treatment for pollution control, 2nd Ed. Tata McGraw-Hill Publishing Co., New Delhi.

Ardern E., Lockett W.T., 1923. Activated Sludge Process. Withington Works. J. Soc. Chem. Ind. 225-230.

Armaselu A., Popescu A., Apostol I., Ardelean I., Damian V., Iordache I., Sarchizian I., Apostol D., 2011. Passive nonspecific labeling of cyanobacteria in natural samples using quantum dots. Optoelectronics and Advanced Materials-rapid communications, 5(10): 1084-1090.

Bensaid A., Thierie J., M Penninckx, 2000. The use of the tetrazolium salt XTT for the estimation of biological activity of activated sludge cultivated under steady-state and transient regimes. Journal of microbiological methods, 40 (3): 255–226

Bitton G., 1999. Wastewater Microbiology. Wiley-Liss, New York.

Burton G., Stensel F.L., 2003. Engineering, Treatment, Disposal and Reuse. Tata McGraw-Hill Publishing Company, New Delhi, India.

Cheremisino N.P., 2002. Handbook of water and wastewater treatment technologies. Butterworth-Heinemann, Boston.

Curds C.R., Hawkes H.A.,1983. Ecological Aspects of Used-Water Treatment. Academic Press, London.

Droste R. L., 1997. Theory and practice of water and wastewater treatment. Wiley, New York.

Duarte M., Giordani R.B., Carli G.A., Zuanazzi J.A., Macedo A.J., 2009. Cytotoxicity of solubilization vehicles for Trichomonas gallinae and Tritrichomonas foetus measured by the resazurin microtiter assay. Vet Parasitol, 166:167-170.

Eikelboom D.H., H.J.J. van Buijsen, 1981. Microscopic Sludge Investigation Manual. TNO Research Institute, The Netherlands.

Ghiță S., Sarchizian I., Ardelean I.I.,2013. Microscopic investigation and automated image analysis of hydrocarbon- tolerant marine cyanobacteria mixed populations cultivated in the absence and presence

of gasoline or diesel. International journal of biology and biomedical engineering, 4(7):164-175
Godeanu S., 1973 Synecological relations in biocenose components in aerotanks. Hidrobiologia, 14: 232-332.
Godeanu S., 2013. Ecologie aplicată. Editura Academiei Române.
Grady C.P.L., Lim H.C., 1980. Biological Waste Treatment: Theory and Applications.
Gray F.N., 2002. Water Technology: An Introduction for Environmental Scientists and Engineers. Butterworth-Heinemann, Oxford.
Guerin T. F., M. Mondido, B. McClenn, Peasley B., 2001. Application of resazurin for estimating abundance of contaminant-degrading microorganisms. Lett. Appl. Microbiol., 32: 340 – 345.
Guterstam B., 1996. Demonstrating ecological engineering for wastewater treatment in a Nordic climate using aquaculture principles in a greenhouse mesocosm. Ecological Engineering, 6 (1-3): 73-97.
Hanel L., 1988. Biological Treatment of Sewage by the Activated Sludge Process. Ellis Horwood, Chichester, U.K.
Larson E.M., Doughman D.J., Gregerson D.S., Obritsch W.F., 1997. A New, Simple, Nonradioactive, Nontoxic In Vitro Assay to Monitor Corneal Endothelial Cell Viability. Investigative Ophthalmology & Visual Science, 38 (10):1930.
Liu D., 1983. Resazurin reduction method for activated sludge process control. Environment Scintific Technology, 17(7): 407-411.
McCluskey C., Quinn, J.P., McGrath, J.W., 2005. An evaluation of three new-generation tetrazolium salts for the measurement of respiratory activity in activated sludge microorganisms. Microb Ecol, 49: 379-387.
McNicholl BP, JW McGrath, JP Quinn, 2007. Development and application of a resazurin-based biomass activity test for activated sludge plant management. Water Research, 41 (1): 127-133
Metcalf X., Eddy X., 1997. Wastewater Engineering: Treatment and Reuse. McGraw Hill, New York.
Mitsch W.J., 1997. Ecological engineering: the roots and rationale of a new ecological paradigm. CRC Press Inc., United States
Moyer, R. H., Campbell J. J. R., 1963. Mechanism of resazurin reduction in milk of low bacterial content. J. Dairy Sci., 46(9): 897–906.
Negulescu M., 1985. Municipal waste water treatment. Ed.Elsevier, Amsterdam, Oxford, New-York, Tokio
Peroni C., Rossi G., 1986. Determination of microbial activity in marine sediments by resazurin reduction. Chem. Ecol., 2(3): 205– 218.
Presură A., Fânaru L., Chichelus E.M., 2014. The Constanta North wastewater treatment plant income generator. Ed. ARA, Urban infrastructure, urban networks, management, 5: 115-125.
Ramsdell G. A., Johnson W. T. Jr., Evans F. R., 1935. Investigation of resazurin as an indicator of the sanitary condition of milk. J. Dairy Sci., 18(11): 705–717.
Roth B.L., Poot M., Yue S.T., Millard P.J.,1997. Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. Appl Environ Microbiol. 63(6):2421-2431.
Sarchizian I., Cîrnu M., Ardelean I.I., 2011. Isolation of cyanobacteria and quantification of their biotechnological potential with respect to redox properties at single cell level. Romanian Biotechnology Journal, 16(6):3-8.
Spellman F.R., 1999. Microbiology for Water/Wastewater Operators. Technomic Publishing Co Inc., Lancaster, USA.
Templeton M.R., Butler D., 2011. An Introduction to Wastewater Treatment.Ventus publishing.
Todd J., 1997. Ecological engineering, living machines, and the visionary landscape. C. Etnier and B. Guterstam (Eds). Ecological engineering for wastewater treatment, 2nd edition. CRC Press Inc.: 113-122.
Vaicum L.M., 1981. Activated sludge wastewater treatment - biochemical bases. Publishing House of the Academy of RS Romania (in Romanian)
Walker, I., M. Davis, 1977. The Relationship Between Viability and Respiration Rate in the Activated Sludge Process. Water Research, 11(7) : 575-578.
Yuan Z., Blackall L., 2002. Sludge population optimisation, a new dimension for the control of biological wastewater treatment systems. Water Res 36(2):482–490
XXX-Determinarea consumului chimic de oxigen STASS SR ISO 6060/1989
XXX-Determinarea continutului de amoniu STASS SR ISO 7150-1/1984
XXX-Determinarea continutului de azotati STASS SR ISO 7890-1/1998
XXX-Determinarea continutului de fosfor STASS SR EN 1189/1996
XXX-Determinarea reziduului uscat si a continutului din namoluri STASS SR EN 12880.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

EFFECTS OF CULTURE MEDIA ON LACCASE PRODUCING WHITE-ROT FUNGI

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Abstract

White rot basidiomycetes are known as producers of several oxidative and hydrolytic enzymes that act together on the degradation of certain components of the plant cell wall. Among these enzymes, laccases represent a family of copper-containing polyphenol oxidases, and can be involved in various processes such as, morphogenesis, pathogenesis, and lignin degradation. Because of their capability to oxidize a wide range of substrates, fungal laccases are currently studied for their potential agricultural, industrial, and medicinal applications. Hence, the aim of this study was to investigate the efficacy of culture media on laccase production by six white rot fungal species: Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var. Florida, and Trametes versicolor. The white-rot fungi were inoculated in PD broth (potato dextrose) and Hwang et al (2008) medium. Extracellular laccase formation by these fungi was recorded by spectrophotometry using guaiacol as substrate. During 30 days of incubation was found that laccase production reached maximum values in the filtrates of both culture media and declined along incubation period. PD medium was optimum for laccase producing white-rot fungi. The maximum laccase activity was obtained from the culture filtrates of Trametes versicolor and Hericium coraloides. The optimal culture medium for laccase producing fungi was PD broth. Also, pH values of the fungal culture media were changed during incubation period. The minimal pH values were recorded in culture filtrates of Laetiporus sulphureus (2.8 and 2.4). The highest laccase activity was detected at pH values between 5.0 and 6.0 in PD medium. A good biomass yield was recorded by Pleurotus ostreatus var. Florida grown on both media tested. It was found that a high production of laccase did not dependent on high biomass yields.

Key words*: laccase activity, guaiacol, macromycetes.*

INTRODUCTION

Laccases (EC 1.10.2.3; benzenediol: oxygen oxidoreductase) are multicopper-containing enzymes, found widely in plants and fungi, especially in white rot fungi which can degrade natural lignin. Laccases can *c*atalyze the oxidation of many aromatic compounds, particularly phenols. Laccase activity has been demonstrated in many fungal species belonging to ascomycetes and basidiomycetes. Among basidiomycetes that produce laccases can be mentioned *Agaricus bisporus* (Wood, 1980), *Pleurotus ostreatus* (Sannia et al., 1986), *Hericium coralloides* (Zou YJ et al., 2012), *Ganoderma lucidum* (Ko et al., 2001) and *Trametes versicolor* (Rogalski et al., 1991). A major role in enzyme activity acts the culture conditions and medium composition. Laccase production by fungi is strongly affected by many parameters like temperature, time of cultivation, stationary or submerged cultures, medium composition (Palmieri et al., 2000). Temperature plays a major role in growth and laccase production of the fungi. In general the most fungi were cultivated at temperatures between 25°C and 30°C for optimal laccase production (Minussi et al., 2007). It was found that the optimal temperature for fruiting body formation and laccase production is 25°C in the presence of light and 30°C for laccase production when the cultures are incubated in the dark (Thurston, 1994). When the temperatures are higher than 30°C, the activity of laccase decreased significantly (Zadrazil et al., 1999). In white-rot fungi laccases are produced as multiple isoenzymes (Solomon et al., 1996). Extracellular laccases production

can be stimulated by a variety of inducing substances especially phenolic compounds related to lignin or lignin derivatives (Hess et al., 2002). *Ganoderma lucidum* produced higher levels of laccases in medium reached in nitrogen and glucose as carbon sources (D'Souza et al., 1999). The aim of the present study was *to investigate the efficacy of culture media on laccase production by six white rot fungal species: Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var. Florida, and Trametes versicolor in submerged cultures.*

MATERIALS AND METHODS

Biological material and culture conditions

Six mushroom species, namely *Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var.* Florida and *Trametes versicolor* were used in this study. The biological material, from the indigenous macromycetes species collection of the Faculty of Biotechnology, was maintained on PDA slants at 4^0C until use. For the experiments the fungal inoculums consisted of 5 mm agar plugs of one week old culture grown on PDA at 25^0C were placed on two variants of liquid media: a PD (potato-dextrose), pH 5.2, and a medium described by Hwang et al. (2008): 20g/l glucose, 2g/l peptone, 2g/l yeast extract, 0,46g/l KH_2PO_4, 1g/l K_2HPO_4, 0.5 g/l $MgSO_4$, pH 6.7. Cultures were incubated at 25^0C in 250 ml Erlenmeyer flasks containing 50 ml of culture medium and agitated at 140 rpm. After 15 and 30 days respectively the samples were analysed for the biomass production, pH and laccase activities in the fungal filtrates. The fungal culture was filtered with Whatman No. 1 filter paper and used as enzyme source. Mycelium, collected and dried at 70°C for 48 h, was used for determination of biomass.

Determination of laccase activity

Laccase activity was determined according Savitha et al. (2011) protocol using the guaiacol as substrate. The reaction mixture contained 3 ml sodium acetate buffer (10 mM, pH 5.0), 1ml guaiacol (2 mM) and 1 ml enzyme source. The mixture was incubated at 30^0C for 15 min. The changes in absorbance due the oxidation of guaiacol in the reaction mixture were recorded by spectrophotometer at 450 nm. One unit of enzyme activity is defined as the amount of enzyme that oxidized 1 µmol of guaiacol per minute. The laccase activity (U/ml) is calculated by the formula: U/ml = (A x Vt x dilution factor) / (t x € x Vs) where A = absorbance at 450nm, Vt = total volume of reaction mixture (ml), Vs = enzyme volume (ml), t = incubation time (min) and € = extinction coefficient of guaiacol at 450nm (12,100 $M^{-1}cm^{-1}$) (Savitha et al., 2011).

RESULTS AND DISCUSSIONS

Basidiomycete isolates of *Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var.* Florida and *Trametes versicolor*, were investigated for the maximum biomass achieving and laccase production in submerged conditions. The experiments were performed using two different culture media: PD and Hwang liquid medium. After 30 days of incubation, the highest fungal biomass quantity was recorded by *Pleurotus ostreatus* var. Florida in the Hwang medium (0.50 g), followed by PD medium (0.48 g) (Figure 1).

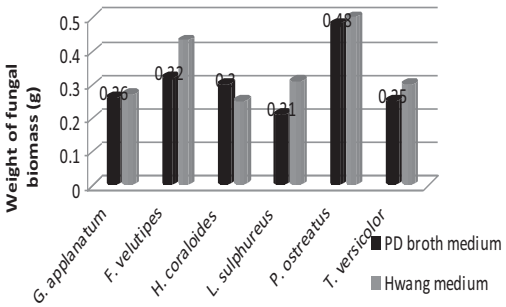

Figure1. Fungal biomass grown on different media after 30 days of incubation

Moreover, Hwang medium offered the optimum chemical composition for mycelium development in all the white-rot fungi tested excepting *Hericium coralloides*: in this case the best biomass production was obtained in PD medium (0.30 g).

During fungal mycelium development in the culture broths (Hwang and PD), pH changes occurred (Figures 2 and 3). Very low pH values were detected in *Laetiporus sulphurous* culture filtrate: pH 2.4 in PD filtrate and pH 2.5 in

Hwang filtrate. These values were closely maintained during the entire period of incubation. Low pH values were also recorded in *Ganoderma applanatum* culture filtrate (pH 4.1 in PD, and pH 3.8 in Hwang medium) and in *Trametes versicolor* culture filtrate (pH 4.2 in Hwang medium) after 15 days of cultivation. Higher pH values were observed in culture filtrated of *F.velutipes* and *P.ostreatus* (Figure 2).

After 30 days of cultivation the pH values were comparable with those after 15 day for *H. coraloides, T. versicolor, P. ostreatus, L. sulfureus* but only in Hwang mediu. For PD medium, the pH values of culture filtrates were generally higher (Figures 3). The highest pH were detected in *F.velutipes* in both media, and in *T.versicolor* cultivated in PD.

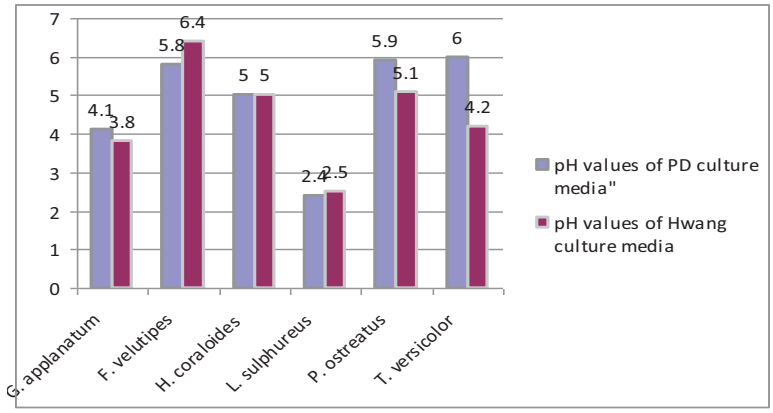

Figure 2. pH changes in the fungal filtrates 15 days after

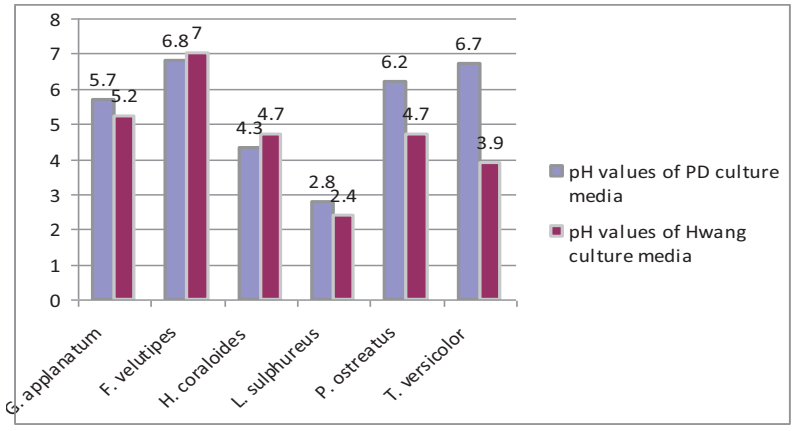

Figure 3. pH changes in the fungal filtrates 30 days after

Regarding the laccase activity, differences among the fungal species cultivated in PD and Hwang liquid media were registered. After 15 days of cultivation, laccase activities were detected in both types of culture filtrates (PD and Hwang medium), with different values depending on fungal strains.

However, PD medium was optimum for laccase producing white-rot fungi. After 15 days of incubation in this medium, *Hericium coraloides* and *Trametes versicolor* presented the highest laccase activities (29.48 U/ml and 17.99 U/ml respectively) (Figures 4).

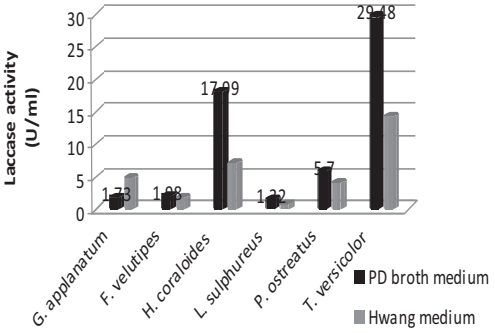

Figure 4. Laccase activity in the fungal filtrates after 15 days of incubation

After 30 days of cultivation the laccase activity decreased, but the best producers remain *H.coraloides* and *T.versicolor*: 12.54 U/ml and 11.07 U/ml, respectively (figure 5). The reduction of the activities was with 27% for *H.coraloides* and more than 60% for *T.versicolor* and could be due to inhibitory compounds released by aged mycelium.

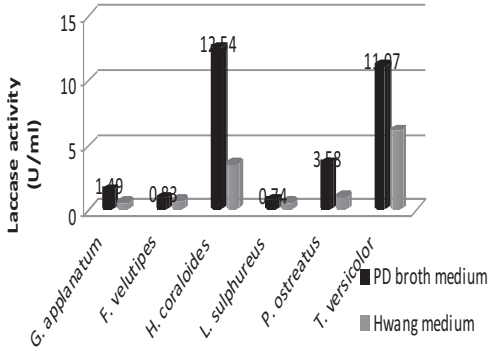

Figure 5. Laccase activity in the fungal filtrates after 30 days of incubation

It was reported that a high production of laccase is not dependent on high biomass yields (Praveen et al., 2011), but the medium formula could influence the enzymatic level (Palmieri et al., 2000; Xavier et al., 2001). Moreover, it was found that laccase production is closely related to the cultivation conditions of the fungi (Praveen et al., 2011; Heinzkill et al., 1998). Among the factors that influence the laccase activity, pH value seem to be very important. Some authors indicated initial pH levels set between 4 and 6 before inoculation, but the pH was not controlled during cultivation period (Vasconcelos et al., 2000; Arora and Gill, 2000). The normal range of pH for typical laccase activity is between 3 and 5, at least for some fungal species (Savitha et al., 2011).

The results obtained in our experiments indicated a correlation between pH value and laccase production: laccase production from the *Trametes versicolor* and *Hericium coraloides* had highest values when the culture filtrate had pH values between 5 and 6 (in PD medium). Similar aspects were observed for *P.ostreatus* cultivated in PD medium. When Hwang medium was used for cultivation, pH had lowest values, and the level of enzymatic activity was also reduced. These results suggested that the medium composition is another factor that influenced the enzymatic activity. However, no correlation between the biomass weight and laccase activity was established.

It is obvious that enhancement of laccase production through optimization of nutritional and physiological conditions during cultivation of white-rot fungi is very important for their utilization at industrial scale (Dhakar and Pandey, 2013). In this order, besides nutritional supplements, some inducers such as organic solvents and metal ions also play important role in production of laccases (Shraddha et al., 2011, Brijwani et al., 2010).

CONCLUSIONS

Indigenous macromycetes isolates of *Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var.* Florida and *Trametes versicolor* were tested for fungal biomass production, pH and laccase production under submerged conditions. The effect of media composition on mycelium growth and laccase activity was also investigated.

During the incubation period was found that laccase production reached maximum values in the filtrates of both culture media after 15 days and significantly decreased after 30 days of incubation. PD medium offered the best nutritional condition for laccase production in almost all the white-rot fungi tested.

Among all mushroom species tested, *Trametes versicolor* and *Hericium coraloides* presented the highest level of laccase activity (29.48 U/ml and 17.99 U/ml).

The influence of pH variation during cultivation on laccase activity was also evaluated. In was shown that the laccase production from the *Trametes versicolor* and *Hericium coraloides* was optimum when the culture media had pH 5-6. No correlation between high production of laccase and high biomass yields was established. Due to the potential biotechnological applications of laccases in various industrial processes and in remediation of soil and contaminated effluents, further studies will be focused on optimization the conditions for enzyme production and recovery.

ACKNOWLEDGEMENTS

This work was made through the "Partnerships in priority areas - PN II" research program, project no. 174/2014, developed with the support of MEN - UEFISCDI.

REFERENCES

Arora, D. S., Gill, P. K., 2000. Laccase production by some white rot fungi under different nutritional conditions. Bioresource Technology, vol. 73, no. 3, pp. 283–285.

Brijwani, K., Rigdon, A., Vadlani, P. V., 2010. Fungal laccases: production, function, and applications in food processing. Enzyme Research, vol. 2010, pg. 1-10.

Dhakar, K., Pandey, A., 2013. Laccase Production from a Temperature and pH Tolerant Fungal Strain of Trametes hirsuta (MTCC 11397). Enzyme Research, vol. 2013, pg. 1-9.

D'Souza, T. M., Merritt, C. S., Reddy, C. A., 1999. "Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*," *Appl. Environ. Microbiol.*, vol. 65, no.12, pp. 5307–5313.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P., Anke, T., 1998. Characterization of laccases and peroxidases from woodrotting fungi (family *Coprinaceae*). *Appl. Environ. Microbiol.*, vol. 64, no. 5, pp. 1601–1606

Hess, J., Leitner, C., Galhaup, C., et al., 2002. Enhanced Formation of Extracellular Laccase Activity by the White Rot Fungus *Trametes multicolour*. *Appl. Biochem. Biotechnol*, 98-100, pp 229 – 241.

Hwang, H.S., Lee, S.H., Baek, Y.M., Kim, S.W., Jeong, Y.K., Yun, J.W., 2008. Production of extracellular polysaccharides by submerged mycelial culture of *L. sulphureus* var. miniatus and their insulinotropic properties. Appl. Microbiol. Biotech.78: 419-429.

Ko, E.-M., Leem, Y.-E., Choi, H. T., 2001. Purification and characterization of laccase isozymes fromthe white-rot basidiomycete *Ganoderma lucidum*. *Appl. Microbiol. Biotechnol.*, vol. 57, no. 1-2, pp. 98–102.

Minussi, R. C., Miranda, M. A., Silva, J. A. et al., 2007. Purification, characterization and application of laccase from Trametes versicolor for colour and phenolic removal of olive mill wastewater in the presence of 1-hydroxybenzotriazole. African J. Biotechnology, vol. 6, no. 10, pp. 1248–1254.

Palmieri, G., Giardina, P., Bianco, C., et al., 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol., vol. 66, no. 3, pp. 920–924.

Praveen, K., Viswanath, B., Usha, K. Y. et al., 2011. Lignolytic enzymes of a mushroom *Stereum ostrea* isolated from wood logs. *Enzyme Research*, vol. 2011, Article ID 749518.

Rogalski, J., Lundell, T., Leonowicz, A., Hatakka, A., 1991.Production of laccase, lignin peroxidase and manganese-dependent peroxidase by various strains of *Trametes versicolor* depending on culture conditions. *Acta Microbiologica Polonica*, vol. 40, pp. 221–234.

Sannia, G., P. Giardina, M. Luna, 1986. Laccase from *Pleurotus ostreatus*. *Biotechnology Letters*, vol. 8, no. 11, pp. 797–800.

Savitha S. D., Gururaj B. T., Nityanand, A.C. et al., 2011. Isolation of laccase producing fungi and partial characterization of laccase. Biotechnol. Bioinf. Bioeng., 1(4):543-549.

Shraddha, R. Shekher, S. Sehgal, M. Kamthania, Kumar, A., 2011. Laccase: microbial sources, production, purification, and potential biotechnological applications, Enzyme Research, vol. 2011, pg. 1-11.

Solomon, E.I., Sundaram, U.M., Machonkin, T.E., 1996. Multicopper Oxidases and Oxygenases. Chem Rev. Nov 7; 96 (7), 2563-2606.

Thurston, C. F., 1994.The structure and function of fungal laccases. Microbiology, 140 (1), pp. 19–26.

Vasconcelos, A.F.D., Barbosa, A. M. R., Dekker, F. H., Scarminio,I. S.,Rezende, M. I., 2000. Optimization of laccase production by Botryosphaeria sp. in the presence of veratryl alcohol by the response-surface method. Process Biochemistry, 35 (10), pp. 1131–1138.

Wood, D. A., 1980.Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J. Gen. Microbiol.*, vol. 117, pp. 327–338, 1980.

Xavier, A. M. R. B., Evtuguin, D. V., Ferreira, R. M. P., Amado, F. L., 2001. Laccase production for lignin oxidative activity, in *Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry*, pp. 4–8, Helsinki, Finland, June 2001.

Zadrazil, F., Gonser, A., Lang, E., 1999. Influence of incubation temperature on the secretion of extracellular ligninolytic enzymes of *Pleurotus* sp. And *Dichomitus* squalens into soil, in Proceedings of the Conference on Enzymes in the Environment: Activity, Ecology and Applications, pp. 12–16, Granada, Spain, July1999.

Zou, Y.J., Wang, H.X, Ng TB, Huang, C.Y., Zhang, J.X., 2012. Purification and characterization of a novel laccase from the edible mushroom *Hericium coralloides*. J Microbiol.,50 (1):72-8.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

VARIATION OF METHANE YIELD IN A BIOGAS PLANT BY USING DIFFERENT SUBSTRATES

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Abstract

Many biogas systems are too small to handle the available supply of substrates. Knowing the quality and retention time at which maximum methane is produced will help in selecting the mixture of best substrates during co-digestion. In such manner, there will be no need for any increment of infrastructure to raise the capacity of a Biogas plant.
The Biogas in an Anaerobic Digestion Plant is produced from different substrates which vary in terms of quantities and quality. Approximately 55% methane and 45% carbon dioxide is issued alongside of other gases which makes difficult the prognoses of methane production for transforming it into electricity and feeding it to grid in order by optimizing the substrates fed on the fermenter.
The objective of this study is to assess the variation of methane and carbon dioxide yield with methane production, retention time and different quality substrates in a biogas plant.
A good productivity of methane (52,5% by biogas volume) has been obtained from a mixture of corn silage and rye, occurred on the 3rd day of digestion. Additional treatment with 4% fats and 2% leftovers of the total daily feeding substrates produced even higher methane. The production of methane was hindered by the presence of some trace gases as hydrogen sulfide which limited the methane producing bacteria.

Key words*: anaerobic digestion, methane, substrates, hydrogen sulphide.*

INTRODUCTION

Biogas is a mixture of methane and carbon dioxide, produced by the breakdown of organic waste by bacteria without oxygen (anaerobic digestion) [1]. Production of methane-rich biogas through anaerobic digestion of organic materials provides a versatile carrier of renewable energy, as methane can be used in replacement for fossil fuels in both heat and power generation and as a vehicle fuel, thus contributing to cutting down the emissions of greenhouse gases and slowing down the climate change.
Anaerobic degradation or digestion involves the breakdown of biomass by a concerted action of a wide range of microorganisms in the absence of oxygen. The general principle of anaerobic digestion is the degradation of organic materials (e.g. carbohydrates, protein and fats) under anaerobic condition, where bacteria convert the organic material to methane, carbon dioxide and water. Acetate is the most important intermediate for methane biosynthesis in the anaerobic environment; it forms approximately 55% of the methane, while the remaining 45% of methane is formed directly from hydrogen and carbon dioxide
Ideally Biogas production would benefit from a consistent mix of feedstock materials, chopped and blended to ensure optimum methane yield, mostly coming from agriculture as energetic plants.
In practice, Anaerobic Digestion (AD) may also take into consideration a consistent proportion of food waste. This is a largely beneficial to environment; however as a single feedstock, agriculture products can present challenges because of the continuous rising market price and therefore income.
Yield is an overriding consideration for efficient energy substrate. The key is to use a feedstock mix that allows the digestion

process to function effectively and maximize methane output, given the size, layout and capability of the operation.
The digestion rate of different feedstocks [2,3] within a bio-digester varies from 2 days to two months. Material that has a high level of sugar or starch is quicker to ferment than feedstocks which have more lignin or cellulose (Figure 1) [1].

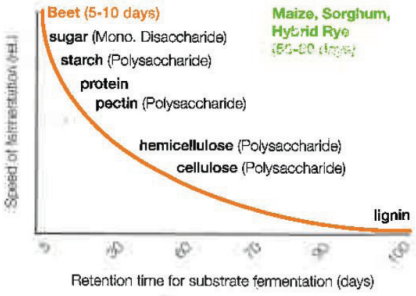

Figure 1. Biogas: relative fermentation characteristics by content

Thuse, substrates like energy beet, used vegetable oil and so will have a shorter retention time[2] in the digester and release more gas over a shorter period of time than wholecrop cereals.
Each individual feedstock component has advantages and disadvantages[5,6]

Table 1. Advantages of different feedstock [5,6]

Feedstock	Advantages	Disadvantages
Mais	- High methane yield/ha - Easy storage and feedout	-Relatively slow retention time
Rye	-High wholecrop yield with high DM -Good in mixture with maize	- Lower methane yield/ha
Manure	Useful starter and mixture product	Low methane output
Waste*	- Pre-treatment necessary - Methane vary much on different type of waste	- Low cost when close to the biogas plant

** to be detailed in the present report*

Table 1 ilustrates some examples of potential mixes in the biogas plant. This shows that, per tone of fresh mass (FM) produced, the most effective mixes for maximum methane yield should comprise maize and some sort of waste[4].
However, the mixture utilization of the traditional agriculture substrates and waste has a positive effect on methane output mostly by keeping the necessary micronutrients of the fermentation process into the required range[4]. As well, care does not need to be taken to ensure that the viscosity of the mix enables good functionality of the plant.
The fast conversion rate of some feedstock helps to grow the gas production, raising the pH inside fermenters plant, encouraging bacterial conversion of the complete feedstock to methane. Furthermore, waste produces a cleaner source of biogas than agriculture feedstock which enables more efficient conversion from methane to electricity&heat trough the Combined heat and power (CHP) unit.
Plant operators will also find that there are significant benefits from the synergies provided by using multiple feedstock types. Such synergies are difficult to quantify and will vary with plant type. However, the experience on biogas plant operations by using different wet or dry substrates proves that are significant benefits in terms of methane production by synergy results.

MATERIALS AND METHODS

In order to highlight the variation of methane yield of the Anaerobic Digestion plant by using different substrates, the following substrates have been used: fish oil, gelatin – pharmaceutical industry, yeast – beverage industry, sludge – waste treatment plant, wheat draff – alcohol industry, sugar beet pulp – sugar industry, corn silage – agriculture.
The methane content of the biogas has been measured by a multi-channel measuring device with an integrated gas conditioning unit for analyzing methane, oxygen, carbon dioxide and hydrogen as well as the concentrations of hydrogen sulfide. The device, SSM 600 Classic, produced by PRONOVA (see picture 2), is designed for both discontinuous and continuous operation for up to four internal measuring points.

Picture 1: SSM 600 Classic PRONOVA

Samples of each mentioned above have been sent to a lab for reveal the potential in terms of biogas and methane as single feedstock source by measuring the pH, biogas yield, [Nl/kg FM], methane yield [Nl/kg FM].
In order to determine the potential of feedstock mixture and benefit of using a combination of feedstock materials, experimental tests have been done at Genesis Biotech Biogas Plant in Romania.
Genesis Biotech Biogas is a 1MWel cogeneration biogas plant, owned by Genesis Biopartner Group and placed in Prahova County, Filipestii de Padure Village.
The plant is daily feeding a quantity of 50 to of biomass, different provenience, agriculture and food industry.
All measured parameters of the test have been registered separately during a biomass feedstock trial and results were implemented in the same objective, in order to rise the productivity of the plant (Picture 1)

Picture 2: Genesis Biotech Biogas Plant

RESULTS AND DISCUSSIONS

The results of the batch test of each substrate sample can be shown in Table 2, below:

Table 2: Biogas/methane yield of feedstock reference substrates

Sample	pH	biogas yield	methane yield	Methane from biogas
	-	Nl/kg FW	Nl/kg FW	%
fish oil	7,58	584	395	68
gelatine - food industry	7,64	245	146	60
Yeast - beverage industry	7,30	89	62	70
sludge - waste treatment plant	7,25	239	117	49
wheat draff	7,21	186	108	58
sugar beet pulp	3,50	110	57	52
Rye	6,9	200	120	60
corn silage	6,80	220	117	53

As shown on Table 2, the methane values vary from 49% to 70% by using different FM substrates but, of course, these substrates cannot ne used as single feedstock, except the corn silage because of the micronutrients content (Cobalt, Cooper, Iron, Manganese, Molybdenum, Selenium – the most important elements[3]) which can only be found on a wide range into corn silage.
Therefore, it is demanded to find the optimum ratio between corn silage and the rest of waste types nominated above.
In the graphics below it can be seen Dthe cumulative biogas/methane yield of the substrates from waste categories which are going to be mixed with corn silage in order to determine the mixture synergy.

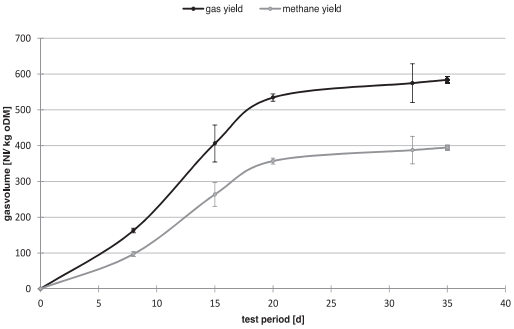

Fig. 2: Dynamics of methane production from fish oil

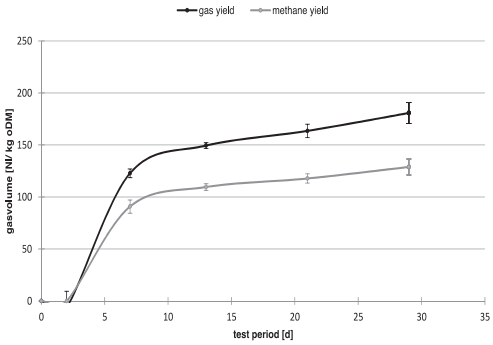

Fig. 5: Dynamics of methane production from sludge

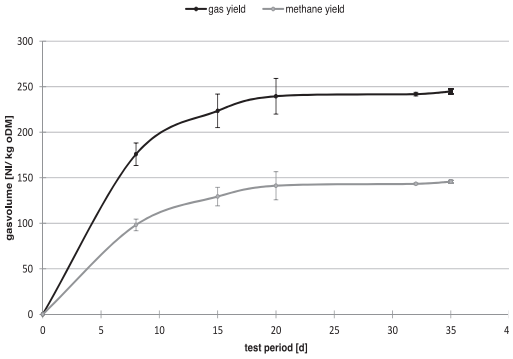

Fig. 3: Dynamics of methane production from gelatin

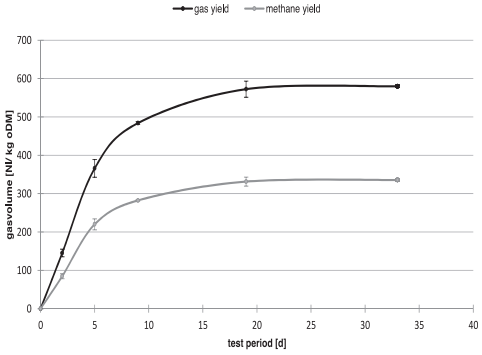

Fig.6: Dynamics of methane production from wheet draff

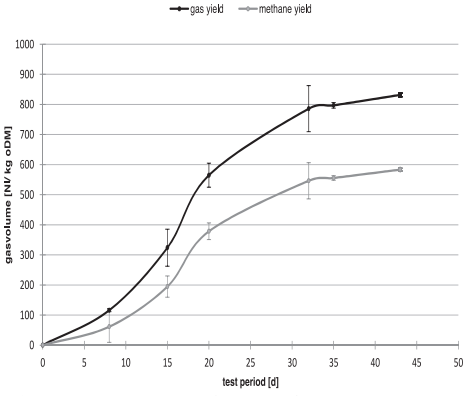

Fig.4: Dynamics of methane production from yeast

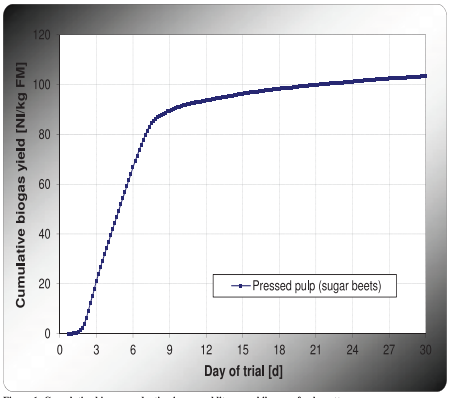

Fig. 7: Development of biogas production – sugar beet pulp

The tests which have been done into the Genesis Biogas Plant revealed some increment of biogas volume as well as methane quantity by mixing them in a certain balance as follows:
Four Mixture types have been tested during 60 days each, performing the following proportion:

Mixture A.
1. Corn silage 88,9%
2. fish oil 3,7%
3. yeast 7,4%

Mixture B.
1. Corn silage 82,5%
2. gelatin 2,5%
3. wheat draff- 15%

Mixture C.
1. Corn silage 72,5%
2. gelatin 2,5%
3. sugar beet pulp - 25%

Mixture D
1. Corn Silage – 75%
2. Rye – 25%

Table 3:Mixture Feedstock to be analyzed

Mixture	biogas yield (theoretic)	biogas yield (measured)	Biogas increment by mixture	Methane yield
	Nl/kg FW	Nl/kg FW	%	%
A	223,7	226,5	1	54,3
B	215	231	7	52,6
C	193	220	14	52,2
D	215	249	15,8	53,5

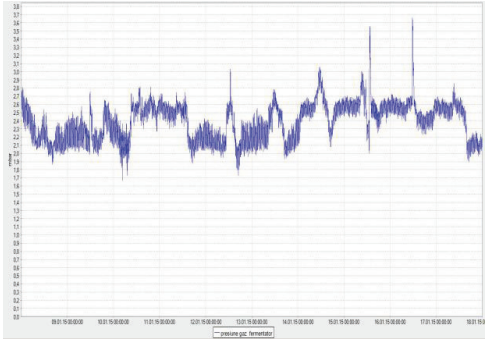

Fig. 8: Feddstock mixture A (corn silage – 88,9%; fish oil – 3,7%; Yeast – 7,4%)

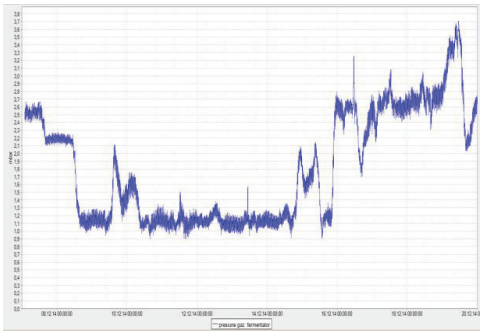

Fig. 9: Feddstock mixture B (corn silage – 82,5%; Gelatine– 2,5%; wheat draff – 15%)

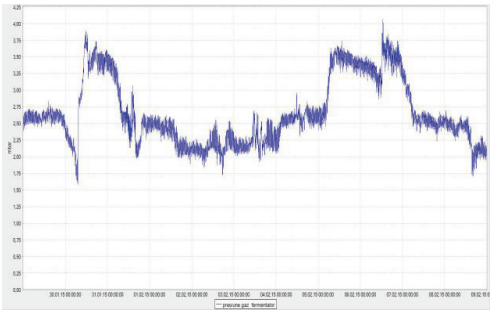

Fig. 10: Feddstock mixture C (corn silage – 72,5%; Gelatine– 2,5%; sugar beet pulp – 25%)

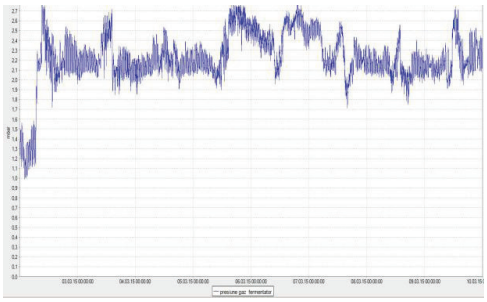

Fig. 11: Feddstock mixture D (corn silage – 75%; Rye– 25%)

CONCLUSIONS

The four test which has been performed on the mentioned feedstock mixtures as shown above, have been performed by replacing the 100% corn silage quantity by an equivalent quantity in terms of biogas calorific power production. The results are as following:
Mixture A: corn silage – 88,9%; fish oil – 3,7%; Yeast – 7,4% - According to Table 3, fig. 8 there is a slight increment on biogas volume of 1% and an improvement of

methane concentration (54,3%) which brings the Cogeneration Heat and Power Central to a better efficiency compared with full corn silage feeding.

Mixture B: corn silage – 82,5%; Gelatine– 2,5%; wheat draff – 15% - According to Table 3, fig. 9 there is a consistent increment on biogas volume of 7% and a decrement of methane concentration (52,6%) which brings the Cogeneration Heat and Power Central to diminish its efficiency compared with full corn silage feeding.

Mixture C: corn silage – 72,5%; Gelatine– 2,5%; sugar beet pulp – 25% - According to Table 3, fig. 10 there is a good increment on biogas volume of 14% and a decrement of methane concentration (52,2%) which brings the Cogeneration Heat and Power Central to diminish its efficiency compared with full corn silage feeding.

Mixture D: corn silage – 75%; Rye– 25% - According to Table 3, fig. 11 there is a very good increment on biogas volume of 15% as well as an increment of methane concentration (53,5%) which brings the Cogeneration Heat and Power Central to diminish its efficiency compared with full corn silage feeding.

As a general conclusion of the present research it can be seen that there are a lot of possibilities to identify the right synergy between mixture feedstock ratio in order to improve the gas output in terms of methane and volume on a AD Biogas plant but what should also be taken into consideration is the cost of waste collection compared with cost of usual feedstock as energetic plants as corn, rye, sorghum, triticale which is strongly dependent by the cost of transport an the biogas gate, not often very cheap.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765

REFERENCES

[1] John Burgess, Simon Witheford. 2013 Biogas in Practice, London, UK

[2] Keymer, U. 2004 Biogasausbeuten verschiedener Substrate, Institut fur Landliche Strukturentwicklung, Betriebswirtschaft und Agrarinformatik, Munchen.

[3] Staubmann, R., Foidl, G., Foidl, N., Gübitz, G.M., Lafferty, R.M., Arbizu, V.M., Steiner, W., 1997. Biogas production from Jatropha curcas press cake.

[4] Lindorfer, H. 2007 Optimised digestion of energy crops and agricultural residues in rural biogas plants. PhD Thesis. University of Natural Resources and Applied Life Sciences, Vienna.

[5] Lindorfer, H. & Ramhold, D. 2008 Possibilities for increasing efficiency in the anaerobic digestion of organic wastes. In: Proceedings Waste-to-Energy Conference Bremen, 253–257.

[6] Lossie, U. & Puetz, P. 2008 Targeted control of biogas plants with the help of FOS/TAC. Practice Report.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PERSPECTIVES IN KEROSENE PRODUCTION FROM *CAMELINA SATIVA* OIL

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Abstract

Global aviation industry is facing some significant challenges on reducing emissions of persistent greenhouse gases and on reducing 33% of total operating costs, represented by oscillating price of fuels. Thus, identifying new sources of biofuels is essential for the future of the industry. One promising research direction is provided by biofuels coming through processing oil, extracted from the seeds of Camelina sativa plant. Camelina sativa presents a lot of interest because of ecological plasticity, low-growing needs (low inputs, minimal nutrients) and the fact that it does not interfere with food resources of the population. The technology of obtaining biokerosen out of Camelina oil is partially similar to that of obtaining biodiesel. Camelina oil is initially subjected to a hydrodeoxigenation operation. Linear alkanes obtained are isomerized and are catalytic selective cracked, in order to obtain branched alkanes. These are more stable in aviation navigation areas, having lower boiling point than diesel biofuel. The last step is the catalytic closure of aromatic rings derived from existing paraffins. It can be avoided the expensive last step, by mixing the branched alkanes with conventional kerosene oil (which contains 25% aromatic hydrocarbons, in particular alkylbenzenes and alkylnaphthalenes). Also, by-products appear, such as natural pesticides, additives in the plastics industry, nutraceutical, forages etc. (alkanolamides, fatty alcohols, isopropyl esters, glycerol etc.). Advantages of using kerosene obtained from Camelina sativa oil are: the significantly low level of greenhouse gases coming from burning, compared with burning of fossil fuels, large base of unpretentious crops of Camelina sativa and highly pure biofuels, similar to fossil fuels. Disadvantages of using kerosene obtained from Camelina sativa oil are: high costs of technological equipment, low oil yields relative to plant production/hectare, large amount of hydrogen required, produced by high consumption of fossil energy and also, competition for the same resources as in the production of biodiesel .

Key words*: biofuels, Camelina sativa, greenhouse gases, kerosene, technology for obtaining kerosene.*

INTRODUCTION

Camelina sativa is a plant cultivated since ancient times (bronze and iron ages). It is not clear why since the Middle Ages, the crops of *Camelina sativa* were replaced by others.

The recent interest for this plant is supported by its reduced claims to a range of nutrients, claims which materialize into smaller amounts of inputs, but also in the ecological plasticity of the plant, which is capable to grow on half-arid soils, with low fertility (Zubr, 1997).

Certain features of *Camelina sativa* oil make it interesting for use as biofuel. Initially, as with other oilseeds, researchers efforts were directed towards obtaining biodiesel fuel.

In 2009, Japan Airlines and KLM Royal Dutch Airlines have successfully tested a mixture of aviation fuel, which contained 50% of a product derived from *Camelina sativa*.

In 2011, similar tests were performed by Honeywell and Boeing, Iberia company (using an A320 aircraft on the route Madrid - Barcelona) and Porter (Q400 aircraft on the route Montreal - Toronto) conducted similar tests on passenger flights.

The interest for the use of biofuels in aviation coming from *Camelina* starts from a series of studies according to which the products derived from this plant promise a decrease by 80% of the toxic impact over the environment (in particular by reducing greenhouse gas emissions, long cycle life), compared with their counterparts obtained from oil (Shonnard et. al., 2010).

In addition, the aviation industry is very vulnerable to world fluctuations in oil prices, fluctuations which are very common in the recent years. Prices vary depending on the geo-strategic policies of the great powers and the states in the Middle East, on the global financial market developments, factors that determine high volatility and instability. Considering that about 33% of the operational costs of the aviation industry are determined by fuel prices, fuel identification of additional resources is essential for the future of the industry.

In this paper we intend to conduct a study on the state of art regarding the potential use of *Camelina sativa* plant, in obtaining biofuels for aviation industry

MATERIALS AND METHODS

In order to accomplish this study were used data from specialized literature, and a series of official statistics (European Commission, reports of aviation companies etc.).

RESULTS AND DISCUSSIONS

Extensive studies concerning the technologies of culture and ecology of the plant *Camelina sativa* took place in several countries in the temperate regions of the world.

It was thus found, that although *Camelina* has reduced soil fertility claims, compared to the most oil-bearing crops, the treatments with nitrogen fertilizers affect the performance of the plant crop. The best results were obtained at additions of nitrogn fertilizers between 78.4 and 100.7 kg/ha, in Montana (USA) and 120 kg/ha in Germany (Eidhin et al. 2003; Frohlich and Rice 2005; Gilbertson et al., 2007; Shukla et al., 2002, Agegnehu and Honermeier 1997). Nitrogen fertilizers are critical for oil content in seeds, which decreases with the increase of nitrogen applied (Agegnehu and Honermeier 1997; Jackson 2008). This was revealed by a study conducted in Canada's maritime provinces by Urbaniak et al. (2008), that emphasized among others, the importance of choosing the variety of *Camelina*, for success or failure of the crop.

Camelina sativa responds to phosphorus fertilizer application, when its soil concentration exceeds 12 ppm (Jackson, 2008). Production per hectare reported by various researchers, is variable. Thus, in 2006 scientists from Huntley MT, obtained production of 1067 to 1093 kg/ha, at a sowing rate of 6.6 to 8.6 kg/ha. In Germany, Agegnehu and Honermeier (1997) reported a production of 2057 kg/ha for a sowing rate of 5.85 kg/ha.

Camelina sativa has reduced needs regarding pests control work. As potential pests we can mention *Phyllotreta cruciferae* (small fleas of cruciferous), which has a higher affinity for other cruciferous and *Peronospora camelinae* downy mildew. Some studies have shown that the *Camelina sativa* plant is competitive against weeds. In 1980, Lovett and Jackson have even suggested that *Camelina sativa* produces a series of allelopathy phytoherbicides, effective in growth stopping of *Linum usitatissimum* (flax).

The most important product of Camelina is oil, obtained by crushing and pressing the seeds. Oil content of seeds, reported to the dry matter, is ranging from 30 to 40% (Strasil, 1997). It is estimated that more than 50% of the composition of such oils is represented by polyunsaturated fatty acids. The contribution of the polyunsaturated fatty acids, in the composition of the oil, varies according to the applied phytotechny, but the main fatty acids identified are linoleic (18: 2) and α - linolenic acid (18: 3ώ3) (Eidhin et al., 2003). Most studies estimate that the content of erucic acid is less than 4% (Vollmann et al., 1996).

α - linolenic acid of *Camelina sativa* differs from its counterparts, extracted from other plants, by greater stability to oxidation. Wastes of oil extraction are an excellent source for animal forages, containing more than 5% α – linolenic acid. US Food and Drug Administration approved in 2009 the introduction of such forages in the chickens and cattle nutrition, in an amount up to 10% from the total ration.

Although polyunsaturated fatty acid composition makes interesting the *Camelina* oil for food consumption (source of omega 3 unsaturated fatty acid), must be considered that its stability is lower than usual edible oils from rapeseed, olive, corn, sesame or sunflower (Matthäus et al., 2004).

The fact that *Camelina* oil does not present popularity as food, makes it an interesting resource as biofuel, because it does not compete with crops for human consumption.

One of the most common fears, regarding the cultivation of certain plants for biofuel production, is the fact that most of the proposed plants have some agronomic traits, which can turn them into invasive plants (drought resistance, tolerance to soil fertility conditions, short cycle development, rapid accumulation of biomass etc.) (Raghu et. al. 2006; Barney and DiTomaso 2008). These native qualities can become overwhelming, in case these plants are widely cultivated (Minton and Mack, 2010). Although researches on invasive potential of *Camelina sativa* are limited, due to the high variability of the factors that must be taken into account, the findings of the current studies tend to evaluate this risks as being rather low (Davis et al., 2011). The quality of *Camelina sativa* seeds is significantly influenced by both phenotype and genotype factors. Vollman et al. (2007) showed that there is a significant number of genotypes of *Camelina sativa*, which can promise the simultaneous selection of superior qualities cultivars, both in terms of production achieved and seed oil content.

However, this selection does not have to take into account the increase in the size of the seeds, since this parameter correlates negative very significant both with total oil content, and the concentration of certain fatty acids (linolenic acid). This production sensitivity to phenotypic factors showes that the estimates regarding the amount of oil / hectare from Camelina crop, varies significantly from author to author, depending on geographic area of reference. In any case, most of the studies place the oil yield / ha of *Camelina*, under the oil yield / ha of rapeseed, over the oil yield / ha of soybeans and within range of variation of the sunflower crop (Table 1).

Table 1. Comparison between yields of different oilseeds (after Oil World Annual 2009, quoted by Moser, B., R., 2010)

Specification	Camelina	Rapeseed	Soybean	Sunflower
Production (t/ha)	0.9 – 2.24	2.68 – 3.39	2.14 – 2.84	1.44 – 1.70
Oil content (%, dry matter)	35 – 45	40 – 44	18 – 22	39 – 49
Oil yield (l/ha)	106 - 907	965 - 1342	347 - 562	505 - 750

The main constituent of *Camelina* oil fatty acids is linolenic acid (32-40% of dry weight), followed by linoleic, oleic and 11 - eicosenoic acids (Table 2).

Table 2. Fatty acids composition of Camelina oil (%), according to various authors

Fatty acid (No. of C)	Authors			
	Leonard (1998)	**Moser şi Vaughn (2010)**	**Zuhr şi Malthaus (2002)**	**Frohlich şi Rice (2005)**
Palmitic (16:0)	5.3	6.8	5.4	5.4
Stearic (18:0)	2.5	2.7	2.5	2.6
Oleic (18:1)	12.6	18.6	14.9	14.3
Linoleic (18:2)	15.6	19.6	15.2	14.3
α – Linolenic (18:3)	37.5	32.6	36.8	38.4
Arachidic (20:0)	1.2	1.5	1.3	1.4
11– Eicosenoic (20:1)	15.5	12.4	15.5	16.8
Eicosadienoic (20:2)	2.0	1.3	1.9	-
Eicosatrienoic (20:3)	1.7	0.8	1.6	-
Behenic (22:0)	0.3	0.2	0.3	0.2
Erucic (22:1)	2.9	2.3	2.8	2.9
Others	2.9	1.2	1.8	3.7

Initially, as with other oilseeds, researchers efforts were directed towards obtaining biodiesel fuel. Biodiesel is defined by the American Society for Testing and Materials (ASTM) as a mixture of monoalkyl esters of long chain fatty acids and is usually obtained by transesterification of the lipids, in the presence of an alkaline catalyst and an excess of methanol, at high temperature (600^0 C).

Another way of obtaining biodiesel from lipids, derived from biological materials, involves their hydrodeoxigenation, under pressure and high temperatures (40-150 atm, $350\text{-}450^0$ C), in the presence of hydrogen and heterogeneous catalysts. It is obtained a mixture of paraffins (linear alkanes with different carbon chain lengths), that are subject in the next stage, to isomerization operations. Isomerization serves to improve the flow properties to cold and also significantly decrease cetane number. The resulting mixture is made up of paraffins with 15-18 carbon atoms, and properties similar to the compounds obtained from counterparts oil.

There are several directions of research to obtain biofuels for aviation. These include

starchy crops, lignocellulosic biomass (wood crops, residues from agriculture or forestry, algal biomass or municipal wastes) and oilseed crops. The conventional kerosene is a mixture of hydrocarbons consists of molecules that usually contain from 8 to 16 carbon atoms, obtained by refining crude oil. This must correspond to very strict standards related to safety in the aviation industry and to some essential goals, namely: providing a large amount of energy per unit of mass or volume; stability at low temperatures, to avoid freezing or gelling in thermic conditions existing at aircraft flying altitudes; compatibility with the materials used in aircraft.

In accordance with these objectives, kerosene must meet criteria that are related to a number of physico-chemical parameters, such as viscosity, surface tension, volatility, lubricity, sulfur amount, combustion properties, etc. The technical characteristics of kerosene, used in aviation industry, are defined by ASTM D1655 standards in United States or DEF STAN 91-91 in Europe (Table 3)

Table 3. Main technical specifications for kerosene used in aviation, in American standards (ASTM D 1655) and European (DEF STAN 91-91)

Fuel	**Jet A**	**Jet A - 1**
Standard	**ASTM D 1655**	**DEF STAN 91 - 91**
Acidity (mg KOH/g)	0.10	0.015
Aromatics (% vol, max.)	25	25
Sulphur, total (% wt)	0.30	0.30
Mercaptan (% wt)	0.003	0.003
Distillation temperature (^{0}C):		
Initial boiling point	-	Report
10% recovery, max	205	205
50% recovery, max	Report	Report
90% recovery, max	Report	Report
Final boiling point	300	300
Flash point (^{0}C, min)	38	38
Density (15^{0}C, kg/m^3)	775 - 840	775 – 840
Freezing point, (^{0}C, max)	- 40	- 47
Viscosity (- 20^{0}C, mm^2/sec, max)	8	8
Specific energy (MJ/kg, min)	42.8	42.8
Smoke point (mm, min)	18	19
Naphtalenes (vol %, max)	3	3
Copper strip (2h, 100^{0}C, max.)	1	1
Thermal Stability		
The pressure drop limit for the filter (mm Hg)	25	25
Tube deposit ratting (max)	< 3	< 3
Gum content (mg/100 ml, max)	7	7

Modern fuels used in aviation industry must satisfy a number of criteria associated with: efficiency, reliability and sustainability. Also, modern fuels must not affect the infrastructure in the field (Andriishin et. Al., 2010). Thus, they must: be supplied in good conditions and in constant quantity; have a good pumpability in different conditions of temperature or altitude; evaporate completely in the combustion chamber and burn in a wide range of variation of the fuel / air mixture; have high heat capacity, high density capability to ensure stable combustion; not contain mineral compounds, have no tendency to form soot or other specific deposits at high or low temperatures; not to be corrosive for aircraft components, to be compatible with non-metallic materials; have good anti-wear properties, for protection and cooling; have large storage life and stability to shipping; have low toxicity both for humans and environment.

Technology of obtaining biokerosen from *Camelina* is partial similar to obtaining biodiesel by hydrodeoxigenation of lipids. But isomerization operation is critical, since branched paraffins aims to achieve. The sequence of reactions required to obtain kerosene is summarized in Figure 1.

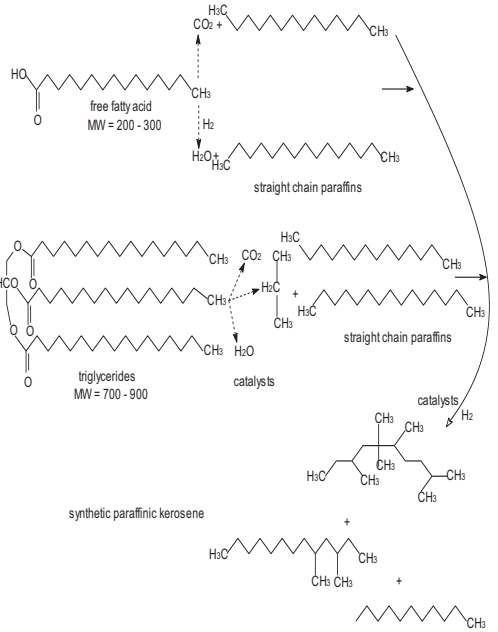

Figure 1. The sequence of chemical reactions for obtaining vegetable oil kerosene

The first reaction involves removing oxygen, with obtaining of linear paraffins, specific for biodiezel. Then, the cracking takes place, to

obtain highly branched paraffins, similar to those components of synthetic kerosene.

In Figure 2 is shown a simplified scheme for industrial production of biokerosen from *Camelina* oil. In the first stage, *Camelina* oil is subjected to a hydrodeoxygenation process (HDO), whose result is obtaining linear alkanes.

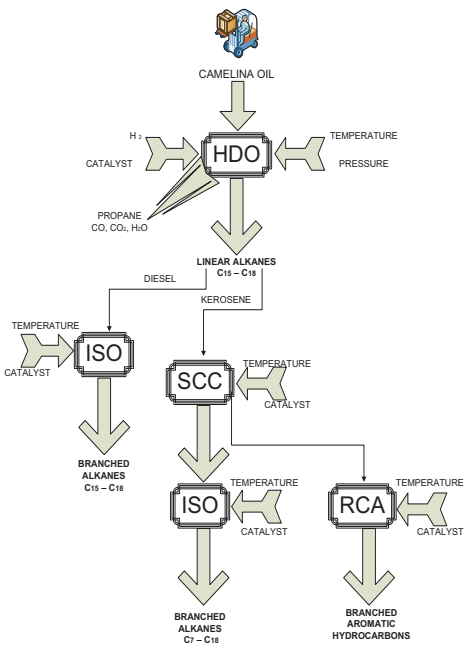

Figure 2. Simplified diagram for obtaining biokerosen of *Camelina sativa* oil (after Moser, 2010)

In second stage, occurs isomerisation (ISO) of linears alkanes to branched alkanes. This process is necessary, as linear alkanes freeze at temperatures encountered in specific areas of air navigation.

The third stage involves selective catalytic cracking (SCC) of the paraffins to shorter-chain hydrocarbons (C7–C18). The aim of this process is to obtain a mixture with a boiling temperature range lower than that of diesel biofuel.

With existing catalyst and reactor technologies, selective catalytic cracking and isomerization can be accomplished in a single stage.

Finally, the last step is the catalytic closure of the aromatic rings, for a part of existing paraffins, operation which for economic reasons may be replaced with conventional kerosene, mixed with biodiesel obtained from oil (biodiesel contains 25% aromatic hydrocarbons, especially alkylbenzenes or alkylnapthalenes).

Some studies have focused on improving the rate of transesterification of the fatty acids from *Camelina* oil. Thus, Patil et al (2011) have achieved a higher rate of production of fatty acid methyl esters (FAME) from *Camelina* oil, coupling catalysts based on metal oxides with heat treatment. With this treatment, two-fold higher yields of fatty acids methyl esters were obtained, using the catalyst of BaO and SrO and microwave heating, compared to conventional treatments. It was observed that BaO and SrO catalysts generated higher FAME yields than the CaO and MgO catalysts. A comparison between the conventional heating and the microwave-assisted transesterification processes, showed that the reaction rate constant obtained in the microwave-assisted transesterification process, are of two orders of magnitude higher than those obtained with the conventional heating method.

In Table 4 is shown the profile of fatty acid methyl esters identified in biokerosene obtained by methanol transesterification of *Camelina* oil (after Llamas et al., 2012; Matas et al., 2012).

Table 4. Profile of methyl esters of fatty acids identified in biokerosen, obtained by transesterification with methanol of *Camelina* oil (after Llamas et al., 2012)

Ester metilic	**CAM 100**
Methyl caprylate C8:0	-
Methyl caprate C10:0	-
Methyl laurate C12:0	0.84
Methyl miristate C14:0	0.41
Methy palmitate C16:0	5.83
Methyl stearate C18:0	2.97
Methyl oleate C18:1	15.83
Methyl linoleate C18:2	18.85
Methyl linolenate C18:3	33.99
Methylarachidate C20:0	1.54
Methyl-cis-11-eicosenoate C20:1	16.03
Methyl behenate C22:0	2.16
Methyl eructate C21:1	1.44
Iodine value	148.8
Mean formula	$C_{19.23}H_{34.97}O_2$
Molecular weight (g mol^{-1})	298.153
Stoichiometric air/fuel ratio	12.508
C (%)	77.45
H (%)	11.82
O (%)	10.73

(CAM = percent of *Camelina* oil)

The results of Llamas et al. (2012), in terms of biokerosen main physical - chemical parameters, obtained by transesterification with methanol of *Camelina* oil, but also with mixtures of *Camelina* oil and various proportions of conventional kerosene, led to the conclusion that only a mixture of 10% biokerosen and 90% conventional kerosene is feasible for use in the aviation industry (Table 5).

Table 5. Physico-chemical characteristics of the biokerosen obtained by transesterification of Camelina oil with methanol and various mixtures with conventional kerosene (as Llamas et al., 2012)

Parameter / Blends	CAM 0 K1 100	CAM 5 K1 95	CAM 10 K1 90	CAM 20 K1 80	CAM 100 K1 0
Color and aspect	clear	clear	clear	clear	clear
Elemental composition (%, wt)					
C	85.9	85.29	8.23	83.86	77.09
H	13.88	12.99	1.,61	12.45	11.09
N	0.02	0.02	0.02	0.02	0.02
S	0.17	0.05	0.03	0.03	0.06
O		1.65	3.11	3.64	11.74
Another properties					
Density (15^0C, kg/m^3)	793	806.7	810.8	819.8	889.3
Viscosity (45^0C, mm^2/s)	-	1.2591	1.3075	1.4539	4.3395
Viscosity (100^0C, mm^2/s)	-	0.6799	0.7053	0.7663	1.7077
Viscosity(-20^0C, mm^2/s)	-	3.11	3.20	3.80	6.96
Higher heating value (MJ/kg)	47.7	45.3	45.0	44.8	40.0
Lower heating value (MJ/kg)	44.44	42.52	42.30	42.14	37.71
Cold filter plugging point (^{0}C)	-	-31	-29	-26	-5
Cloud point (^{0}C)	-42.5	-26	-26	-22	1
Pour point (^{0}C)	-	-40	-32	-31	-5
Oxidative stability (hours)	-	>8	> 8	> 8	1.4

(CAM = percent of *Camelina* oil)

In addition to the products of interest resulted from the process of biokerosen obtaining, a number of other substances and residues can be exploited in different directions. This is a significant hope for economic profitability of the technology.

By-products can be used as: natural pesticides, additives in the plastics industry, nutraceuticals, forages and so on (alkanolamides, fatty alcohols, isopropyl esters, glycerol etc.).

It is estimated that the presented technology has a number of advantages, such as: obtaining products with a level of greenhouse gas emissions significantly lower, compared with fossil counterparts; large base of crops that can be processed in this direction and obtaining biofuels of high purity and chemical composition similar to that of fossil fuels. But the technology presents a number of disadvantages, which include: high costs of raw materials procurement and technological facilities, low yields relative to oil production/hectare of crops, competing for the same resources used in the production of biodiesel, high necessary of hydrogen produced today by a significant consumption of fossil energy.

Table 6 presents a summary of key aspects of technologies for obtaining biokerosen, currently proposed: Kerosen Parafinic Sintetic with hydrotreated esters and fatty acids (HFA-SPK), Kerosen Parafinic Sintetic Fischer - Tropsch (FT – SPK) and Kerosen Parafinic Sintetic from alcohol (ATJ – SPK). These technologies belong to the findings of a study by SINTEF ENERGI AS (Norway) for AVINOR, completed in 2012.

Table 6. Comparative analysis of the main constituent elements of the proposed technologies for obtaining biokerosen (SINTEF Energi I study for Avinor, 2012)

	HEFA - SPK	FT-SPK	ATJ-SPK
Feedstock	**Conventional oil crops**: soybean, palm oil, rapeseed, Coconut, corn; **New oil crops:** jatropha, camelina şi halophyte; **Microalgae**	**Lignocellulosic biomass**: energy crops, agricultural and forestry residues, wastes	**Sugars**: sugarcane, sugar beets, molasses, and fruits **Starches:** corn, cassava, potatoes, and root crops **Lignocellulosic biomass**: energy crops, agricultural and forestry residues
By-products	Diesel, fractions of propane, naphtha and LPG, natural pesticides, nutriceuticals plastics, animal feed, heat and chemicals	Diesel, gasoline, naphtha, chemicals (hydrogen, methanol,)	**From sugars and starches**: diesel (from alcohol production), Proteins and fats (from jet-fuel production); **From lignocellulosic biomass**: diesel (from alcohol production), lignin and small amounts of proteins (from jet-fuel production)

	HEFA - SPK	FT-SPK	ATJ-SPK
Costs	Low CAPEX, High OPEX: high feedstock prices, low yields (little oil content in the crop), large hydrogen requirement	High CAPEX (gasification, gas cleaning and FT steps), Low OPEX (use of residues as feedstocks, high conversions)	Low CAPEX High OPEX: micro-organisms and pre-treatments
Certification	Certification since July 2011 by the ASTM D1655 standard, up to 50% blending with petroleum- based jet kerosene	Certification since September 2009 by the ASTM D1655 standard, up to 50% blending with petroleum-based jet kerosene	Under ASTM certification. Expected to be approved as fully synthetic aviation fuel with 100% replacement of the petroleum-based jet kerosene by 2014
Commercialization	Pilot plants under construction	Pilot plants under construction	Pilot plants under construction
Advantages	Wide range of feedstocks can be processed; Product life cycle emissions significantly lower compared to fossil fuels (80-85% including only biomass conversion processes); Very pure and high quality product with a chemical composition similar to conventional jet-fuel	Wide spectra of potential products; Large feedstock flexibility; Product life cycle emissions much lower compared to fossil fuels (90-95% including only biomass conversion processes); High conversions; Relatively low external hydrogen requirement when applying certain gasification systems (indirect gasification)	All steps necessary to convert alcohol to jet-fuel are at commercial scale in the petrochemical industry; Large feedstock flexibility; ATJ-SPK does not require blending with petroleum-derived jet-fuel; Little amount of external hydrogen required; High specificity when processing biomass to alcohols through fermentation
Challenges	High investment cost of the plants; High feedstock prices; Feedstock availability (competing with biodiesel producers for the same feedstock); Sustainability concerns; Low oil yields; Large amounts of hydrogen required	High capital costs; Biomass gasification still requires optimization, particularly with regards to tar minimization; Large amounts of hydrogen required	High alcohols production costs, particularly from lignocellulosic biomass; Limited experience with alcohols other than methanol/ethanol; Low production rates when working with microorganisms; High sensitivity of microorganisms towards impurities;

* CAPEX, Capital Expenditure, refers to investment expenditure required, ie all those costs that are amortized over the whole life of the investment;
** OPEX, operating expense refers to expenses necessary to support current activities

CONCLUSIONS

Since the technology for obtaining kerosene from vegetable oils, by transesterification, has already been certified, it is expected that its implementation will take place in the shortest time. There are a number of uncertainties that could influence the evolution of this technology, such as oil price relative to the price of the main raw materials of oilseeds.
It is expected that a decrease in the price of oil, or an increase of the raw materials of oilseeds, to limit investors enthusiasm. On the other hand, new technologies are proposed and enthusiastically embraced by researchers, as getting kerosene from microalgae.
Given the fact that *Camelina sativa* plant is not part of the crops which interfere with food resources of the population, its use for obtaining biokerosen may confer an advantage in discussed issue.
In addition, as we have shown, the technology for obtaining kerosene from oil plants can be improved, in terms of economical profitability, by increasing the use of the byproducts, which occur in the main reaction pathways of this technology.

ACKNOWLEDGEMENTS

This research work was carried out with the support of project: Romania's participation in FP7 Programme - Initiative Towards a sustainable Kerosene for Aviation (ITAKA), Contr.no. 236/EU/04.09.2013

REFERENCES

Agegnehu M., Honermeier B., 1997. Effects of seeding rates and nitrogen fertilization on seed yield, seed quality and yield components of false flax (*Camelina sativa* Crtz.). Die Bodenkultur. 48: 1520.

Andriishin M. Ya. M., Boichenko S., & Ryabokon, L., 2010. Gaz Prirodnyi, Paliva ta Olivy. Astroprint, Odessa.

Barney J. N., & DiTomaso J. M., 2008. Nonnative species and bioenergy: are we cultivating the next invader? Bioscience, 58(1), 64-70.

Davis A. S., Wiedenmann R. N., Simberloff D., & Mack, R. N., 2006. Adding biofuels to the invasive species fire?, Science-New York then washington, 313(5794), 1742.

Davis P. B., Menalled F. D., Peterson R. K., & Maxwell B. D., 2011. Refinement of weed risk assessments for biofuels using *Camelina sativa* as a model species, Journal of Applied Ecology, 48(4), 989-997.

Eidhin D. N., Burke J. Lynch B. O'Brine D., 2003. Effects of dietary supplementation with camelina oil on porcine blood lipids, J. Food Sci. 68, 671–679.

Frohlich A., Rice B., 2005. Ind. Crops Prod., 21, 25 – 31.

Gilbertson P. K., Johnson B. L., Berti M. T., & Halvorson, M., A., 2007. Seeding date and performance of specialty oilseeds in North Dakota. Issues in new crops and new uses. ASHS Press, Alexandria, 105-110.

Jackson G. D., 2008. Response of camelina to nitrogen, phosphorus, and sulfur. Fertilizer Facts. Montana State Univ., Ext. Serv., Western Triangle Ag. Res. Center, Conrad.(online) Available from http://landresources.montana.edu/fertilizerfacts/pdf/FF,2049.

Leonard E.C., 1998. Inform, 9, 830 – 838.

Llamas A., Al-Lal A. M., Hernandez M., Lapuerta M., & Canoira L., 2012. Biokerosene from babassu and camelina oils: production and properties of their blends with fossil kerosene, Energy & Fuels, 26(9), 5968-5976.

Lovett J. V., & Jackson H., F., 1980. Allelopathic activity of *Camelina sativa* (L.) Crantz in relation to its phyllosphere bacteria. New Phytologist, 86(3), 273-277.

Matas B., Bugge M., Kempegowda R. S., Anthe G., Paap S., 2012. Benchmark of conversion and production technologies for synthetic biofuels for aviation, Report SINTEF Energy Research for Avinor, https://avinor.no/globalassets/_konsern/miljo-lokal/miljo-og-samfunn/delrapport-1-teknologi.pdf

Matthäus W., Leindotteröl M., 2004. Ein altes Pflanzenöl mit neuer Zukunft?, Ernähr.-Umsch. 51, 12–16.

Moser B. R., 2010. Camelina (*Camelina sativa* L.) oil as a biofuels feedstock: Golden opportunity or false hope?. Lipid technology, 22(12), 270-273.

Moser B., R., Vaughn, S., F., 2010, Bioresour. Technol, 101, 646 – 653.

Patil P., Gude V. G., Pinappu S., & Deng S., 2011. Transesterification kinetics of *Camelina sativa* oil on metal oxide catalysts under conventional and microwave heating conditions. Chemical Engineering Journal. 168: 1296-1300.

Raghu S., Anderson R. C., Daehler C. C., Minton M. S., & Mack R. N., 2010. Naturalization of plant populations: the role of cultivation and population size and density, Oecologia, 164(2), 399-409.

Shonnard D. R., Williams L., & Kalnes T. N., 2010. Camelina-derived jet fuel and diesel: Sustainable advanced biofuels, Environmental Progress & Sustainable Energy, 29(3), 382-392.

Shukla V. K. S., Dutta P. C. & Artz W. E., 2002. Camelina oil and its unusual cholesterol content. Journal of the American Oil Chemists' Society,79(10), 965-969.

Strasil Z., 1997. Content of oil and individual fatty acids in some species of alternative oil-bearing crops, Rost. Vybroba, 43, 59–64.

Urbaniak S. D., Caldwell C. D., Zheljazkov V. D., Lada R., & Luan L., 2008. The effect of cultivar and applied nitrogen on the performance of *Camelina sativa* L. in the Maritime Provinces of Canada.Canadian Journal of Plant Science,88(1), 111-119.

Vollmann J., Damboeck A., Eckl A., Schrems H., Ruckenbauer P., 1996. Improvement of *Camelina sativa*, an underexploited oilseed. p. 357-362. In: J. Janick (ed.), Progress in new crops. ASHS Press, Alexandria, VA.

Vollmann J., Moritz T., Kargl C., Baumgartner, S., & Wagentristl, H., 2007. Agronomic evaluation of Camelina genotypes selected for seed quality characteristics, Industrial Crops and Products, 26(3), 270-277.

Zubr J., 1997. Oil seed crop: *Camelina sativa*, Ind. Crop. Prod., 6, 113-119.

*** ASTM Standard Specification D 1655-11b for Aviation Turbine Fuels "Jet A-1", http://www.exxonmobil.com/AviationGlobal/Files/WorldJetFuelSpec2008_1.pdf

***British Ministry of Defence Standard DEF STAN 91-91/Issue 7 Amendment 1, 16 December, 2011 for Turbine Fuel, Kerosene Type, Jet A-1, NATO Code F-35, Joint Service Designation: AVTUR, http://www.seta - analytics.com/documents/ Defstan_91-91_R7.pdf

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MICROBIAL SCREENING FOR LIPASE AND AMYLASE PRODUCTION USING NEWLY ISOLATED STRAINS FROM VARIOUS BIOTOPES

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Abstract

This paper presents the results of preliminary experiments in order to obtain biologically active substances through biosynthesis, by using microorganisms isolated from various biotopes in Romania. The main research objective is defined by a bioprospecting study on some bacterial, yeast and fungi strains, aiming at the selection of those with biotechnological potential in producing lipolytic and amylolytic enzymes. After collecting and processing the various nature originated samples (soil, sand, mud, water, plant material) for the isolation of industrial importance microorganisms, a total of 104 microbial strains, including 70 bacteria, 10 yeasts and 24 fungi, were obtained. For the isolation and identification of the microorganism groups/species, the decimal serial dilutions technique on specific agar media was used; some bacterial strains were identified by MALDI-TOF mass-spectrometry. In order to select lipase and amylase producing microorganisms, some screenings were performed, using different solid media formulas containing inductors such as Tween80 and Tributyrin, starch respectively. Positive results were noticed for 66 strains - clear or opaque areas were observed around the colonies, formed due to enzymatic hydrolysis. Further experiments are to be conducted only with those microorganisms that demonstrated enzymatic activity (considering the diameter of the clear or opaque areas of hydrolysis) for all of the culture media compositions.

Key words*: microorganisms, lipase, amylase, bioprospecting, mass-spectrometry.*

INTRODUCTION

The exploitation of microorganisms is widely used in many different industry sectors, such as agricultural, chemical, environmental, food, health etc. Heretofore, only a part of the total microbial population has been revealed. Considering this fact, exploiting the microbial potential will offer a possibility to increase the biotechnological uses of microorganisms and also to discover new compounds. White biotechnology has applications in industrial processes for production of enzymes, vitamins, flavours, pesticides, fuels and food. New technologies have evolved in enzyme screening, challenging the scientists all over the world to deliver even more diverse products. Different industries require enzymes which can be used as organic catalysts in various processes on a commercial scale. Certain microbial enzymes are of special interest, including lipases and amylases. The technological progress in this field started along with Dr. Takamine's research about fungal enzymes, in 1894 and also with Boidin and Efront's studies about bacterial enzymes (Underkofler et al., 1958).

Nowadays, many enzymes from microbial sources, with industrial applications, are obtained on large scale (commercial use of different enzymes is forecast to reach $ 7.6 billion this year) (Turki, 2013). These biomolecules act as biocatalysts to perform

economic and environmental-friendly reactions, successfully replacing the conventional chemical catalysts (Singh, 2013). Selected strains of bacteria, fungi and yeast have been intensively studied for their uses in enzyme's biosynthesis.

The microbial strains were identified using MALDI-TOF mass-spectrometry (MS) – a "shotgun" type proteomics technique for direct protein fingerprinting of bacteria (Lay, 2001) and another microorganisms (Dingle and Butler-Wu, 2013).

These enzymes found promising applications in various industrial fields, such as: *α-amylase* in starch liquefaction, food (syrups, brewing, baked products), paper, textile (desizing of cotton) and pharmaceutical industry (Sivaramakrishnan et al., 2006; Singh et al., 2011; Singh et al., 2014; Suribabu et al., 2014); *lipases* in hydrolysis of triglycerides to free fatty acids and glycerol – food and feed (flavour development for dairy products - butter, cream and beverages), detergents (approx. 1000 tons of lipase/year is sold), cosmetics and personal care products, polymers synthesis, pharmaceuticals, agrochemical industry and environmental sector (bioremediation – oil spills, degradation of polyester waste) (Sharma et al., 2001; Sharma et al., 2011; Singh, 2013).

The literature describes numerous studies using different strains of bacteria (*Bacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Lysinibacillus* sp.), yeasts (*Saccharomyces cerevisiae, Candida rugosa, Yarrowia lipolytica, Rhodotorula glutinis*) and fungi (*Rhizopus* sp., *Aspergillus* sp., *Mucor* sp., *Penicillium* sp.) for the production of these extracellular enzymes (Zarnea et al, 1980; Banu, 1987; Walker, 1998; Annamalai et al, 2011; Kumar et al, 2013; Rajesh et al., 2013; Singh, 2013; Khannous et al., 2014).

The main purpose of this study is bioprospecting on microorganisms isolated from various natural biotopes, aiming at the selection of some microbial strains with biotechnological potential. This paper describes a rapid screening of 104 newly isolated microbial strains for the ability to produce extracellular lipases and amylases, using agar media containing specific substrates.

MATERIALS AND METHODS

Sampling sites

In the present study, various samples collected from diverse natural biotopes, such as: soil, sand, mud, water, rock green moss, spruce cones and beechnuts were considered. Microbiological diversity was ensured by sampling in different Romanian counties.

Growth media and cultivation conditions

Specific isolation liquid media were used for bacteria (IPS), yeasts and fungi (YMPG), in which equal amounts of the samples were added: 1 ml of water, 1 gram of soil/sand/sludge/fragments of muscle, cone and beechnuts (comminute to the appearance of fine powder), respectively. 100 ml of each sterile media were added to the Erlenmeyer flasks (500 ml capacity) and then inoculated; growth conditions were considered suitable: 220 rpm, 24h at 30º-31ºC for bacteria and 72h at 27º-28ºC for yeasts and fungi.

Serial dilutions of the collected samples were carried out and 1 ml of dilutes was pour plated on nutrient agar (NA) and YMPG supplemented with chloramphenicol. The NA plates were incubated at 30º-31ºC for 48h, while YMPG plates were incubated at 27º-28ºC for 72-96h. Morphological appearances of the microorganisms were observed and distinct colonies were subcultured to obtain pure isolates, which were then maintained on their specific media (NA, YMPG and PDA) and stored at 4ºC.

IPS medium contained (% w/v): glucose 2.0, yeast extract 0.2, KH_2PO_4 0.2, citric acid 0.1, $MgSO_4*7H_2O$ 0.05; pH adjusted to 7.5.

The other media used for cultivation the microorganisms are described in ATCC or DSMZ catalogues as: YMPG (DSMZ no. 186), NA - Nutrient Agar (ATCC no. 3), PDA - Potato Dextrose Agar (ATCC no. 97).

Morphological characterisation

Microscopic examination was performed using a conventional microscope, OPTIKA B-600 Ti; Gram staining and methylene blue coloration were used for bacteria and yeasts, while the fungi were investigated by native preparation.

Identification of newly isolated bacterial strains

Prior to MS analysis, the collected strains were prepared according using the method of ethanol treatment followed by extraction with formic acid and acetonitrile (Freiwald & Sauer, 2009). Microflex LT, a MALDI-TOF mass-spectrometer manufactured by Bruker Daltonics Inc. (Billerica, MA, USA), equipped with nitrogen laser, was used to acquire the mass-spectra from fresh colonies. Based on the specifity of the mass spectrum for a large number of bacteria, fungi and yeasts (Ryzhov & Fenselau, 2001; Marvin et al., 2003; Suarez et al., 2013), the dedicated MALDI Biotyper software identify microorganisms by analysing the expression of the most abundant ribosomal proteins from the acquired mass spectra. The pattern of ribosomal protein expression is automatically compared by the software with a large number of reference patterns from its database. MALDI Biotyper analysis generates a characteristic mass and intensity distribution of those proteins and uses them to identify unknown samples by comparing their "fingerprint" with the patterns included in its open database.

Screening of the isolated strains for extracellular lipase and amylase production on solid media

Plate detection containing different inductors considered as carbon sources (tributyrin, tween 80 and soluble starch) were used to screen the microbial strains for lipase and amylase production ability (Kumar et al., 2012). The agar plates containing seven different screening media were spot inoculated with all the 104 isolated strains and incubated at 30º-31ºC (48h) for bacterial growth and at 27º-28ºC (72-120h) for yeast and fungi. The media compositions are presented in Table 1.

The plates were observed every 24 hours, measuring the dimensions of the clear/ opaque areas around the colonies, formed due to enzymatic hydrolysis; the amylolytic activity was noticed after the plates were flooded with Lugol's solution (1.3%).

RESULTS AND DISCUSSIONS

A total of 104 microbial strains, including 70 bacteria, 10 yeasts and 24 fungi were newly isolated from environmental samples (see Table 2) and screened for their lipase and amylase producing ability on solid media. The collection of isolates used in this study was deposited as both, vegetative conserve and lyophilized, in the Culture Collection of Industrial Importance Microorganisms (CMII) of the National Institute for Chemical-Pharmaceutical Research and Development, Bucharest.

Table 1. The solid screening media compositions for extracellular lipase and amylase activity (% w/v)

Medium	Carbon source	Nitrogen source	Growth factor				Other
			1	2	3	4	
Bacteria – lipase screening media							
TBA	*Tributyrin 1.0*	*Bacto-peptone 0.5*	*Beef extract 0.3*	-	-	-	-
T80	*Tween80 1.0*	*Bacto-peptone 1.0*	*NaCl 0.5*	-	-	-	*$CaCl_2$ 0.01*
M4.B	*Tween80 1.0*	*Tryptone 1.0*	*Yeast extract 0.5*	-	-	-	*$CaCl_2$ 0.1; neutral red*
Yeast and fungi - lipase screening media							
M4.D	*Glucose 2.0; Tween80 1.0*	*Tryptone 1.0*	*Yeast extract 0.5*	-	-	-	*$CaCl_2$ 0.1; neutral red*
YS	*Glucose 2.0; Tween80 1.0*	*Bacto-peptone 0.5*	*Yeast extract 0.5*	-	-	-	*$CaCl_2$ 0.01*
Bacteria – amylase screening medium							
GA	*Soluble starch 1.0*	*Bacto-peptone 0.1*	*Beef extract 0.3*	*NaCl 0.5*	-	-	-
Yeast and fungi - amylase screening media							
CDA	*Soluble starch 1.0*	*$NaNO_3$ 0.2*	*KCl 0.05*	*$MgSO_4*7H_2O$ 0.05*	*K_2HPO_4 0.01*	*$FeSO_4*7H_2O$ 0.001*	-

Table 2. Environmental sampling sites and their natural source in Romania

Sample type	Sample location	Coordonates
Water samples		
Salted lake	Lacul Negru, Ocna Sibiului	*45°52'29"N 24°4'0"E*
Salted lake	Lacul Randunica, Ocna Sibiului	*45°52'29"N 24°4'0"E*
Natural dam lake	Lacul Rosu, Harghita	*46°47'34"N 25°47'35"E*
Cold spring water	Piatra Neamt, Neamt	*46°55'39"N 26°22'15"E*
Soil, mud and sand samples		
Mud from salted lake	Lacul cu namol, Ocna Sibiului	*45°52'29"N 24°4'0"E*
Soil from an oil field	Videle, Teleorman	*44°16'N 25°32'E*
Soil from a limestone and marlstone extraction region	Tasca, Neamt	*46°52'49"N 25°58'52"E*
Soil	Constanta	*44°10'24"N 28°38'18"E*
Soil	Fetesti, Ialomita	*44°23'10"N 27°50'38"E*
Soil from cultivated field	Mircesti, Olt	*44°40'53"N 24°36'48"E*
Soil from cultivated field	Petresti, Dambovita	*44°38'59"N 25°20'27"E*
Garden soil	Nicolae Balcescu, Tulcea	*44°59'45"N 28°35'11"E*
Beach sand	Constanta	*44°10'24"N 28°38'18"E*
Plant materials		
Green moss from a rock	Bicaz-Chei, Neamt	*46° 91'08"N 26° 09' 11"E*
Spruce cones	Piatra Neamt, Neamt	*46°55'39"N 26°22' 15"E*
Beechnuts	Piatra Neamt, Neamt	*46°55'39" N 26°22' 15"E*
17 samples	**15 locations**	

In Figure 1 is graphically represented the percentage frequency of the different microbial groups (bacteria, yeasts and fungi) isolated from the collected samples. Bacterial strains were the predominant microorganisms and the most frequent genus was *Bacillus* sp. However, there was noticed a distinct distribution of microbial groups which might indicate that their growth is depending on specific environmental conditions (e.g. saline environments).

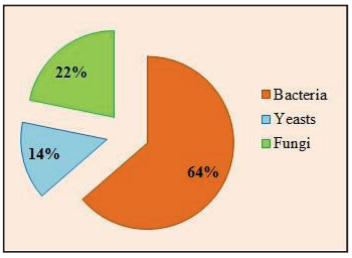

Figure 1. Microbial diversity of the newly isolated strains

In order to compare the bacterial diversity within all the 17 samples, some of them indicated that the bacterial population were the least diverse in beach sand and in plant materials and most diverse in soil and water. Fungi and yeasts were also predominant in soil and water samples (Figure 2).

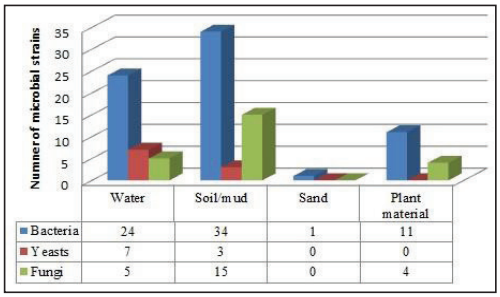

Figure 2. Ecology and microbial biodiversity of the newly isolated strains

The microorganisms' isolation is considered to be a necessary approach to obtain novel microbes and physiological characteristics for understanding their ecophysiological and environmental functions, and for their potential applications (Dang et al., 2009).

After isolation and purification, the microbial strains were morphologically characterised, noting their micro- and macroscopic aspects (e.g. diffusible pigment, size, shape and colour of the colonies; shape of cells, hyphae or spores were recorded). Regarding bacterial diversity, 56% of isolates (39 strains) were represented by Gram-negative strains and 44% (31 strains) by Gram-positive ones (Figure 3).

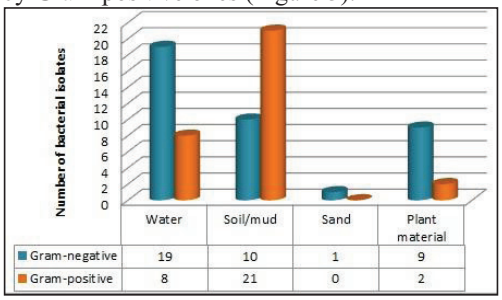

Figure 3. Gram-negative and Gram-positive isolates distribution in environmental collected samples

Screening of the isolates for enzymatic activity was performed using seven different culture media. 74% of the microbial strains were

considered positive for lipolytic activity and 26% for amylolytic activity, respectively.

The occurrence of clear/opaque zones around the colonies happened on all three lipase screening media only for few isolates. Extracellular lipolytic activity of tested microorganisms was noticed for 98% on tributyrin agar, 52% on tween80 agar and 25% on M4.B media (Figure 4 and 5). Areas of saponification with forming crystals were observed on YS medium and opaque zones with red to yellow colour variation were noticed for four yeast strains (Figure 6).

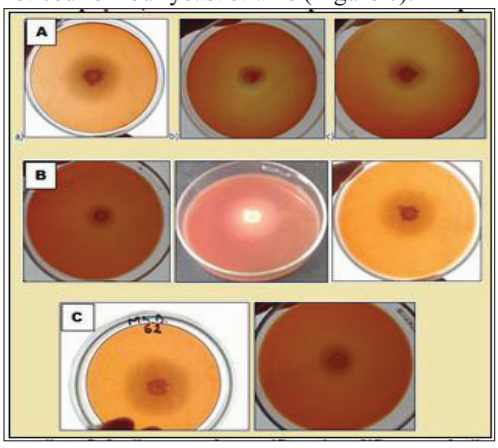

Figure 4. Different bacteria isolates on M4.B medium; opaque halos around colonies and colour variation formed due to the lipolytic activity

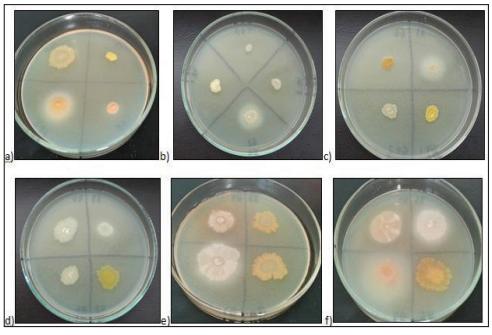

Figure 5. Different bacterial isolates on T80 medium; opaque halos around the colonies formed due to the lipolytic activity

Fungal strains were tested on the same media as yeasts, YS and M4.D, but the colonies could be observed only during the first 48 hours of incubation; after this time the fungal colonies were expanded and possible areas of opacity did not reveal.

Figure 6. Isolated yeast strains on YS medium; opaque halos around the colonies formed due to the lipolytic activity.

Screening using tween and tributyrin agar plates is frequently done for differentiation of lipase/esterase producers, but it can't be known for sure if the microorganisms are true lipase synthesizers. In the literature, tweens (fatty acid esters of polyoxyethylene sorbitan) are widely used as substrates for the detection of lipase/esterase producing microorganisms in solid media. The screening method is based on the precipitation as calcium salt of the fatty acids released by hydrolysis of tweens. Tween80 is mostly used as substrate, because it contains esters of oleic acids, which can be easily hydrolyzed by lipases and rarely by esterases. The liberated fatty acids bind the calcium ions incorporated into the medium (e.g. as $CaCl_2$); this complex can be observed as insoluble crystals around the microbial colonies (Kumar et al., 2012). Some divalent cations were reported to stimulate or to inhibit lipase production, including Ca^{2+} which is a metal cofactor able to stimulate the lipase activity of *Bacillus, Pseudomonas, Chromobacterium* and *Acinetobacter* sp. (Lu et al., 2013). However, the existence of triacylglycerol acylhydrolases [E.C 3.1.1.3] has to be verified by applying a biochemical method (e.g. titrimetric test – using olive oil as an inexpensive lipase substrate) (Kouker and Jaeger, 1987).

The most relevant results for lipolytic activity were noticed at 16 of the bacterial strains (isolation codes: 8, 12, 13, 15, 25, 28, 30, 45, 46, 49, 50, 54, 62, 63, 64, 92) and for three yeast strains (isolation codes: 78, 80, 86).

In Figure 7, some of the microbial strains which showed amylolytic activity on CDA and GA screening media are presented.

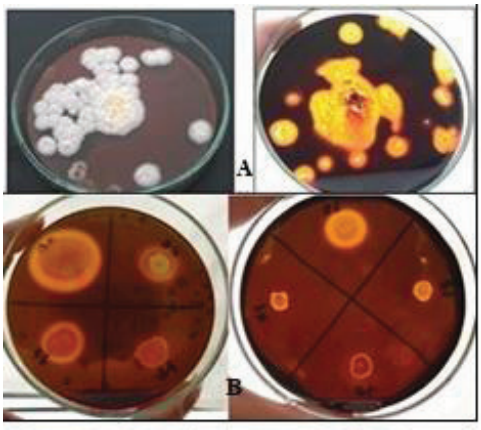

Figure 7. A) Fungal strain (isolation code 117) on CDA medium. B) Bacteria on GA medium. Plates flooded with Lugol's solution; clearly halos around colonies due to the amilolytic activity

Positive results for the amylolytic activity were noticed for 18 bacterial strains (isolation codes: 8, 9, 10, 16, 25, 28, 29, 35, 49, 50, 51, 54, 61, 70, 81, 89, 91 and 93) and for two fungal strains (isolation codes: 106, 117).

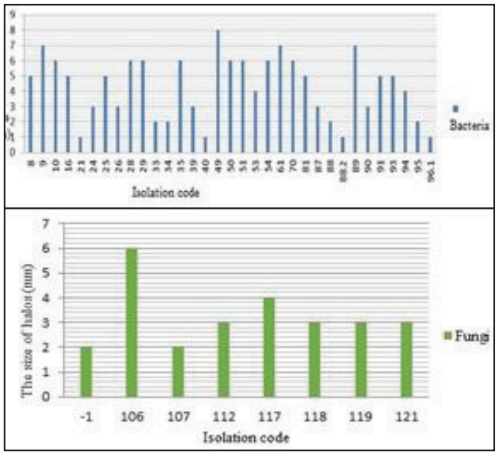

Figure 8. Bacteria and fungi – the size of halos (mm) corresponding to starch hydrolysis by amylase production

In figure 9 is presented the MALDI-TOF MS-dendrogram for some of the identificated microbial strains; also it can be noticed that *Bacillus sp.* is the predominant genus isolated from nature.

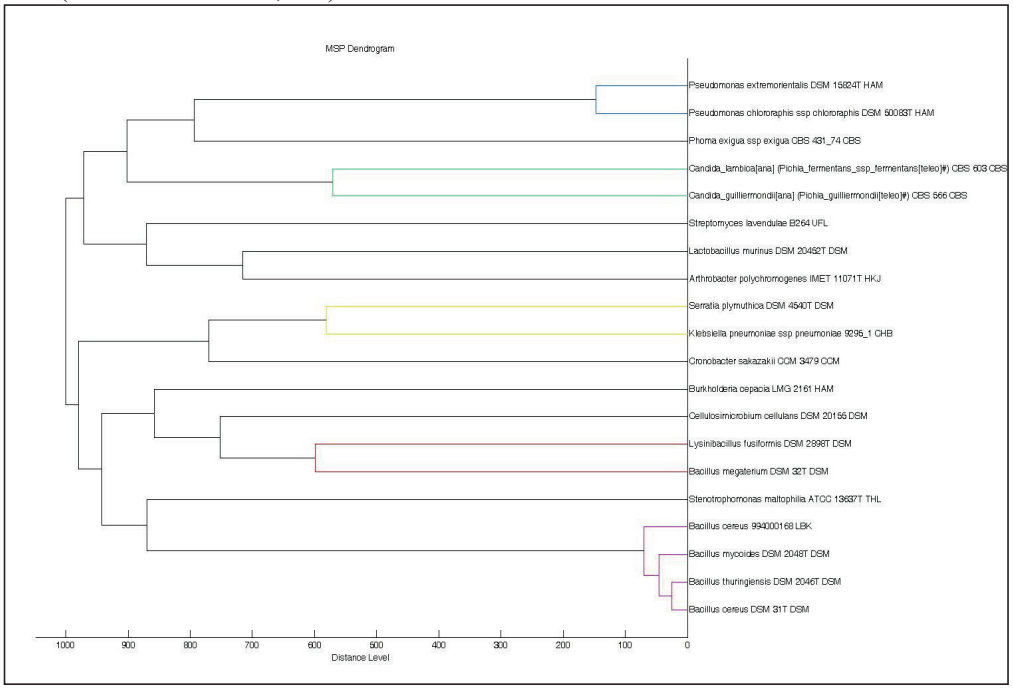

Figure 9. Identification of strains: the corresponding MALDI-TOF MS dendrogram

CONCLUSIONS

The present study reveals the importance of newly isolated microorganisms, wild strains from different natural environments, which can be a sustainable resource for bioprospecting novel bioactive molecules.

Therefore, it is important to consider their value as part of the continuously developing biotechnology. Thereby, 104 strains were isolated and screened for enzymatic activity on solid media: 54 strains were considered to have positive results for lipase production and 19 microorganisms showed clear zones of starch hydrolysis. Further studies were conducted and 22 isolates were identified by MALDI-TOF mass-spectrometry, including bacterial strains from diverse genera such as: *Bacillus* (the most predominant), *Pseudomonas*, *Serratia*, *Lysinibacillus*, *Cronobacter*, *Cellulosimicrobium* (former *Oerskovia*) and *Klebsiella* sp. (Figure 9).

One of the newly isolates, a yeast-like strain was identified by 18S ribosomal RNA sequencing and BLAST analysis (within another research collaboration) as *Galactomyces geotrichum* (to be published).

ACKNOWLEDGEMENTS

This research work was carried out with the support of the Ministry of Education and Scientific Research and National Institute for Chemical-Pharmaceutical Research & Development, Bucharest; the studies were conducted in the frame of the PN 09 11-04-03 grant project (2014-2015).

REFERENCES

Agrawal B., Mishra S., 2013. Extremophiles: Sustainable resources and biotechnological implications. First Edition, edited by Om V. Singh, published by Jhon Wiley&Sons, Inc., 291-315.

Annamalai N., Thavasi R., Vijayalakshmi S., Balasubramanian T., 2011. Extraction, purification and characterization of thermostable, alkaline tolerant α-amylase from *Bacillus cereus*. Indian Journal of Microbiology, 51(4): 424-429.

Banu C., 1987. Biotehnologii in industria alimentara. Ed. Tehnica, Bucuresti.

Dang H., Zhu H., Wang J., Tiegang L., 2009. Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough. World J. Microbiol. Biotechnol., 25: 71-79.

Dingle T.C., Butler-Wu S.M., 2013. MALDI-TOF mass spectrometry for microorganism identification. Clin. Lab. Med., 33(3):589-609.

Freiwald A., Sauer S., 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. Nat. Protoc., 4(5):732-742.

Khannous L., Jrad M., Dammak M., Miladi R., Chaaben N., Khemakhem B., Gharsallah N., Fendri I., 2014. Isolation of a novel amylase and lipase-producing *Pseudomonas luteola* strain: study of amylase production conditions. Lipids in Health and Disease, http://www.lipidworld.com/content/13/1/9, p.1-9.

Kouker G., Jaeger K.E., 1987. Specific and sensitive plate assay for bacterial lipases. Applied and Environmental Microbiology, 53(1): 211-213.

Kumar D., Kumar L., Nagar S., Raina C., Parshad R., Gupta V.K., 2012. Screening, isolation and production of lipase/esterase producing Bacillus sp. Strain DVL2 and its potential evaluation in esterification and resolution reactions. Arch. Appl. Sci. Res., 4(4):1763-1770.

Kumar S.G., Gurramkonda C., Rather G., Muniramanna G.S.C., Mangamuri U.K., Podha S., Choi Y.L., 2013. Glucoamylase from a newly isolated *Aspergillus niger* FME: detergent-mediated production, purification, and characterization. J. Korean. Soc. Appl. Biol. Chem., 56:427−433.

Lay J.O.Jr., 2001. MALDI-TOF mass spectrometry of bacteria. Mass Spectrom. Rev., 20(4):172-194.

Lu J., Brigham C.J., Rha C., Sinskey A.J., 2013. Characterization of an extracellular lipase and its chaperone from *R. eutropha* H16. Appl. Microbiol. Biotechnol., 97: 2443-2454.

Marvin L.F., Roberts M.A., Faya L.B., 2003. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in clinical chemistry. Clin. Chim. Acta, 337(1-2):11-21.

Rajesh B., Usha D.C., Aruna B.D., Bhaskar R.I., 2013. Screening and microbial characterization of lipase producing organic solvent tolerant *Lysinibacillus fusiformis* C5 (MTCC 11801). International Journal of Scientific & Engineering Research, 4(1): 427-431.

Ryzhov V., Fenselau C., 2001. Characterization of the protein subset desorbed by MALDI from whole bacterial cells. Anal. Chem., 73(4):746-750.

Sharma D., Sharma B., Shukla A.K., 2011. Biotechnological approach of microbial lipase: a review. Biotechnology, 10(1): 23-40.

Singh P.N., 2013. Review. Microbial enzymes with special characteristics for biotechnological applications. Biomolecules, 3: 597-611.

Singh S., Bali V., Sharma L., Mayala J., 2014. Production of fungal amylases using cheap, readily available agriresidues, for potential application in textile industry. Bio Med Research International, p. 1-9.

Singh S., Sharma V., Lal Soni M., 2011. Biotechnological applications of industrially important amylase enzyme. International Journal of Pharma and BioSciences, 2(1): 486-496.

Sivaramakrishnan S., Gangadharan D., Nampoothiri K. M., Soccol C. R., Pandey A., 2006. α-Amylases from

Microbial Sources. An overview on recent developments. Food Technol. Biotechnol., 44(2): 173-184.

Suarez S., Ferroni A., Lotz A., Jolley K.A., Guérin P., Leto J., Dauphin B., Jamet A., Maiden M.C., Nassif X., Armengaud J., 2013. Ribosomal proteins as biomarkers for bacterial identification by mass spectrometry in the clinical microbiology laboratory. J. Microbiol. Methods, 94(3):390-396.

Suribanbu K., Govardhan T.L., Hemalatha K.P.J., 2014. Characterisation of α-amylase producing *Bacillus mycoides* strains from Bay of Bengal, Visakhapatnam. Int. J. Pure App. Biosci., 2(1): 266-271.

Turki S., 2013. Towards the development of systems for high-yield production of microbial lipases. Biotechnol. Lett., 35: 1551-1560.

Underkofler L.A., Barton R.R., Renert S.S., 1958. Production of microbial enzymes and their applications. Appl. Microbiol., 6(3): 212-221.

Walker G.M, 1998. Yeast. Physiology and Biotechnology. Ed. John Wiley & Sons LTD, England, p. 305-306.

Zarnea G., Mencinicopschi G., Bragarea S., 1980. Bioingineria preparatelor enzimatice microbiene. Ed. Tehnica, Bucuresti.

FOOD SAFETY

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE PRESENCE OF SOME HEAVY METALS IN EDIBLE MUSHROOMS PACKAGED FOR COMMERCIALIZATION

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Abstract

Edible mushrooms are foods with a great nutritional value of which chemical composition differs from one species to another depending on the nutritive substrate, the development stage and microclimate conditions. The accumulation of heavy metals can take place in different phases of the technological process of growing, harvesting, conditioning, packaging as it becamed necessary to determine the acumulation of heavy metals from edible mushrooms.
The researches were done by using samples with commercial mushrooms (Agaricus and Pleurotus) packaged in bottle recipes, tin cans and on fresh samples, purchased from public sales units.
Determinations were done by using optical emission spectrometry with inductively coupled plasma ICP – OES, after the disaggregation with a microwave oven for digestion Berghof.
In the case of fresh mushrooms, the higher concentrations of zinc (4,16 mg/kg) and copper (3,15 mg/kg) can be explained by introducing them as zinc sulphide and copper sulphide in the nutritive substrate.
The mushrooms packaged in glass jars presented higher quantities of lead (0,44 mg/kg), this fact can be explained by the intense traffic in the vicinity of the mushroom farms or by soil composition where were grazing animals from which was obtained the fertilizer used in the substrat of culture.
The samples of mushrooms packaged in cans presented a higher quantity of tin (0,39 respectively 0,40 mg/kg) released probably from cans walls as a result of their deterioration. All the heavy metals existing in the samples were under the maximum admissible limits established by the legislation in force. In all the mushroom farms were identified residues of copper, zinc and lead. Tin was present just in the mushrooms packaged in cans.

Key words: *mushrooms, heavy metals, packages, commercialization.*

INTRODUCTION

Edible mushrooms are considered as foods with high nutritional value, their chemical composition differs from one species to another depending on many factors of which the nutritive subtrate, the development stage and microclimate conditions represents the most important factors. Mushrooms can be consumed both fresh (raw food or after cooking) and preserved.
Regarding the food value, mushrooms are very suitable to be associated with different foods or ingredients, taking part in this way in improving the food quality. Regarding the content of organic substances, edible mushrooms contain proteins (3-5%), glucides (1-3%), lipids (0,5-1%), non nitrate substances (1,5-7%), vitamins A, B1, B2 and D. It contains also malic, tartric and citric acid as well as tanning substances, ethereal oils and enzymes. Therefore, mushrooms represents a hybrid between vegetable and animal protein, and are very rich in Amino Acids (especially glutamic acid) wich gives them a similar taste to meat (Mecinicopschi, 2013). In the Table 1 can be observed the content in essential Amino Acids of the mushroom compared to other basic foods.

Table 1. The essential Amino Acids present in some basic foods reported to fresh substance (by INS)

Foods / Amino Acids	Milk	White bread	Rice	Cabbage	Lettuce	Potatoes	Mushrooms
Isoleucine	200	300	350	40	-	90	200
Leucine	350	600	600	60	-	100	200
Lysine	300	170	300	70	70	100	200
Methionine	90	120	130	13	4	20	50 - 200
Phenylalanine	170	400	400	30	-	90	30
Tryptophan	60	70	80	10	12	30	10
Valine	200	300	60	40	-	100	50 - 200
% proteins	10	10	7.5	1.4	1.2	2	2.5 – 3.5

Because of their chemical composition, mushrooms combat the tiredness, prevent the cardiovascular diseases and even cancer. In addition, it has a special taste and flavour, and can be used both for healthy and diabetic people (because it does not contain starch).

From an economic perspective the mushroom culture creates the opportunity to obtain substantially incomes by producers, using cheap raw materials (e.g. horse dung, cereal straw). Recovery mushroom culture is ensured throughout the year, because of the fact that the market is not saturated and the sale price is convenient for producer, the offer being smaller compared to the demand. Also mushrooms can be raw materials for canning factory (Mateescu, 1982).

In our country can be prepared the mycelium (material for inoculation) for ten species of mushrooms including two species of *Agaricus* (Champignon) and six species of *Pleurotus*. There are some species of mushrooms that are cultivated strictly for their medicinal properties, for obtaining drugs (penicillin, favicina, streptomycin) or are used as raw materials for the extractraction of some colorants (*Coprinus, Rusulla*).

The acumulation of heavy metals can take place in different phases of the technological process of growing, harvesting, conditioning, packaging as it becamed necessary to determine the acumulation of heavy metals from edibles mushrooms in order to establish the conformity of their content in heavy metals reported to the maximum limit legally allowed (Petcu, 2014). Also, it becamed notorious the ability of mushrooms to absorb heavy metals from the environment (known as biosorption) and the possibility of decontamination of the environment (of the soil) using this method, by cultivating on a large scale the macroscopic mushrooms (Das, Vimala, Karthika, 2008).

MATERIALS AND METHODS

The study was performed on sixteen samples of mushrooms from four types of packing such as: four samples of fresh mushrooms, four samples of cutted mushrooms packed in metal tins, four samples of whole mushrooms in cans belonging to the genus *Agaricus* and four samples of mushrooms in glass jars of the genus *Pleurotus*. For all of sixteen samples determinations were made in order to find lead, mercury, cadmium, zinc, tin, arsenic and copper. There have been used four samples (belonging to the lot) from each type of mushrooms that were taken from public sales units.

The samples were analyzed using optical emisson spectrometry with inductively coupled plasma (ICP – OES), which allows the determination of the concentration or of the total quantity of an element from the sample.

The determinations were done after a previously preparation of the samples, as the disaggregation with a microwave oven for digestion Berghof, according to the method described in the article „*Evaluation of Various*

Sample Preparation Procedures for the Determination of Chromium, Cobalt and Nickel in Vegetable" published in the Journal of Analytical Atomic Spectrometry in 1997 (Carlosena, Gallego, Valcarcel).

There was weigh from each type of mushrooms four samples having the weight mentioned in the Table 2, then were subjected to the digestion. After that the samples were let to cool for two hours and were made dilutions with bidistilled water then the samples were analyzed with a spectrometer.

Table 2. The weight of the 16 samples of mushrooms obtained after dissagregation and weigh

Type / Sample	Type 1	Type 2	Type 3	Type 4
S_1	$m_1 = 0,2993$	$m_1 = 0,3010$	$m_1 = 0,3017$	$m_1 = 0,2993$
S_2	$m_2 = 0,2878$	$m_2 = 0,3005$	$m_2 = 0,3015$	$m_2 = 0,2995$
S_3	$m_3 = 0,2990$	$m_3 = 0,3010$	$m_3 = 0,3010$	$m_3 = 0,2995$
S_4	$m_4 = 0,2995$	$m_4 = 0,3008$	$m_4 = 0,3015$	$m_4 = 0,2994$

The prepared standard samples were in concentration of 0,001 ppm, 0,1 ppm and 50 ppm for each element. Based on this standards was drawn the calibration curve of the device. In the preparation of the standards was used ultrapure water. The standard solutions of the used chemical element are inserted into the device, in this way being measured the intensity of the characteristic emission for each element. When is measured the intensity of the emission, it is compared with the specific calibration curve for measure the concentration corresponding to the intensity (Figure 1).

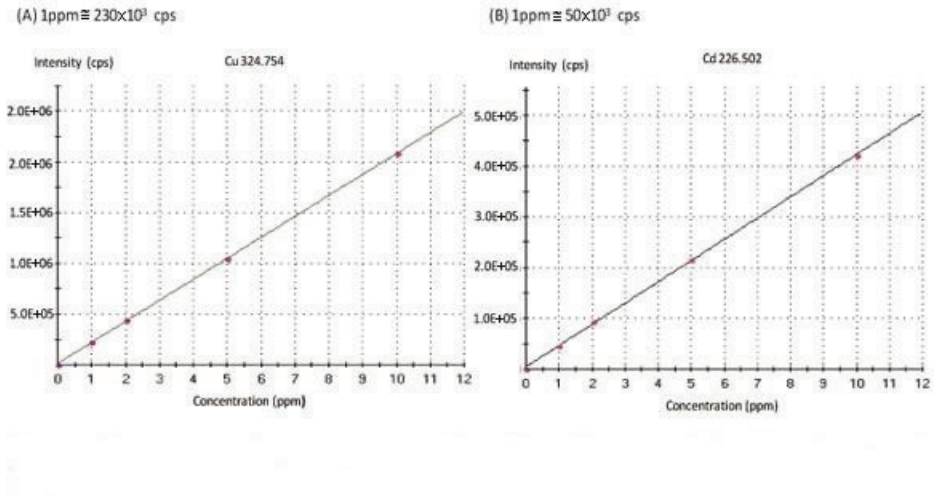

Figure 1. Standard curves for copper and cadmium used to convert between counts per second (cps) and concentration (ppm) (by Tonon, Oliveira, Soriano & Colepicolo, 2011)

RESULTS AND DISCUSSIONS

The determinations performed according to the methodology presented above, highlighted the following results for lead, mercury, cadmium, zinc, tin, arsen and copper as follows:

Table 3. Results obtained by analyzing the *Champignon* fresh mushroom samples

Mushrooms / Metals	S_1 mg/kg	S_2 mg/kg	S_3 mg/kg	S_4 mg/kg	Average mg/kg	LMA mg/kg
Pb	0.1222	0.2411	0.1833	0.1774	0.1810	0.5
Hg	0.0023	0.0025	0.0011	0.0016	0.0018	0.05
Cd	0.0511	0.0311	0.0500	0.0420	0.0435	0.1
Zn	4.5111	3.4911	4.5011	4.1663	4.1677	15
Sn	-	-	-	-	-	-
As	0.2122	0.2333	0.1997	0.2210	0.2150	0.5
Cu	3.1665	3.3000	2.9999	3.1496	3.1540	5.0

Regarding the Champignon fresh mushrooms can be observed the fact that all the determined values are situated below the maximum limit allowed by the regulations in force and that, compared to other analyzed metals, were detected higher levels of zinc and copper (Table 3). This fact can be explained by the presence of zinc sulphide and copper sulphide added in order to achieve the culture substrate necessary for mushroom growth.

Table 4. Results obtained by analyzing the *Pleurotus* mushroom samples packaged in glass containers

Mushrooms / Metals	S_1 mg/kg	S_2 mg/kg	S_3 mg/kg	S_4 mg/kg	Average mg/kg	LMA mg/kg
Pb	0.440	0.440	0.440	0.441	0.440	0.5
Hg	-	-	-	-	-	-
Cd	0.001	0.001	0.001	0.001	0.001	0.1
Zn	2.616	2.620	2.616	2.618	2.617	15
Sn	-	-	-	-	-	150
As	-	-	-	-	-	0.5
Cu	1.453	1.455	1.455	1.454	1.454	50

Regarding the *Pleurotus* mushroom samples packaged in glass jars, can be observed higher levels of zinc, copper and lead but that are also situated below the maximum allowed limits, as it can be seen in Table 4. A possible explanation for the lead can be given by the presence of some polluting sources near the place were are cultivated the mushrooms or for it presence in the culture substrate and in the manure coming from animals that were grazing on lands polluted with lead.

Table 5. Results obtained by anlyzing the integer *Champignon* mushroom samples packaged in metallic boxes

Mushrooms / Metals	S_1 mg/kg	S_2 mg/kg	S_3 mg/kg	S_4 mg/kg	Average mg/kg	LMA mg/kg
Pb	0.397	0.398	0.398	0.396	0.397	0.5
Hg	-	-	-	-	-	-
Cd	0.008	0.008	0.008	0.008	0.008	0.1
Zn	5.502	5.505	5.514	5.509	5.507	15
Sn	28.43	28.44	28.48	28.46	28.45	150
As	-	-	-	-	-	0.5
Cu	1.972	1.973	1.976	1.975	1.974	5.0

Regarding the mushroom samples packaged in metallic boxes, both in case of the integer and the sliced mushrooms can be observed higher quantities of tin even if it is below the maximum allowed limits (Table 5 and 6). An explanations of it presence can be given by the

release of tin from box lining as a result of the deterioration of the can. It appears when the inner layer is not perfectly adherent to the metal surface so that can take place the migration of the metal in the food that preserves. If the inner wall of can has a dark colour, gray to black, it means that tin or another element from the box compositon was released and was combined with the food.

Table 6. Results obtained by analyzing sliced *Champignon* mushroom samples, packaged in metallic boxes

Mushrooms / Metals	S_1 mg/kg	S_2 mg/kg	S_3 mg/kg	S_4 mg/kg	Average mg/kg	LMA mg/kg
Pb	0.400	0.400	0.400	0.400	0.400	0.5
Hg	-	-	-	-	-	-
Cd	0.025	0.025	0.041	0.039	0.032	0.1
Zn	6.047	6.118	6.072	6.063	6.075	15
Sn	33.351	34.000	33.335	33.349	33.508	150
As	-	-	-	-	-	0.5
Cu	1.829	1.828	1.912	1.907	1.869	5.0

In all samples obtained from all the mushroom types were observed significant lead concentrations but that were framed in the maximum allowed limits. The higher concentration was observed at the mushrooms packaged in glass containers and the lowest at fresh mushrooms.

The presence of heavy metals in the mushroom samples can be explained and by its bioabsorption ability, by its ability to retreive the heavy metals from the environment, the presence of cadmium, arsen and mercury in case of fresh mushrooms it can be attributed to this phenomenon.

CONCLUSIONS

All the heavy metals existing in the samples were below the maximum allowed limits established by the legislation in force.

In all the mushroom farms were identified residues of copper, zinc and lead, coming from the mushrooms culture substrate.

Tin was present just in the mushrooms packaged in metallic boxes, resulted from the crossing of the metal from the mushroom wall cans.

Mercury, cadmium and the arsen were present in the samples in lowest quantities in some cases below the detectable limits of the device.

REFERENCES

Carlosena A., Gallego M. & Valcarcel M., 1997. Evaluation of Various Sample Preparation Procedures for the Determination of Chromium, Cobalt and Nickel in Vegetable. Journal of Analytical Atomic Spectrometry, 12: 479-486.

Das N., Vimala R. & Karthika P., 2008. Biosorption of heavy metals - an overview. Indian Journal of Biotechnology, 7: 159-169

Lee, M. Susan, Metals in foods. A literature survey, No. 12, The British Food Manufacturing Industries Research Association, Surrey, UK, 1990.

Mateescu N., 1982. Producerea ciupercilor. Editura Ceres Bucuresti

Mecinicopschi, Gh., 2013. Cipercile- carnea sănătoasă a pământului. www.doctormenci.ro/sanatate/page/2

Petcu, C.D., 2014. Ambalaje utilizate în industria alimentară. Editura Granada Bucuresti.

Tonon A., Oliveira M., Soriano E. & Colepicolo P., 2011. Absorption of metals and chemical elements present in three species of Gracilaria (Gracilariaceae) Greville: a genus of economical importance. Revista Brasileira de Farmacognosia, 21(2).

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

OBSERVATIONS REGARDING THE LEVEL OF SOME MINERALS IN PARTIALLY DECARBONATED WATER

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Abstract

Water represents the essential natural resource needed for all biological processes in nature, having a particular importance for sustaining life. Nowadays drinking water has as main source, sourface waters and groundwaters that are the source of natural mineral waters. Mineral waters have a very important weight in our alimentation being intended both for daily consumption as for treating certain diseases. In order to satisfy consumers preferences, the producers resort to diversify the assortments of natural mineral water from the market, either by partially or totally degassing or by impregnating with extra carbon dioxide.

The study was performed on three assortments of partially decarbonated mineral water, aiming the level of nitrates, nitrites, chromium, copper, nickel by spectrophotometer method, using a UV-VIS SPECORD 205 spectrophotometer, respectively absorbtion spectrophotometer with graphite furnace.

After the determinations carried out, reading and interpretation of the results, it was noted that the case of assortment 3 at the studied batch, the level of nitrates (2,01 mg/l) was larger than that shown on the label, and nickel level was considerable higher (0,014 mg/l) compared with the level of other minerals determined.

All studied parameters were situated under maximum allowable limits, even if in case of assortment 3 at analyzed batch was noted a higher level of nitrates compared with the one shown on the label. In case of assortments 1 and 2 at analyzed batch the levels of nitrates, chromium, copper, nickel were situated under the values shown on the label. Even if the researched parameters are under the maximum allowable limits by the legislation in force, is needed a carefully monitoring by the competent institutions.

Key words: *heavy metals, minerals, partially decarbonated water.*

INTRODUCTION

ater represents the essential natural resource needed for all biological processes in nature, having a particular importance for sustaining life. Most of the water that exist around the world (approximately 97%) is represented by seas and oceans. In the ice caps exists more than 2% from the total water, and the stream, rivers, lakes, phreatic canvas and the atmosphere represents the rest of 1%. But unfortunately, this 1% usually represents the human supply source of water.

Data from WHO shows that, the minimum quantity of water necessary for the human body is 5 liters per day, from which approximately, 1,5 – 2 liters is represented by the consumed water. The perfomed estimations shows that at approximately 15 years the human water consumption became double, in the future being provided a global fresh water crisis.

The drinking water is a part of the water cycle in nature and has as sources surface waters and

groundwaters, from which comes the natural mineral waters (Figure 1) (Evans, Perlman, 2014). It has an important weight in alimentation being intended both for daily consumption as for treating certain diseases. Those waters comes from streams approved for these purposes, that are regularly verified (at least once a year) for the Ministry of Health bodies, and are containing in solution different minerals dissolved from the rocks through are circulating, due to which it has some specific therapeutic properties.

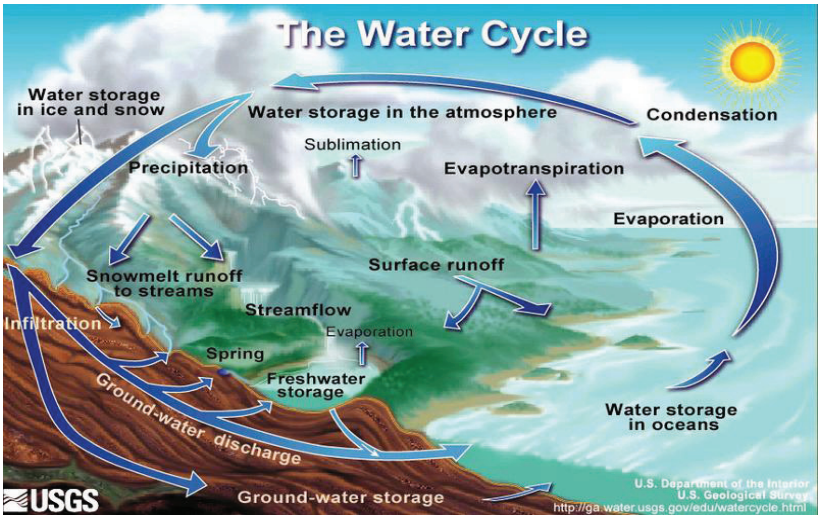

Figure 1. Water cycle in nature (by US Geological Survey, 2014)

Natural mineral water is the microbiologically pure water that comes from a phreatic canvas or from a underground aquifer deposit and comes from a source exploited by natural emerging or by drilling. It can be in a clearly way distinguished by the ordinary drinking water by its nature – characterized by the mineral components and as the case by some effects, and through its original purity that are maintained intact by the underground storage that protects against any pollution risk.

All the natural mineral water that are at one time on the market must be officially recognized by publishing in the official bulletins, at national and european level by mentioning the trade name, the extraction source and the bottling place.

In order to satisfy the consumers preferences the producers resort to diversify the assortments of natural mineral water from the market, either by partially or totally degassing or by impregnating with extra carbon dioxide (Caragea, 2010). In this way the owners of the sources of carbonated natural water are trying, using the degassing procedure (perfectly legal) to enter on the market with a product closer to plain water, while those who own sources of plain water impregnate the water with carbon dioxide (equally legal), entering in this way on the carboned water market which consumption is traditional mostly in Romania (Bărbulescu, 2003; Zamfirescu 2012).

Given the risk of pollution in the different stages of technological process - capture, degassing , packaging (Mănescu, 1991), considering human intervention on the environment (excessive soil fertilization with manure and synthetic) that led to the contamination of groundwater (especially with nitrates) (Goran, 2009; Jin 2004), regarding the necessity of being in accordance with standards , we undertook this study on the level of some minerals in partially decarbonated water.

MATERIALS AND METHODS

The study was performed on three assortments of partially decarbonated mineral water, packaged in PET, from different producers. There were used four samples (belonging to the same lot) from each assortment, collected after their purchasing from public sale units. The samples were analyzed aiming the level of nitrates, nitrites, by spectrophotometer method, using a UV-VIS SPECORD 205 spectrophotometer, and the level of chromium, copper, nickel using the absorbtion spectrophotometer with graphite furnace.

Figure 2. Spectrophotometer Specord 205

Figure 3. Spectrophotometer with graphite furnace

Determinations were performed according to ISO 7890-3 standard from 2000 that establish the method of determination of the nitrate ion and, acording to SR ISO 6777 standard from 2002 that establish the method of determination of the nitrates from the drinking water. With regard at chromium, copper and nickel, it were determined according to SR EN ISO 15586 standard from 2008 after the disaggregation with nitric acid specified in ISO 15587-2.
Were prepared standards for each analyte. Based on this standards was drawn the calibration curve of the device. After that were read the values of the concetration of substances and were reported the results for water, in micrograms per liter (µg/l). For the samples where was not obtained a detectable signal, the results were reported as being „below the detection limit".

RESULTS AND DISCUSSIONS

The determinations performed according to the methodology presented above, highlighted the following results for nitrates, nitrites, chromium, copper and nickel.

Table 1. Results obtained by analyzing water samples for nitrates

Type / Sample	Type 1 (mg/l)	Type 2 (mg/l)	Type 3 (mg/l)
S1	0,31	-	2,03
S2	0,30	-	2,07
S3	0,28	-	1,99
S4	0,35	-	1,95
Average	0,31	-	2,01

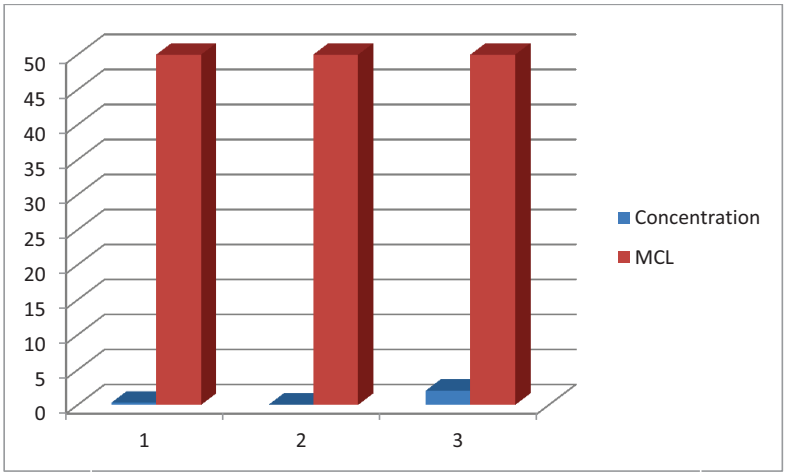

Figure 4. Nitrates concentration in the samples analyzed

It can be observed that the assortment no. 3 of partially decarbonated water is the one that has the higher content of nitrates that is a lot below the maximum limit allowed by the regulations in force (50 mg/l). Also it can be observed that in case of type no. 2 of analyzed water, the nitrate is below the detection limit of the device. Also, I could notice that in case of the type no. 3 the content of nitrates was higher than the one written on the label.

The determinations performed for nitrites and chromium shown that these two analytes were situated below the detection limit of the device.

From Table 2 results that all the three assortments of water anlayzed using the absorbtion spectophotometer with graphite furnance method to determine copper, is below the maximum allowed limits allowed by the legal norms, even if no. 3 was having a higher content (0,00464 mg/l).

It can be observed that from the three water types analyzed for the determination of nickel concentration, type no. 3 has the higher value (0,01424 mg/l) but is situated below the maximum allowed limit (0,02 mg/l).

The quantities of nitrate highlighted in the analyzed samples are corresponding to lowest limits compared to the detected values in the researches performed in Italy (1997) and India (2010).

The content of nitrites is undetectable in the case of our decarboned water samples compared to the researches performed in Italy where the content of nitrites exceeded the maximum limit allowed by the law (Montagna, 1997).

Higher concentrations were detected in our case at nickel 14,24 µg/l compared with lowest concentrations of nickel discovered in Serbia (4,00 µg/l) in 2012(Rajkovic).

Table 2. Results obtained by analyzing water samples for copper

Type / Sample	Type 1 (mg/l)	Type 2 (mg/l)	Type 3 (mg/l)
S1	0,0003	0,00107	0,00464
S2	0,0007	0,00104	0,00469
S3	0,0098	0,00109	0,00459
S4	0,0005	0,00108	0,00465
Average	0,0004	0,00107	0,00464

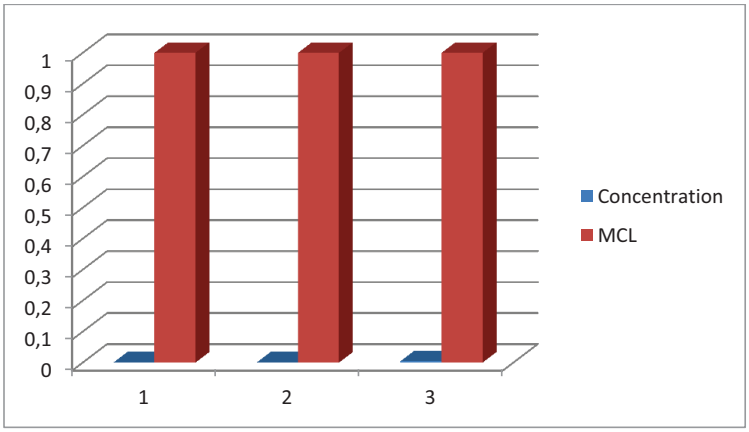

Figure 5. Copper concentration in the samples analyzed

Table 3. Results obtained by analyzing water samples for nickel

Type / Sample	Type 1 (mg/l)	Type 2 (mg/l)	Type 3 (mg/l)
S1	0,00123	0,00120	0,01429
S2	0,00125	0,00122	0,01424
S3	0,00120	0,00125	0,01418
S4	0,00124	0,00121	0,01427
Average	0,00123	0,00122	0,01424

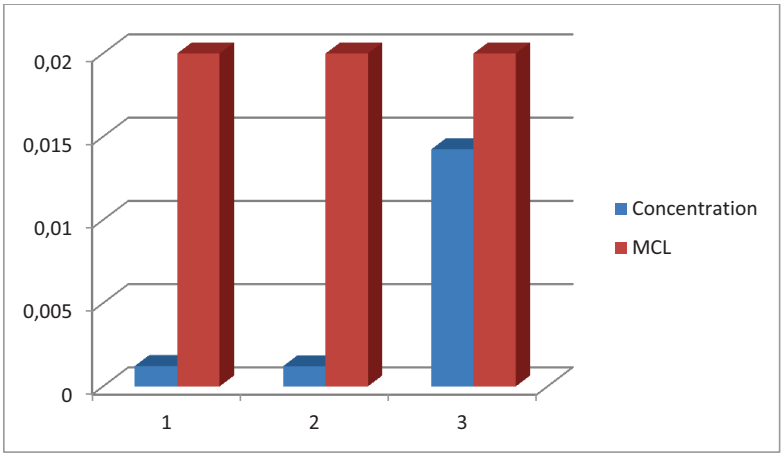

Figure 6. Nickel concentration in the samples analyzed

CONCLUSIONS

In all assortments of partially decarbonated water, all studied parameters presented values below the maximum limit allowed by the regulations in force.

The highest nitrate concentration was highlighted in the third studied type of mineral water. In this case the determined concentration was higher than the one written on the label.

In all cases nitrites and chromium were situated below the detectable limit of the device. Assortment no. 3 presented also the highest quantities of copper and nickel.

REFERENCES

Bărbulescu, Georgeta, 2003, Merceologie alimentară, Editura Didactică si Pedagogică Bucuresti

Caragea, Nela, 2010, Băuturi alimentare, Editura Terra Nostra, Iasi

Evans J., Perlman H., 2014, The water cycle, US Geological Survey

Goran G.V., 2009, Toxicologia produselor alimentare, Editura Printech, Bucuresti

Jin Z., Chen Y., Wang F., Ogura N., 2004. Detection of nitrate sources in urban groundwater by isotopic and chemical indicators, Hangzhou city, China. Environmental Geology 45:1017–1024

Mănescu S., Tănăsescu Gh., Dumitrache S., Cucu M., 1991, Igienă, Editura Medicală, Bucuresti

Montagna M.T., Signorile G., De Donno A., Bagordo F., Carrozzini F., 1997, Groundwater quality in Southern Salento. Journal of Preventive Medicine and Hygiene, 38: 5-9.

Rajkovic M., Sredovic Ivana, Racovic M., Stojanovic Mirjana, 2012, Analysis of Quality Mineral Water of Serbia: Region Arandjelovac, Journal of Water Resource and Protection, (4) 783- 789

Zamfirescu F., 2012, Ghidul apelor minerale naturale, Editura Novis, Cluj Napoca

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

EVALUATION OF ANTIFUNGAL ACTIVITY OF SELECTED LACTIC ACID BACTERIA STRAINS AGAINST SPOILAGE MOULD *PENICILLIUM EXPANSUM*

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Abstract

Various spoilage fungi that contaminate fruits, vegetables and other food commodities produce economic losses as well as harmful effects on human and animal health caused by ingestion of mycotoxins The public concern about the use of chemical fungicides during cultivation and storage of fruits and vegetable determined the search for new strains of lactic acid bacteria (LAB) able to control the fungal growth of mycotoxigenic species. The effect of 8 LAB strains have been assayed on the growth of Penicillium expansum isolated from infected apples. Interaction between LAB strains and selected spoilage fungi was tested by overlay assay method. Discrete spots of liquid cultures of tested LAB strains were placed on MRS agar and after incubation, overlaid with soft PDA containing fungal propagules. Data concerning the evolution of the diameters of clear visible inhibition zones of fungal growth around the LAB strain spots were monitored for 10 days. A number of 5 strains have shown antifungal activity with clear inhibition zone developed at 5 days, the effect of 2 strains being sustained until the 10th day of monitoring. P. expansum spores were cultivated with 3 efficient LAB strains on PD liquid medium and monitored for 17 days. The inhibition of spore forming lasted for 2 strains until the end of monitoring period and for the other strain until the end of the first week. Completely sporulated mycelium with powdery aspect was evidenced in control flask with pure culture of P. expansum after 5 days. Probably chemical interactions appeared between fungus and LAB strains that changed the color of culture media towards reddish-brown comparatively to control. The results could be used in biotechnological methods for the control of spoilage fungi with application in fruits preservation.

Key words: *antifungal activity, inhibition zone, lactic acid bacteria, Penicillium expansum.*

INTRODUCTION

A wide spectrum of filamentous fungi can contaminate various food commodities, with important economic losses around the world.

Fungal infection causes food spoilage with high impact on organoleptic properties. The harmful effects on human and animal health is determined by the contamination with mycotoxins produced by fungi belonging mainly to genera *Aspergillus, Fusarium* and *Penicillium* (Gerez et al., 2009; Bryden, 2012).

Penicillium expansum, the blue mold of apples is a mycotoxigenic species recognized for the capacity to produce patulin and citrinin as secondary metabolites with toxic effect when ingested (Watanabe, 2008).

The effects of highly toxic metabolites synthesized by fungal species on human health include carcinogenic, teratogenic, immunotoxic, neurotoxic, hepatotoxic and nephrotoxic (Bryden, 2007; Wild and Gong, 2009).

Even though several physical and chemical methods have been developed to control fungal growth, some moulds acquired resistance to chemical treatment and preservatives.

In the last decades, biopreservation, the control of one organism by another, has become an alternative to conventional methods motivated

by general public demands for preservatives-free food and feed (Schnurer and Magnusson, 2005).

Lactic acid bacteria have been considered natural biological antagonists for mycotoxigenic fungi that contaminate various fruits and vegetables (Trias et al., 2008). Blagojev et al. (2012) reviewed the possibility of using various species of lactic acid bacteria as biopreservatives for the control of mycotoxigenic fungi and the main mechanisms involved in antimicrobial efficiency of lactic acid bacteria such as: the yield of organic acids, competition for nutrients and production of antagonistic compounds. Species belonging to genera *Lactococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* are recognized for their ability to synthesize bacteriocins (microbial proteins) with antifungal effect against various mycotoxigenic fungi (Erginkaya et al., 2011). Muhialdin et al. (2011) listed a series of antifungal compounds produced by lactic acid bacteria and their spectrum range of inhibitory activity against mycotoxigenic species. Cyclic dipeptide released by *Pediococcus pentosaceous* inhibited *Penicillium expansum*, the bacteriocins produce holes in the membrane of the cell causing content leakage and alteration of the trans-membrane potential. Recent studies showed that lactic acid bacterial biofilms rich in exopolysccharides could be used as biocontrol agents (Ünal et al., 2011).

The aim of this work was to assess the effect of different lactic acid bacterial strains on growth of mycotoxigenic fungal species *Penicillium expansum* and to select performant strains as biopreservatives for fruits.

MATERIALS AND METHODS

The 8 strains of lactic acid bacteria were obtained from dr.Medana Zamfir, Institute for Biology Bucharest and have been assayed on the growth of possible mycotoxigenic fungus *Penicillium expansum* isolated from infected apples. The fungus was isolated by plating decimal dilutions of heavily infected apple fragments on PDA (produced by Merck KGaA Germany), after incubation at 25 ^{o}C for 5 days. Pure cultures were streaked on PDA in test tubes. Microscopic observation and photographs have been carried out for morphological characterization. Taxonomic identification was done according to determinative manual for food-borne fungi (Samson and van Reenen-Hoekstra, 1988). Interaction between lactic acid bacteria and selected fungi was tested by overlay assay method (Magnusson et al., 2003). Discrete spots of 48 hours liquid cultures of the 8 lactic acid bacterial strains were placed on MRS agar (Liofilchem Italy) and after 24 h incubation at 30^{o}C, overlaid with soft PDA containing propagules of test fungi. Data were collected after 72 hours incubation at 25^{o}C concerning the diameters of clear visible inhibition zones of fungal growth around the lactic acid bacterial strain spots. Values obtained represented the mean of three replicates per assay. The Petri dish cultures were monitored for the persistence of the inhibition zones over a period of 10 days. A reversion of the zone of inhibition after 96 h was recorded as a fungistatic action while those with inhibition zone for at least 7 days were recorded as being fungicidal in action (Adebayo and Aderiye, 2010). Fungal spores of *Penicillium expansum* were cultivated with 3 efficient strains of lactic acid bacteria on PD liquid medium and monitored for 17 days. Fifty milliliters of sterile PD broth containing 10^5 spores of *Penicillium expansum* mL^{-1} and 2 mL each of the LAB isolates were added into triplicate 100 mL Erlenmeyer flasks. In the control experiment, only 2 mL of sterile MRS broth was added to the PD broth containing 10^5 spores of *Penicillium expansum*. The flasks were incubated at 25°C for 5 days.

The percentage inhibition was determined by comparing the growth of the control with the treated fungi. The dry weight was determined

by drying the mycelia in an oven at 70°C for 48 hours. The data obtained represent the mean of 3 replicates per variant. The percent inhibition was calculated using the formula:

$$GI\ (\%) = C_o - C_F/C_o \times 100$$

where C_o was the dry weight of the control and C_F was the dry weight of fungal mycelium after inhibition by lactic acid bacteria (Hamed et al., 2011).

RESULTS AND DISCUSSIONS

Penicillium expansum isolated in pure culture from heavily infected apples (Figure 1) presented blue-green colonies with yellowish reverse and fruity odour. Microscopical aspects from Figure 2 reveal the specific conidiophores branching, the cylindrical metulae bearing 5-8 phialides and greenish subglobose conidia, smooth-walled.

The effect of 8 lactic acid bacterial strains on *Penicillium expansum* assayed by overlay method ranged from no visible inhibition to clear visible inhibition zone as shown in the aspect of petri plates. The assay results (Figure 3) showed that after 3 days 5 lactic acid bacterial strains (13, 15, 43, Lpa and Lpl) had inhibition zone diameter over 25 mm. Two strains (53, LCM5) had no inhibitory effect on mycotoxigenic fungus *Penicillium expansum* and one strain (64) failed to grow. The persistence of clear inhibition zones around 4 strains (13, 15, 43 and Lpa) until the 10th day suggest the fungicide effect against test fungus. The strain Lpl presented reversion of the inhibition zone after 6 days showing a potential fungistatic effect. The results are in accordance with data obtained in other studies: 9 of the 17 lactic acid bacterial strains isolated from indigenous Nigerian fermented foods exhibited a high fungicidal activity against spoilage fungi *Penicillium citrinum*, *Aspergillus niger*, *Aspergillus flavus*, as there was no reversion of the delay in growth caused by these strains before and after 7 days incubation (Adebayo and Aderiye, 2010).

Figure 1. *Penicillium expansum* - pure culture from infected apple

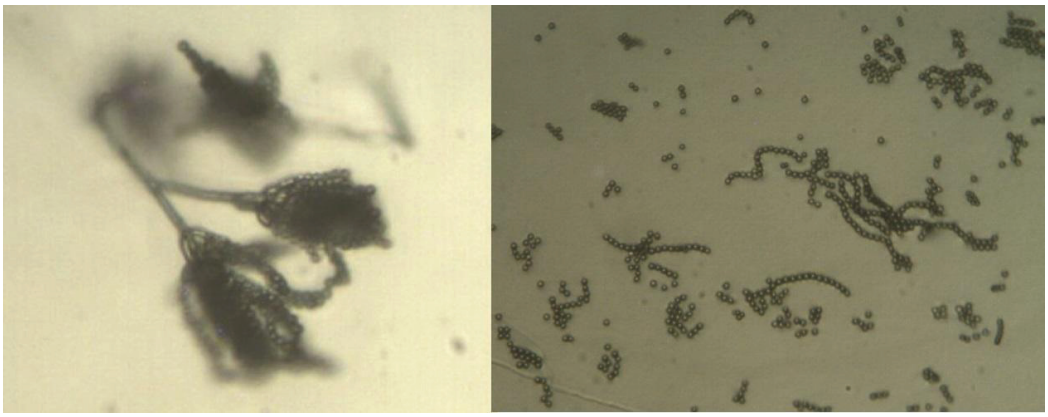

Figure 2. Microscopical aspect of conidiophores ramification (left) and chains of greenish conidia (right)

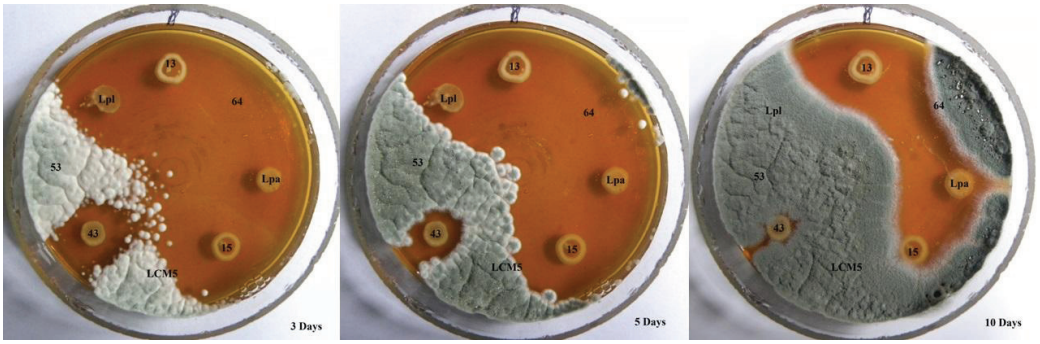

Figure 3. Evolution of inhibitory effect of lactic acid bacteria against *Penicillium expansum*

Our research by optic microscopy evidenced hyphal alterations of *Penicillium expansum* (Figure 4) with frequent loss of cellular content, fragmentation and delays in sporulation process as a consequence of the antifungal activity in the inhibition zone around lactic acid bacteria spots.

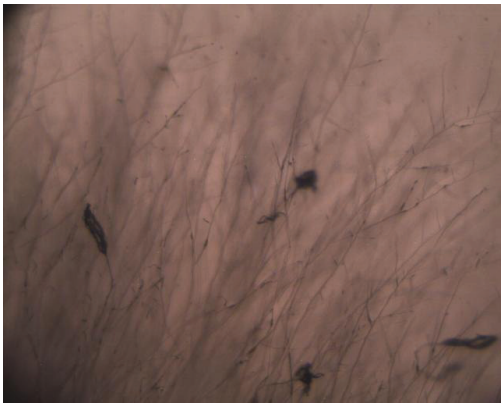

Figure 4. Microscopic aspect with altered fungal hyphae from inhibition zone

Results are in concordance with co-cultivation assay carried out by Ström (2005) when mycelial growth of mycotoxin-producing *Aspergillus nidulans* was 36% inhibited by *Lactobacillus plantarum* and increased vacuolization, disturbed branching and swollen hyphal have been described.

Trias et al. (2008) showed that 4 lactic acid bacterial strains isolated from fresh fruits and vegetables inhibited the development of infection produced by *Penicillium expansum* on apples by 20%, due to high capacity to produce organic acids. Concentrated culture filtrate of *Lactobacillus plantarum* 21B isolated from sourdough, with increased production of phenyllactic and 4-hydoxy-phenyllactic acid, presented efficient antifungal activity against 7 mycotoxigenic fungi including *Penicillium expansum* (Lavermicocca et al., 2000).

Antifungal activity of 3 lactic acid bacteria (strains 15, 43, Lpa) on fungal mycelia growth and sporulation was assessed in liquid media (Figure 5). All strains inhibited the sporulation of test fungus until the 5th day as compared with the control represented by pure culture of *Penicillium expansum* completely sporulated. Perhaps biochemical interactions appeared consequently to the beginning of fungal sporulation when co-cultivated with lactic acid bacterial strain Lpa, determining the color change of the medium towards reddish-brown compared with the control. The inhibition of spore forming lasted for the strains 15 and 43 until the end of monitoring period.

The growth inhibition percent of *Penicillium expansum* by the three lactic acid bacterial strains ranged from 3.40 % for Lpa to 21.96 % for strain 15 (Table 1). Literature also cites data from *in vitro* assay where various isolates of *Lactobacillus* showed a broad spectrum of antifungal activity ranging from no inhibition against *Rhizoctonia solani* to 75 % against *Fusarium oxysporum* (Hamed et al., 2011).

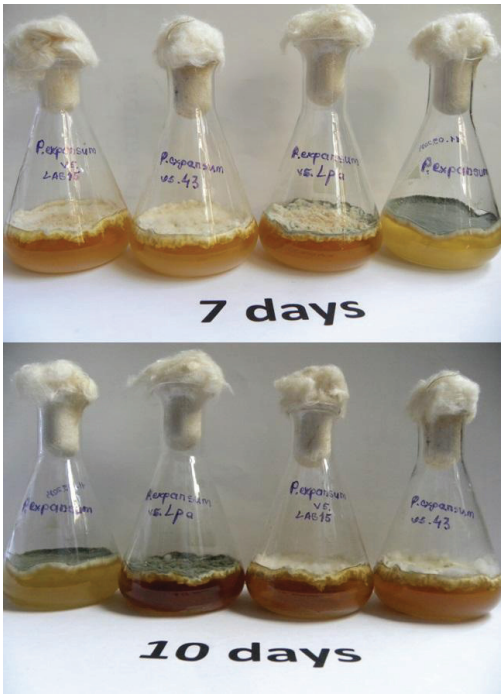

Figure 5. Evolution of antifungal effect of lactic acid bacteria against *Penicillium expansum*

Table 1.The dry weight and percentage growth inhibition of *P. expansum* by 3 strains of lactic acid bacteria

Variant	Dry weight (mg)	Growth inhibition (%)
P. expansum	2.64	Control
P.expansum - Strain 43	2.18	17.42
P.expansum - Strain 15	2.06	21.96
P.expansum- Strain Lpa	2.35	3.40

Other research reported partial (inhibition of conidia formation) or total (inhibition of conidial and aerial hyphae formation) inhibition of *Penicillium* expansum in the presence of yeasts isolated from apple or other fruits, as well as a ten times reduction of quantity of patulin (Taczman-Bruckner, 2005).
Our results obtained on liquid media confirmed the prolonged antifungal effect from overlay assay on solid media in concordance with data reported by Adebayo and Aderiye (2010) in experiments with lactic acid bacteria assayed for antifungal effect of cell free supernatants by agar well diffusion method and co-cultivation in liquid media.

CONCLUSIONS

A number of 5 lactic acid bacterial strains have shown antifungal activity on *Penicillium expansum.*
The lactic acid bacterial strains 13, 15, 43 and Lpa presented fungicidal activity against potential mycotoxigenic fungus.
The strain Lpl of lactic acid bacteria presented potential high fungistatic activity.
The effect of lactic acid bacteria in liquid media consisted in delaying the sporulation process and reduced dry weight of mycelium.
The results could be used in biotechnological methods for the control of spoilage fungi with application in fruits preservation.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

REFERENCES

Adebayo C.O. and Aderiye B.I., 2010. Antifungal Activity of Bacteriocins of Lactic Acid Bacteria from Some Nigerian Fermented Foods. Research Journal of Microbiology, 5, 1070-1082.

Blagojev N., Skrinjar M., Veskovic-Moracanin S., Soso V., 2012. Control of mould growth and mycotoxin production by lactic acid bacteria metabolites. Romanian Biotechnological Letters, 17, 7219-7226.

Bryden W.L., 2007. Mycotoxin in the food chain: Human health implications. Asia Pacific Journal of Clinical Nutrition, 16, 95-101.

Bryden W.L., 2012. Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. Animal Feed and Science Technology, 173, 134-158.

Erginkaya Z., Unal E., Kalkan S., 2011. Importance of microbial antagonisms about food attribution. In:

Science against microbial pathogens: communicating current research and technological advances A. Mendez-Vilas (Eds.), Adana, Turkey, 1342-1348.

Gerez C.L., Torino I.M., Rollan G., Fond de Valdez G., 2009. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. Food Control, 20, 144-148.

Hamed H.A., Moustafa Y.A., Abdel-Aziz S.M., 2011. *In vivo* Efficacy of Lactic Acid Bacteria in Biological Control against *Fusarium oxysporum* for Protection of Tomato Plant. Life Science Journal, 8(4): 462-468.

Lavermicocca P., Valerio F., Evidente A., Lazzaroni S., Corsetti A., Gobertti M., 2000. Purification and characterization of novel antifungal compounds from sourdough *Lactobacillus plantarum* strain 21 B. Applied and Environmental Microbiology, 66, 4084-4090.

Magnusson J., Strom K., Roos S., Schnurer J., 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology letters, 219, 129-135.

Samson A.R. and van Reenen-Hoekstra E., 1988. Introduction to food-bourne fungi, (Eds.) CBS Netherlands, 1-209.

Savadogo A., Ouattara C.A.T., Bassole I.H.N., Traore S.A., 2006. Bacteriocins and lactic acid bacteria – a minireview. African Journal of Biotechnology, 5, 678-683.

Schnurer J., Magnusson J., 2005. Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science and Technology, 16, 70-78.

Ström K., 2005. Fungal inhibitory Lactic Acid Bacteria-Doctoral thesis. Swedish University of Agricultural Sciences, Uppsala, 23-25.

Taczman-Bruckner A., 2005. Inhibition of fruit and vegetable spoilage mould *Penicillium expansum* with yeasts – Doctoral thesis. Corvinus Univeristy of Budapest, 6-8.

Trias R., Baneras L., Montesinos E., Badosa E., 2008. Lactic acid bacteria from fresh fruit and vegetables as biocontrol agensts of phytopathogenic bacteria and fungi. International microbiology, 11, 231-236.

Ünal E., Kalkan S., Erginkaya Z., 2011. Use of lactic acid bacteria biofilms as biocontrol agents. Science and Technology Against Microbial Pathogens, 207-209.

Watanabe M., 2008. Production of mycotoxins by Penicillium expansum isolated from apple. Journal of Food Protection, 71(8): 1714-1729.

Wild C.P., Gong Y.Y., 2009. Mycotoxins and human disease: a largely ignored global health issue. Oxford Journals-Carcinogenesis,31,71-82.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

RESEARCH REGARDING THE INFLUENCE OF DOUGH PREPARATION PROCESS ON THE ACRYLAMIDE LEVEL IN BREAD

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Abstract

The influence of the dough preparation process on the acrylamide level formed in different bread types was studied. For each preparation process – direct (PD) and indirect (PI), four types of bread: "Pan bread", "Simple bread stick", "Olive bread stick" and "Onion bread stick" were obtained.Water content, acrylamide level and chromatic parameters, CIELab (L, a*, b*) for each type of bread were measured. Acrylamide analysis was performed by GC/MS/MS-SRM, by using the internal standard method of labelled acrylamide (1,2,3-^{13}C), min. 99% purity.The results show that, acrylamide level obtained for the bread samples prepared through PI process is lower than the acrylamide level obtained for the bread samples prepared through PD process. Preparation of dough through PI process decreases the acrylamide level, in all tested samples, with 6.29% to 31.63% compared with the PD process.Regarding color parameters (L*, a*, b*), samples obtained through both PD and PI process, had similar variations. Also it was found no correlation between CIELab color parameters and the level of acrylamide.*

Key words**:** *acrylamide, bread, process, GC/MS/MS.*

INTRODUCTION

Since 1994, International Agency for Research on Cancer (IARC) classified acrylamide as potential carcinogen to humans (group 2A), and in 2001, Scientific Committee on Toxicity, Eco-toxicity and Environment demonstrated health risks and toxic properties of acrylamide such as neurotoxicity, genotoxicity, carcinogenicity and reproductive toxicity (IARC, 1994; Dybing et al., 2003; Wilson et al., 2006; Keramat et al., 2011).

In April 2002, scientists from National Food Administration in Sweden along with scientists from Stockholm University have raised fears, discovering that people consume acrylamide through their diet by eating common foods, such as bread, biscuits, chips, coffee, etc., at levels much higher than the dose allowed in drinking water (SCF, 2002). Their results were quickly confirmed by working groups consisting of experts from the World Health Organization, Food and Agriculture Organization, National Center for Food Safety and Technology (FAO/WHO, 2002).

In February 2005, The Joint FAO/WHO Expert Committee on Food Additives (JECFA) made an assessment of acrylamide in terms of food safety concluding its risks to human health and the need for efforts to be done in order to reduce the exposure to acrylamide (JECFA, 2005).

Acrylamide content in food varies from one type to another, depending on the raw material and technological process (343 mg/kg in cereals and cereal-based products, 477 mg/kg in potatoes and processed potato products, 509 mg/kg in coffee and green tea, 19 mg/kg in meat and offal, 17 mg/kg in dehydrated fruits and vegetables). In most countries, food products that contribute to acrylamide intake by dietwere: French fries (16-30%), potato chips (6-46%), coffee (13-39%), bakery products (10-30%), and

confectionery products (10-20%) (Svensson et al., 2003; Konings et al., 2003; Hamlet et al., 2005; Amrein et al., 2007; Olmez et al., 2008).

Processing conditions, such as fermentation time, baking time, and temperature as well as food matrix also influence formation and reduction of acrylamide.

This paper aim was to investigate the influence of dough preparation process (direct and indirect) on acrylamide content in several bread types.

By using direct process for dough preparation in obtaining bread processes like activation and adaptation of yeast to dough environment, yeast cell multiplication, lactic acid accumulation through lactic fermentation and flavor substances accumulation through alcoholic and acid fermentation are determined.

Obtained products were analyzed regarding CIELab chromatic characteristics in an attempt to correlate the color of products with acrylamide content.

MATERIALS AND METHODS

Dough preparation is one of the key steps in bakery products technological process. The quality of dough, after mixing and fermentation directly influence the quality of final products.

Two types of dough preparation processes were used: direct or mono-phase process (dough) – PD and indirect or bi-phase process (sponge – dough) – PI. For each process were obtained four types of products: "Pan bread", "Simple bread stick", "Olive bread stick" and "Onion bread stick" using the same 480 wheat flour type.

The main operations in dough preparation process were ingredient selection and scaling, mixing and fermentation.

Direct process (PD) of dough preparation consisted in mixing and kneading, in a single step, of all the raw and auxiliary materials: 2 kg flour, 300 mL sodium chloride solution (10% w/v), 400 mL of fresh yeast emulsion (15% w/v) and 590 mL of water. All components were mixed in a mixer for 10 minutes.The obtained dough was left in a fermenting room at a temperature of 30°C for 90 minutes. After fermentation time ended, corresponding ingredients were added: divided black olives and dehydrated onion, rehydrated in advance. Each type of bread was kneaded again for another 5 minutes. Dough was divided in pieces of 110 grams each for bread sticks and 600 grams for pan bread. Divided dough was properly modeled and placed in rectangular trays (20 mm x 100 mm) and were left in a steam fermenting room at 30°C and 85% relative humidity for 30 minutes (bread stick) respectively 45 minutes (pan bread). Bread types were baked in an oven with two overlapped chambers, with controlled temperature and time. "Bread sticks" were baked at 220°C for 40 minutes, while "Pan bread" was baked at 230°C for 40 minutes.

Indirect process (PI) of dough preparation consisted insponge preparation, which was then used to prepare the dough. Sponge preparation role is to obtain a favorable environment for both yeast cells propagation and fermentation for several compounds production: primarily lactic acid, which helps to improve dough characteristics and also taste and flavor of bread. Sponge was prepared using flour, water and yeast. Flour amount used was half of total amount of flour used for dough preparation. Flour: water ratio was approximately 2:1. All yeast amounts were used to obtain the sponge which was left for 2 hours at 30°C to ferment. After fermentation, remaining flour was added together with water and salt and then was mixed for about 10 minutes. Obtained dough was transferred in a fermenting room for 45 minutes at 30°C. After fermentation time was completed corresponding ingredients were added: divided black olives and dehydrated onion, rehydrated in advance. Each type of bread was kneaded again for another 5 minutes. Dough was divided, modeled and baked in the same way as in direct process.

Experimental samples obtained both through direct and indirect processes are presented in Table 1. Samples were obtained in 3 batches of 4 types prepared by 2 processes. For acrylamide analysis was used a homogenous sample from all 3 batches.

Table 1. Experimental variants for obtainingbread through direct and indirect process

Sample name	Ingredients, kg	Process type						
		Direct process (PD)			Indirect process (PI)			
		Dough preparation		Fermentation time, min.	Dough preparation			Fermentation time, min.
		Dough	Total	Dough/ Final fermentation	Sponge	Dough	Total	Sponge/ Dough/ Final fermentation
Pan bread (code 1 – PD) (code 2 – PI)	Flour	2.0	2.0	90/45	1.0	1.0	2.0	120/45/45
	Yeast	0.06	0.06		0.05	-	0.05	
	Salt	0.03	0.03		-	0.03	0.03	
	Water	1.20	1.20		0.6	0.6	1.20	
Simple bread stick (code 9 – PD) (code 10 – PI)	Flour	2.0	2.0	90/30	1.0	1.0	2.0	120/45/40
	Yeast	0.06	0.06		0.05	-	0.05	
	Salt	0.03	0.03		-	0.03	0.03	
	Water	1.20	1.20		0.6	0.6	1.20	
Olive bread stick (code 5 – PD) (code 6 – PI)	Flour	2.0	2.0		1.0	1.0	2.0	
	Yeast	0.06	0.06		0.05	-	0.05	
	Salt	0.03	0.03		-	0.03	0.03	
	Water	1.2	1.2		0.6	0.6	1.20	
	Olives	0.24	0.24		-	0.24	0.24	
Onion bread stick (code 7 – PD) (code 8 – PI)	Flour	2.0	2.0		1.0	1.0	2.0	
	Yeast	0.06	0.06		0.05	-	0.05	
	Salt	0.03	0.03		-	0.03	0.03	
	Water	1.2	1.2		0.6	0.6	1.20	
	Onion	0.24	0.24		-	0.24	0.24	

Equipment. Bread samples were obtained by using the following equipments: oven with two chambers and controlled time and baking temperature (Mondial Forni), dough mixer (Diosna), manual divider (Vitella), molding machine (Kohler). For acrylamide analysis a gas chromatograph (Trace GC Ultra) coupled with a triple quadruple mass spectrometer (TSQ Quantum XLS) purchased from Thermo Fisher Scientific, USA was used.

Methods. Moisture and acidity were analyzed according to SR 90:2007, lipids content and carbohydrates content were analyzed according to SR 91:2007, protein content was determined according to SR EN ISO 20483:2007, ash content was analyzed according to SR EN 2171:2010 and crude fiber content was determined according to SR EN ISO 6865:2002.

Acrylamide was analyzed using an internal GC/MS/MS method (Negoita et al., 2014; Negoita et al., 2015) adapted after Pittet et al., 2004; Nemato et al., 2002; Cheng et al., 2006.

Acrylamide concentration was calculated with the following formula:

$$C = [440 \cdot C_0 \cdot (100 - U1)]/[w \cdot (100 - U_2)] \ \mu g/kg \ bread$$

where:

440 Total sample volume extract (400 µL ethyl acetate + 40 µL triethylamine), µL

C_0 Acrylamide concentration measured by GC/MS/MS fromfood matrix, mg/L

$$[C_0 = \frac{1}{b} * (\frac{A_{aaN}}{A_{aaM}} * F_{ISTD} - a)]$$

w Amount of breadcrumbs used, g

U_1 Fresh matrix humidity, %

U2 Breadcrumbs humidity, %

b Calibration curve slope

A_{aaN} Area of corresponding acrylamide signal of food matrix

A_{aaM} Area of corresponding acrylamide signal of internal standard

$\frac{A_{aaN}}{A_{aaM}}$ Ratio between analyte signal area and internal standard signal area is defined as response factor corresponding to the

analyte from food matrix

F_{ISTD} Correction factor of internal standard, defined as ratio of matrix internal standard concentration and calibration internal standard concentration

a Intercept

Color evaluation of samples was made at room temperature, using a HunterLab colorimeter and Universal Software V4.01 Miniscan XE Plus. The CIELab'76 parameters were evaluated:

L* - Color luminance: 0 – black and 100 – white

a* - red-green coordinate: positive values are red, negative values are green and 0 is neutral

b*- yellow-blue coordinate: positive values are yellow, negative values are blue and 0 is neutral.

RESULTS AND DISCUSSIONS

Raw and auxiliary materials were analyzed regarding physico-chemical characteristics. The results are presented in Table 2.

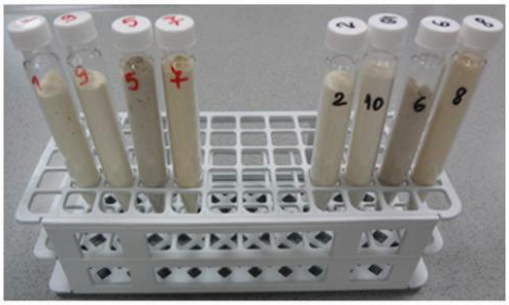
Table 2. Physico-chemical characteristics of raw and auxiliary materials

Characteristic	White flour, type 480	Sliced black olives	Dehydrated onion
Moisture, %	12.71	55.0	14.5
Ash, %	0.42	-	-
Proteins, %	10.04	2.4	10.5
Lipids, %	0.88	22.8	1.6
Carbohydrates, %	64.24	17.2	70.0
Fiber, %	0.61	-	-
Acidity, grades	2.44	-	-

All fourth bread types: "Pan bread", "Simple bread stick", "Olive bread stick", "Onion bread stick", obtained through both processes, were analyzed regarding acrylamide content and chromatic characteristics *CIELab*. Obtained samples (Figure 1) were transformed in breadcrumbs (Figure 2) by drying and grinding.

Pan bread (code 1 and 2) | **Simple bread stick** (code 9 and 10) | **Olive bread stick** (code 5 and 6) | **Onion bread stick** (code 7 and 8)

Figure 1. Experimental samples



1 - Pan bread **(PD)**
9 - Simple bread stick **(PD)**
5 - Olive bread stick **(PD)**
7 - Onion bread stick **(PD)**
2 - Pan bread **(PI)**
10 - Simple bread stick **(PI)**
6 - Olive bread stick **(PI)**
8 - Onion bread stick **(PI)**

Figure 2. Breadcrumbs of experimental bread samples

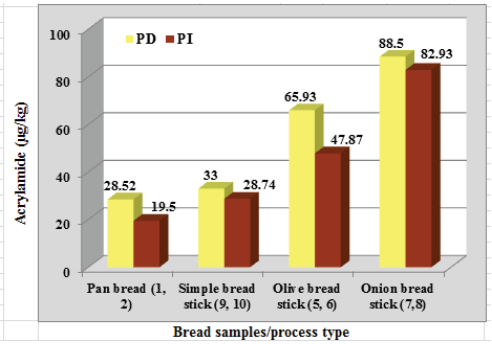


Figure 3. Acrylamide content in bread samples obtained by direct and indirect process

In Figure 3 are presented the results for acrylamide analysis in obtained bread samples.
Obtained results are according to other international researches (Fridriksson et al., 2004; CAC/RCP, 2009) which showed that a long time for dough fermentation induce a lower acrylamide level. This can be explained by the fact that both asparagine content and carbohydrates, main precursors in forming acrylamide, are consumed during fermentation. Asparagine is the source for nitrogen nutrition of yeasts while carbohydrates represents carbon source for yeasts growth and development.
Both samples obtained by direct (PD) and indirect process (PI) showed the same variations of the *CIELab* color parameters (Figure 4).

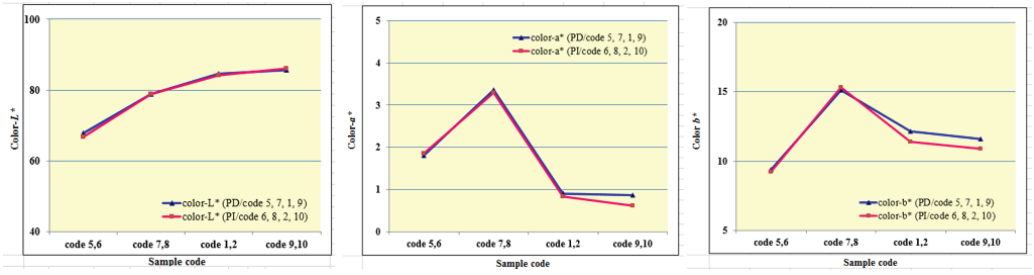


Figure 4. CIELab color parameters of bread types obtained by direct (PD) and indirect process (PI)

"Simple bread stick"(code 9, 10) and "Pan bread"(code 1, 2) samples, obtained by both processes, had the lowest acrylamide content (19.5 and 33 µg/kg) and the highest luminance values (84-86). In the case of "Olive bread stick", obtained by both PD and PI, acrylamide level do not correlate with their color. By evaluation of acrylamide level of bread samples obtained both direct (PD) and indirect process (PI) (Figure 3) with *CIELab* color parameters (Figure 4) it was found no correlation between those two.

CONCLUSIONS

Obtained results revealed that a longer fermentation of the dough, determines lower levels of acrylamide concentration. Thus, in the 4 experimental breads obtained by indirect process (PI) the acrylamide content was with 6.29% to 31.63% lower compared to samples obtained by direct process (PD).
Regarding color parameters, *L*, a*, b**, bread samples obtained through both direct and indirect process had the same variations.
There is no correlation between the acrylamide level of the achieved samples and their *CIELab* color parameters.″Simple bread

stick", "Pan bread" and "Onion bread stick" samples had highest luminosity values, while "Olive bread"samples had the lowest luminosity value, due to the main ingredient: olives.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of National Education – State Authority for Scientific Research, Technological Development and Innovation, by Nucleu Programme PN 12 48, contract 48 N/2012.

REFERENCES

Amrein T. M., Andres L., Escher F., Amado R., 2007. Occurrence of acrylamide in selected foods and mitigation options. Food Additives and Contaminants, 24, 13–25.

Cheng W. C., Hsiao S. W., Chou S. S., Sun-Hwang L., Lu T. J., Yeh A. I., 2006. Determination of Acrylamide in Chinese Foods by GC-Ion Trap MS Using 2-Bromopropenamide and 2-Bromopropenamide-^{13}C3,Journal of Food and Drug Analysis, 14(2), 207-214.

Dybing E., Sanner T., 2003. Risk assessment of acrylamide in foods. Toxicological Sciences, 75, 7-15.

Fredriksson H., Tallving J., Rosen J., Aman P., 2004. Fermentation reduces free asparagine in dough and acrylamide content in bread. Cereal Chemistry, 81(5), 650–653.

Hamlet C. G., Baxter D. E., Sadd P. A., Slaiding I., Liang L., Muller R., 2005. Exploiting process factors to reduce acrylamide in cereal-based foods. Report prepared on behalf of the U.K. Food Standards Agency, RHM Technology, High Wycombe, UK.

Keramat J., LeBail A., Prost C., Jafari M., 2011. Acrylamide in Baking Products: A Review Article, Food Bioprocess Technol., 4, 530–543.

Konings E.J.M., Baars A. J., Van Klaveren J. D., Spanjer M. C., Rensen P. M., Hiemstra M., Van Kooij J. A., Peters P. W. J., 2003. Acrylamide exposure from foods of the Dutch population and an assessment of the consequent risks. Food and Chemical Toxicology, 41, 1569–1579.

Negoita M., Catana M., Iorga E., Catana L., Adascalului A., Belc N., 2014. Determination of Acrylamide in Bread by Gas Chromatography – Tandem Mass Spectrometry, Romanian Biotechnological Letters, 19(4), 9561-9568.

Negoita M., Adascalului A., Iorga E., Catana L., Catana M., Belc, N., 2015. Internal validation of the method for determination of acrylamide in bread by gas chromatography tandem mass spectrometry, Revista de Chimie, 66(4), in press.

Nemato S., Takatsuki S., Sasaki K., Maitani T., 2002. Determination of Acrylamide in Foods by GC/MS Using ^{13}C-labeled Acrylamide as an Internal Standard, Journal of the Food Hygienic Society of Japan, 43(6).

Ölmez H., Tuncay F., Özcan N., Demirel S., 2008. A survey of acrylamide levels in foods from the Turkish market. Journal of Food Composition and Analysis, 21, 564–568.

Pittet A., Perisset A., Oberson J.M., 2004. Trace level determination of acrylamide in cereal-based foods by gas chromatography–mass spectrometry. Journal of Chromatography A, 1035, 123–130.

Svensson K., Abramsson L., Becker W., Glynn A., Hellenäs K.E., Lind Y., Rosén J., 2003. Dietary intake of acrylamide in Sweden. Food and Chemical Toxicology, 41, 1581-1586.

Wilson K. M, Mucci L. A., Cho E., Hunter D. J., Chen W. Y., Willet W. C., 2006. Dietary acrylamide intake and risk of premenopausal breast cancer. American Journal of Epidemiology, 169: 954-961.

***CAC/RCP 67-2009. (Code of Practice for the reduction of acrylamide in foods).

***FAO/WHO. Consultation on the Health Implications of Acrylamide in Food Geneva, 25-27 June (2002).

***IARC (International Agency for Research on Cancer), Lyon, France, 1994. Some industrial chemicals. IARC Monographs on the Evaluation of Carcinogenic Risk for Chemicals to Humans, vol. 60, p. 435.

***JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2005. Sixty-fifth meeting, Geneva.

*** SCF (Scientific Committee on Food), 2002. Opinion of the Scientific Committee on Food on new findings regarding the presence of acrylamide in food.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THERAPEUTICAL AND FREE RADICAL SCAVENGING PROPERTIES OF *CYNARA SCOLYMUS L.* LEAVE EXTRACTS

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Abstract

Cynara scolymus L. is a perennial herbaceous plant of the Asteraceae family and is one of the most common and important medicinal plants used of the Mediterranean etnopharmacy. In folk medicine Cynara scolymus L. leaf extracts have been widely used as astringent, blood cleanser, cardiotonic, detoxifier, digestive stimulant, diuretic and hypocholesterolemic because was proved to inhibit cholesterol biosynthesis and LDL oxidation. In the present study we investigated the antioxidant characteristics, of most chemical important constituents of extracts from leaves of Cynara scolymus L. (phenolic and flavonoid compounds, amino acids,) as well as of selective extracts obtained after technological processes (filtration, concentration, separation, precipitation). Antioxidant properties was studied using the chemiluminescence technique and DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The total flavonoids and polyphenols content was spectrophotometrically determined according to Romanian Pharmacopoea (FR). In addition the viability of cells were detected by MTS - assay that emphasize significant stimulation of the growth of mouse fibroblast 3T3 in a dose-dependent manner.

Key words*:chemiluminescence, Cynara scolymus, pharmacological test.*

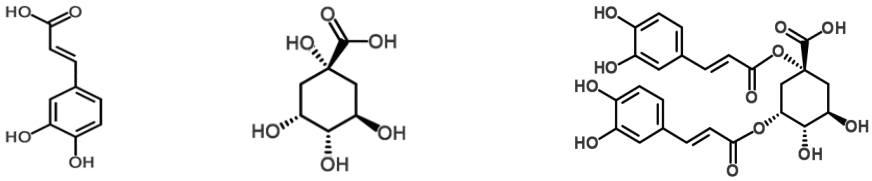
INTRODUCTION

Cynara scolymus L. is a plant herbaceous perennial belonging to family Asteraceae of Mediterranean origin, North Africa, Canary isles and Southern Europe.
It is well adapted to xerothermic conditions of Southern Europe (Moglia, 2008) (Raccuia, Cavallaro, & Melilli, 2004), (Gominho, 2001), (Bianco, 2005;).
A complex of biologically active compounds is responsible for its therapeutic properties.The principles important constituent of *Cynara scolymus* L. leaves include three principal chemical compounds classes:phenolic acids, flavanoids and sesquiterpene lactones. (Nasser, 2012)
Phenolic acids are represented the combinations of caffeic acid (Figure 1a) and quinic acid (Figure 1b) as well as by two important coumpounds cynarin (Figure 1c) and chlorogenic acid (Figure 2). Flavanoids compounds are represented mainly by glycoside of apigenin (apigenin-7-O- glycoside) and luteolin (Figure 3) including luteoline-7- glycoside (cynaroside) and luteoline-7- rutinoside (sculomoside). Sesquiterpene lactones represented by the cynaropicrin (Figure 4), dehydrocynaropicrin, cynaratriol (Figure 5).
(Brand, 1999), (Brand Z. N., 1990) (Nasser, 2012)
In addition, a total of 15 amino acids were found in *Cynara scolymus* L. leaves (aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine (Orlovskaya, 2007) **.**

Leaves of *Cynara scolymus* L. have been used for hepatoprotection and as a choleretic and diuretic for its lipid-lowering, hepato stimulating, and appetite-stimulating actions. Different pharmacological researches about *Cynara scolymus* L. have demonstrated their health-protective potential, especially their anticarcinogenic, hypocholesterolemic, anti-HIV activities, bile-expelling, lipid-lowering effects and antimicrobial agent. (Xianfeng Zhu, 2004)

(Kraft, 1997), (Kirchhoff R. et.al, 1994), (Gebhardt, 1997), (Gebhardt R. , 1998). (McDougall, et al., 1998).

Should be emphasized the fact that, artichoke is a potential good source of antioxidant activity because it contains large amounts of caffeic



a **b** **c**

Figure 1. Chemical structure a) caffeic acid b) quinic acid c) cynarin

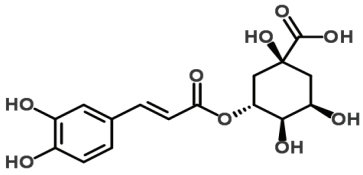


Figure 2 Chemical structure of chlorogenic acid

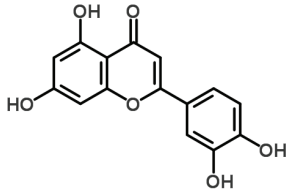


Figure 3. Chemical structure of luteolin

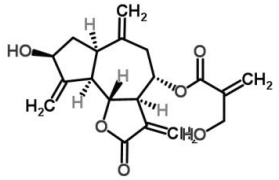


Figure 4. Chemical structure of cynaropicrin

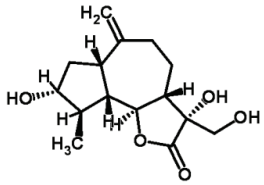


Figure 5. Chemical structure of cynaratriol.

acids. Caffeic acid derivatives are the main phenolic compounds in *Cynara scolymus* L., with a wide range of caffeoylquinic acid derivatives with chlorogenic acid (5-O-caffeoylquinic acid) as the most important of these derivatives. (Liorach, 2002),
(Chen & Ho, 1997) (Tomás-Barberan, Ferreres, & Gil, 2000) (Lattanzio, Cardinali, di Venere, Linsalata, & Palmieri, 1994).
In the present study, we show that *Cynara scolymus* L. extracts possesses the ability to scavenge free radicals and reduce oxidative stress. Phytochemical studies of *Cynara scolymus* L.extracts revealed that several phenolic compounds greatly contribute to the antioxidant activities of this plant. (Sheng-Yang Wang, 2003), (Simonetti, Gardana, & Pietta, 2001).

MATERIALS AND METHODS

Plant materials. The *Cynara scolymus* L. leaves are commercial samples, obtained from FARES: S.C. Romania.
Chemicals. Aluminium chloride, Sodium acetate, Folin-Ciocalteu phenol reagent, Arnow reagent, ethanol, methanol, acetic acid, rutin, quercetin, chlorogenic acid, caffeic acid, glutamic acid, glycine, methionine, leucine, tyrosine, histidine, were purchased from Sigma-Aldrich and ultrapure water (Millipore water system).
Reagents for antioxidant activity/ radical scavenging activity determination : luminol. 5-amino-2,3-dihydrophthalazine-1,4-dione-H_2O_2 in buffer TRIS-HCl, at pH 8.6 (chemiluminescence methods) and DPPH (α,α−Diphenyl−β−Picrylhydrazyl).
Reagents for in vitro pharmacological tests: Dulbecco's Modified Essential Media (DMEM), Fetal Calf Serum (FCS) and antibiotics (penicilin and streptomycin) were purchased

from Sigma-Aldrich (St. Louis, MO). Indicators-MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was procured from Promega GmbH.

Cell cultures: 3T3 fibroblasts obtained from ATCC (LGC Standards, Germany) were cultured in DMEM supplemented with 10% FCS and 1% antibiotics (10,000 units/ml penicillin and 10,000 µg/ml streptomycin in 0.85% saline).

Equipments. Soxhlet extraction system, *Spectrophotometer* UV-Vis, Jasco, Japan V-570 for DPPH method and quantitative determination of flavonoids, polyphenols, polyphenol-carboxylic acids and total hydroxicinnamic derivates .

Digital Rotary Evaporator RE100-Pro LCD (Dragon Laboratory Instruments Limited)

Chemiluminometer (Sirius Luminometer Berthelot - GmbH Germany): for *antioxidant activity* measurements by chemiluminescence tehnique (CL).

Qualitative analysis (LC-ESI-MS *analysis)*. Qualitative information was gathered using a mass spectrometer instrument LCMS Shimadzu 2010 EV, consisting of a single quadrupole analyzer and a diode array detector, operated in the negative electrospray mode and by direct infusion of sample. Ion source parameters were as follows: capillary voltage 1.5kV, interface voltage 2.5kV, source temperature 120^0C, desolvation temperature 250^0C, nitrogen gas flow 1.5l/min and the sample injection flow rate of 10µl/min. The acquisition was made both in a total ion scan mode (from 50 to 600 m/z) and by single ion monitoring (of ions of interest: 179, 353, 431, 447, 515 m/z). Sample solutions were filtered with a 0.45-µm (pore size) disposable syringe filter (Sigma-Aldrich)

Quantitative analysis: The quantitative determination of the total flavonoids content, total polyphenols content, polyphenolcarboxilic acids and the specific physical-chemical indicators were done according to the FR X (Romanian Pharmacopoea, 1993), (Ciulei, Istudor, Palade, Albulescu, & Gard, 1995)

Antioxidant activity

CL method. The antioxidant activity (AA%) of samples (CN1, CN2, CN3, CN4, CN5 –has been determined and compared with that of pure standards: Rutin, quercetin, chlorogenic acid, caffeic acid, glutamic acid, glycine, alanine, methionine, leucine, tyrosine, histidine were purchased from Sigma-Aldrich.

Chemiluminescence method (CL), was applied using luminol - H_2O_2 as generator system, in tampon TRIS-HCl, pH= 8.4 by using Sirius Luminometer Berthelot - GmbH Germany. The antioxidant activity of samples was calculated by using the relation (Iftimie N., 2004)

$$AA\,\% = \frac{I_0 - I}{I_0} \cdot 100$$

where: I_0 =the maximum CL for standard at t=5 s; I= the maximum CL for sample at t =5 s.

DPPH Radical Scavenging Activity

The free radical scavenging activity (SR%) was quantitatively tested using 2, 2'-diphenyl-1-picrylhydrazyl according to the modified method of Brand-Williams *et al.* (Brand-Williams W, 1995). A DPPH solution (80 µM) was freshly prepared in 95% methanol. A volume of 250 µl of this solution was allowed to react with 35 µl sample and the absorbance was measured at 515 nm. The radical scavenging activity (SR%) was calculated as follows:

$$SR\% = 100\left(1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right)$$

The $DPPH^{\bullet}$ assay was repeated three times.

RESULTS AND DISCUSSIONS

Obtaining of vegetal selective extracts from Cynara scolymus L. leaves

The vegetal selective extracts (CN1, CN2, CN3, CN4, CN5) were obtained by a succession of technological stages, consisting in the first stage in the solid-liquid extraction in a Soxhlet installation. Following the extraction procedure (3 cycles of extraction), the vegetal material used was removed, and the obtained filtrates were brought together (crude extract CN1) were processed by vacuum concentration (Digital Rotary Evaporator RE100-Pro LCD, water bath at constant temeperature) until obtaining a residue which, passed through successive precipitations with polar and non-polar solvents, centrifugation, filtering at low pressure and purification. The various fractions extractive (CN2, CN3, CN4, CN5) of non-

hygroscopic, fine powdery samples have been obtained by variation of the operational parameters: crushing degree of the plant, the solvent used for, plant-solvent ratio, extraction time, temperature, type of concentrating, precipitating and purifying.

Qualitative analysis

For crude extract of Cynara scolymus L. obtained by Soxhlet method (CN1), before carrying out the processing, was assessed qualitatively analysis by identifying representative chemical compounds.The direct injection negative ion mode MS spectrum of crude extract of Cynara scolymus L leaves showed the presence of only two pseudomolecular peaks with 191 m/z and 447 m/z (Figure 6). On the basis of their m/z these peaks could be easily ascribed to caffeoylquinic acids that were previously identified in many plants, especially in *Cynara scolymus* L. Also the molecular ion, m/z = 191 is found in fingerprint in the following compounds: quinic acid, 1-O-caffeoylquinic acid, 5-O-caffeoylquinic acid (chlorogenic acid), 3,5-di-O- caffeoylquinic acid, 1,5-di-O-caffeoylquinic acid,belonging to the chemical composition of *Cynara scolymus* L leaves. (Schutz, 2004). In mass spectra (Scan mode TIC- total ion current) were identified at m/z 353 (chlorogenic acid), m/z 515(cynarin) value of the deprotonated molecular ion. The structures of peaks with $[M-H]^-$ at m/z= 133, m/z= 215, m/z= 285, m/z= 327, m/z= 379, m/z= 425, m/z=489 can be attributed to molecular fragments generated from isomers of caffeic acid, quinic acid, dicaffeoylquinic acid etc, and the other compounds. (Kapusta, et al., 2013)

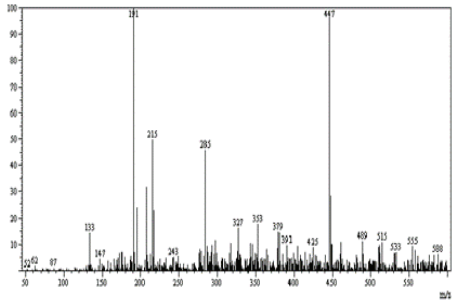


Figure 6. MS fingerprint for crude extract CN1 (SCAN mode by direct injection

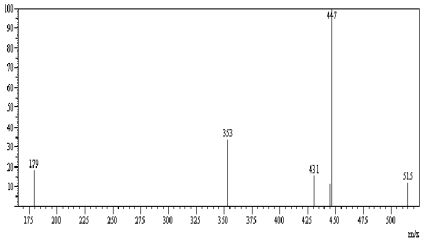


Figure 7. MS fingerprint for crude extract CN1 (SIM mode by direct injection)

Table 1. Chemical compounds identified in crude extract of Cynara scolymus L leaves – SIM mode

Compounds	Molecular formula	Molecular weight g·mol^{-1}	$[M-H]^-$ m/z
Caffeic acid	$C_9H_8O_4$	180.16	179
Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	353
Apigenin-7-O-glycoside	$C_{21}H_{20}O_{10}$	432.38	431
Luteolin-7-O-glycoside	$C_{21}H_{20}O_{11}$	448.38	447
Cynarine	$C_{25}H_{24}O_{12}$	516.45	515

The figure 7 and table 1 show, the compounds identified by ESI-MS in the negative single ion monitoring (SIM).

Quantitative analysis:

Table 2. Physical-chemical characteristics from extract of Cynara scolymus L.

Samples	**CN 1**	**CN2**	**CN3**	**CN4**	**CN5**
Ash %	-	2.14	1.98	2.10	1.87.
Humidity %	-	3.95	3.74	3.21	4.11
Flavonoids, mass % (as rutin)	0.93	2.72	2.41	3.02	2.27
Polyphenols, mass % (as gallic acid)	1.07	3.41	3.56	7.11	4.29
Polyphenol carboxilic acids, mass % (as caffeic acid)	1.14	3.29	2.73	4.89	4.67

Table 2 shows the amount of flavonoids expressed in rutin, total polyphenols expressed in gallic acid and the polyphenol carboxylic acids expressed in caffeic acid for the samples analysed (CN1, CN2, CN3, CN4, CN5)

In the case of crude extract (CN1) is observed smaller amount of flavonoids, total polyphenols, polyphenol carboxylic acids. After application the specific processing, for crude extract it is found a noticeable increase, of the amount of biologically active chemical compounds (CN2, CN3, CN4, CN5).

The total phenolic content ranged in a wide range from 1.07%-7.11%. The flavonoids and polyphenol carboxylic acids contents, followed a similar pattern as the total phenolics, but the increase was not so great (table 2).

Antioxidant activity

The antioxidant activity evaluated by CL and the results of DPPH radical scavenging activities is shown in Figure 9. Both of these methods demonstrated similar values for samples analyzed, as well as, for biological chemical compounds responsible for the therapeutic action. It should be noted antioxidant activity values of *Cynara scolymus* L leaves crude extract (CN1) and high levels for selective extracts. Processing technologies, of crude extract (CN1) led to the enrichment of active principles (flavonoids, total polyphenols, polyphenol carboxylic acids), which generates a very high antioxidant activity between $61.9_{CL}/60.2_{DPPH}$ - $94.5_{CL}/93.7_{DPPH}$. We can mention that the values AA%/SR% obtained for selective extracts (CN2, CN3, CN4, CN5) are comparable to those of standards tested.

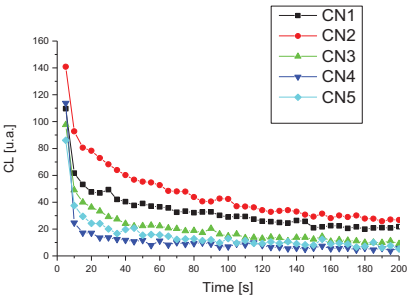


Figure 8.CL evolution in time of the samples CN1, CN2, CN3, CN4

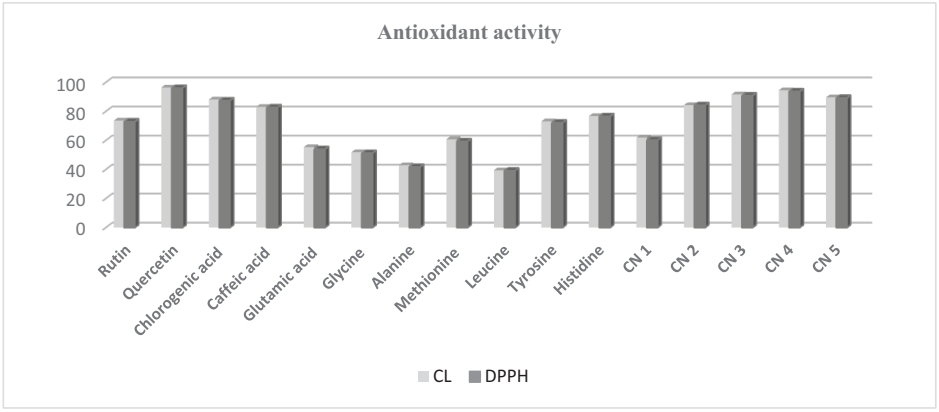


Figure 9. Evaluation of antioxidant activity by CL and DPPH method.

Cell viability

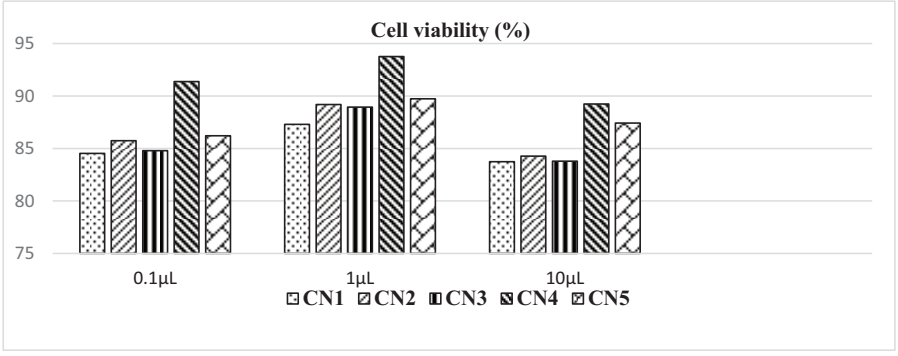


Figure 10. Evaluation of cell viability

The effect of crude extract (CN1) and of various selective extracts of *Cynara scolymus* L leaves (CN2, CN3, CN4) on cell viability in 3T3 fibroblasts cells is shown in Figure 10 .
The cell viability of 3T3 fibroblasts cells was increased with the treatment of various selective extracts. Tests performed with samples CN1, CN2, CN3, CN4, CN5 had a dose-dependent effect on cell viability. The percentage of cells viability at 24 h was between 84.53% - 91.37% in cells incubated. with 0.1µL samples, 87.29% - 93.75% in cells incubated with 1µL samples and respectively 83.74% - 89.23% in cells incubated with 10µL samples. May be notice two aspects, that the best results were obtained from incubation of samples 1 µL and the sample with the most satisfactory results is CN4, which shows the best values of amount of the biologically active chemical compounds, as well as the high antioxidant activity (94.5% $_{CL,}$ 93. 7$_{DPPH}$).

CONCLUSIONS

Qualitative and quantitative analysis, highlights chemical composition of the extracts of *Cynara scolymus* L. leaves. The value of antioxidant activity and phytochemical analysis results suggest that the design of biotechnology-processing of *Cynara scolymus* L. extract can lead to obtain selective extracts enriched in active principles. Antioxidant properties evaluated by the chemiluminescence technique and DPPH(2,2-diphenyl-1-picrylhydrazyl) method emphasized significant values. The total flavonoids and polyphenols content was spectrophotometrically determined according to Romanian Pharmacopoeia (FR). In addition the viability of cells were detected by MTS - assay have emphasized significant stimulation of the growth of mouse fibroblast 3T3 in a dose-dependent manner but were also emphasized of the antioxidant properties and amount of biological compounds.

ACKNOWLEDGEMENTS

This paper was supported by the Romanian National Authority for Scientific Research, Project PN II PCCA No 113/2012 and PN II PCCA No 210/2014.

REFERENCES

Bianco V. V., 2005. Present situation and future potential of artichoke in the Mediterranean basin. Acta Hort, 681: 39-55.

Brand-Williams W. C. M., 1995. Use of a free radical method to evaluate antioxidant activity. Food Sci Technol, 28: 25-30.

Chen J. H., Ho C. T., 1997. Antioxidant activities of caffeic acid andits related hydroxycinamic acid compounds. J. Agric. Food Chem., 45: 2374-2378.

Ciulei, I., Istudor, V., Palade, M., Albulescu, D., & Gard, C. 1995. Pharmacognostic and phytochemistry analysis of vegetable products.(Vol.1).Bucharest: Ed.Medicala.

Gebhardt, R. 1997. Antioxidative and protective properties of extract from leaves of the artichoke (Cynara scolymnus L.) against hydroperoxide-induced oxidative stress in cultured rat hepatocytes. Toxicol. Appl. Pharmacol. ,144: 279-286.

Gebhardt, R. 1998. Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (Cynara scolymnus L.) extracts. J. Pharmacol. Exp. Ther., 286: 1122-1128.

Gominho, J. J., 2001. Cynara cardunculus L. – a new fibre crop for pulp and paper production. Ind. Crops Prod. Ind. Crops Prod., 13: 1-10.

Iftimie N., Herdan J.M., Giurginca M., Meghea A., 2004. Chemiluminescence technique for the evaluation of some mineral and vegetable oils protected by antioxidants. Rev. de Chimie, 55(7): 512-514.

Kapusta I., Szpunar Krok, E., Bobrecka Jamro D., Cebulak T., Kaszuba J., Tobiasz Salach R., 2013. Identification and quantification of phenolic compounds from Jerusalem artichoke (Helianthus tuberosus L.) tubers. Food, Agriculture and Environment, 11(3&4):601-606;

Kirchhoff R. et.al, (1994). Increase in choleresis by means of artichoke extract. Phytomedicine, 1(8), 107–115 .

Kraft K., 1997. Artichoke leaf extract-recent findings reflecting effects on lipid metabolism, liver, and gastrointestinal tracts. Phytomedicine, 4: 369-378.

Lattanzio V., Cardinali A., Di Venere D., Linsalata, V., & Palmieri S. 1994. Browing phenomena in stored artichoke (Cynara scolymusL.) heads: enzymatic or chemical reactions? . Food Chem., 50: 1-7.

Liorach R., Espin J. C., Tomas-Barberan F. A., Ferreres F., 2002. Antichoke byproducts as a potential source of health-promoting. J.Agric.Food.Chem, 50(11): 3458-3464.

McDougall B., King P. J., Wu B. W., Hostomsky Z., Reinecke M. G., RobinsonW. E. 1998. Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. Antimicrob. Agents Chemother., 42: 140-146.

Moglia A. S., (2008). Stress induced biosynthesis of dicaffeoylquinic acids in globe artichoke. J. Agric. Food Chem., 56: 8641-8649.

Nasser A. M., 2012. Phytochemical Study of Cynarascolymus L. (Artichoke) (Asteraceae) Cultivated in Iraq, Detection and Identification of Phenolic Acid Compounds Cynarin and Chlorogenic Acid. Iraqi J Pharm Sci, 21(1):6-13.

Raccuia S. A., 2004. Intraspecific variability in Cynara cardunculus L. var.sylvestris Lam. Sicilian populations: seed germination. J.Arid Environ., 56:107-116.

Raccuia S. A., 2004. Genetic diversity in Cynara cardunculus revealed by AFLP markers: Comparison between cultivars and wild types from Sicily. Plant Breeding, 123:280-284

Romanian Pharmacopoea, (1993)., Bucharest: Ed. Medicala., edition X: 335,779.

Schutz K. K., 2004. Identification and quantification of caffeoylqunic acids and flavonoids from artichoke (Cynara scolymus L.) heads, juice and pomace by HPLC-DAD-ESI/MSn. J. Agric. Food Chem., 52:4090-4096.

Sheng-Yang Wang, Hsing-Ning Chang, Kai-Ti Lin, Chiu-Ping Lo, Ning-Sun Yang, Lie-Fen Shyur, 2003. Antioxidant Properties and Phytochemical Characteristics of Extracts from Lactuca indica, J. Agric. Food Chem., 51:1506-1512.

Simonetti P., Gardana C., Pietta P., 2001. Plasma levels of caffeic acid and antioxidant status after red wine intake. J. Agric. Food Chem, 49: 5964-5968.

Tomás-Barberan F.A., Ferreres F., Gil M.I., 2000. Antioxidant phenolic metabolites from fruit and vegetables and changes during postharvest storage and processing. Studies in Natural Products Chemistry, 23: 739-795.

Orlovskaya T. V., Luneva I. L., Chelombit'ko, V. A., 2007. Chemical Composition Of Cynara scolymus leaves. Chemistry of Natural Compounds, 43(2): 239-240.

Xianfeng Zhu, Hongxun Zhang, Raymond Lo, 2004. Phenolic Compounds from the Leaf Extract of Artichoke (Cynara scolymus L.) and Their Antimicrobial Activities. J. Agric. Food Chem. 52: 7272-7278.

Brand Z. N., (1990). Constituent of cynara folium bitter compound sesequiterpene. Phytother.,11: 169 – 175.

Brand Z. N., (1999). Constituents of cynara folium flavonoids. Phytother., 20: 292 – 302.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

WHY IS LABELING IMPORTANT. APPLICATION OF (EU) No. 1169/2011 IN ROMANIA

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Abstract

The paper aims to present the importance of the new European Food Law regulations regarding the novel labeling practices. (EU) Nr. 1169/2011 must have been applied in all EU countries until 13 December 2014, because 2 years later, all products without nutrition declaration will no longer be allowed to be put for sale. This new regulations contribute to the attainment of a high level of consumer protection, free movement of safe and wholesome food that contributes to health and well-being of citizens. Another important thing that we shall consider is that the application of these new regulations will guarantee that the consumers will be properly informed and any practices that could mislead the consumers will be prevented. In Romania, there are a few food producers that applied these new regulations, and that is a major concern for all potential buyers. In this paper is presented a case study that shows how a new product that needs to be put on the market, can be labeled within the limits of new EU regulation.

Key words*: labeling, food safety, regulations, Romania.*

INTRODUCTION

The new piece of European legislation is called the Food Information for Consumers Regulation (EU FIC). It came into force on 13rd December 2014 and changed the way allergen information appears on labels and on food that is pre-packed, sold loose or served when you are eating outside of the home. The EU FIC brought general and nutrition labeling together into a single regulation to simplify and consolidate existing labeling legislation.

Any of the 14 allergens that are on the regulatory list has to be emphasized on the label, if they are used as ingredients in a pre-packaged food. Businesses can choose what method they want to use to emphasize these allergens, for example, by listing them in bold, italics, highlighted or underlined, to help identify them (Ellis van Diermen, 2014).

Information about allergenic ingredients will be located in a single place, i.e. the ingredients list on pre-packed food. This means that the voluntary use of the current types of allergy boxes (such as: 'Contains nuts') that provide a short cut to allergen information also given in the ingredients list, will no longer be allowed.

Currently, loose foods (that can be bought without packaging) for example in supermarkets, delis, cafes and restaurants; don't have to provide information you need about food allergens. However, starting with 13rd December 2014, information on any of the 14 allergens used as ingredients will need to be provided for these foods (Popa M., 2014).

APPLICATION OF EU 1169/2011 REGULATION ON LABELING

In order to put a new product on the market a producer needs to make sure that the label is corresponding to the new EU Regulation 1169/2011. With the adoption of this Regulation the nutritional information is mandatory part of labeling. Another measure that was adopted is the necessity of the font size to be at a minimal of 1,2 mm. This measure helps the consumers to see what is in their product better, because it is known that the producers tend to put the additives in small fonts. By using a nutritional or health claim on the packaging, the nutritional information should be expanded with the micro (nutritionals) of which the claim is made. Also, the percentage of Recommended Daily Allowances (RDA) should be calculated per 100 g or 100 ml of product. In this new regulation bolding the allergens is a must, and

even if someone has a suspicion that an ingredient that may cause allergic reaction to the end consumer is inside the product, it is necessary to specify that at the bottom of the label (EU Nr. 1169/2011 Directive)

CASE STUDY

In this section of the paper it is presented an example of a label that respects the new requirements of the 1169/2011 EU Regulation. The product is called „Mango-pineapple cookies". In Table 1 it may find the ingredient list of the mango-pineapple cookies. The main goal of this case study is to show how a producer should make a label for its product respecting the new EU regulations (Ellis van Diermen, 2014).

Table 1. List of ingredients for the „Mango-pineapple cookies"

Ingredients	% of end product
Barley flour	33
Sugar	14
Margarine	14
Mango pieces	9
Cereal mix	
- Rye flakes	9
- Oat flakes	9
- Sesame seed	3
Inuline	7
Barn eggs	1
Raising agents: E339 and E500	<0.5
Vitamin pre-mix (B1, B2, B3)	<0.5
Pineapple-flavor (natural)	<0.1

Currently, the rules for pre-packed foods establish a list of 14 food allergens, which have to be indicated by reference to the source allergen whenever they, or ingredients made from them, are used at any level in pre-packed foods, including alcoholic drinks.
The list consists of cereals containing gluten, crustaceans, mollusks, eggs, fish, peanuts, nuts, soybeans, milk, celery, mustard, sesame, lupine and sulphur dioxide at levels above 10mg/kg, or 10 mg/liter, expressed as SO_2.
In our example we have the next list of allergens that are shown in Table 2 (Ellis van Diermen, 2014).

Table 2. List of allergens that are present in the product

eggs	+	soy	+
gluten	+	sesame	+
oat	+	sesame oil	+
rye	+	lupin	**?**
barely	+		

As it can observe all the allergens that are for sure present in the product have the „+" mark near them and the one allergen that is to be believed present has the question mark „?" near it.

Another important thing that must be done in order to make the correct label is to answer to the following questions:

1. Is it allowed to use the product name „mango-pineapple" cookies based on the ingredients presented in table 1?
2. Are the suggested picture of mango and a slice of pineapple allowed on this packaging?
3. Can we state that the product is without artificial flavors?

Here are some basic points that the producers need to pay special attention at when designing a label:

- Mandatory declarations by law regarding labeling, such as legal product name, allergens and ingredient declarations see Regulation 1169/2011, article 9.
- Note down all nutritional value records about energy and (micro) nutrients following the obligations in Regulation 1169/2011, article 30.
- In relation to the nutrition declaration pay attention to the suggested claims (USP's) in the memo (Regulation 1169/2011, article 9, f).
- The nutritional declaration should not be calculated here. Only mentioning the needed (micro) nutrients to be noted on the table (without values) is sufficient.

Having all these information it can proceed in making the actual label. In Figure 1 we can see the actual label with all the regulations applied.

Figure 1. Final form of the label of the product

Cookies with dried mango pieces and pineapple flavor with

240 gram

Ingredients: **barley** flour, sugar, margarine [vegetables oils in varying proportions (sunflower oil, fully hydrogenated rapeseed oil, partly hydrogenated palm oil)], water, salt, emulsifier: **soy** lecithin, vitamins A and D3], 9% dried mango pieces [8.1% mango, sugar, preservatives E223], **rye** flakes, **oats** flakes, dietary fiber (inulin), **sesame** seed, barn **egg**, rising agents (E339 and E500), natural pineapple flavoring, vitamins B1, B2, B3. May contain traces of lupine.

Average nutritional value	Per 100 g	Per cookie (40g)
Energy	2140 kJ / 510 kcal	855 kJ / 205 kcal
Fat	26.1 g	10.4 g
of which saturated	6.8 g	2.7 g
Carbohydrates	58.5 g	23.4 g
of which sugar	24.0 g	9.6 g
Fiber	5.6 g	2.2 g
Protein	7.8 g	3.1 g
Salt	0.3 g	0.12 g
Vitamin B1	0.4 mg 36% NRV	0.16 mg 15% NRV
Vitamin B2	0.4 mg 30% NRV	0.17 mg 12% NRV
Vitamin B3	4.8 mg 30% NRV	1.92 mg 12% NRV

NRV – Nutrient Reference Values

Best before: see front of pack.
Store in a cool and dry place.

Legal claims

As stated earlier there are a number of legal claims that need attention if the producers want the label to be appropriate.

1. Is it allowed to use the product name „mango-pineapple cookies„ based on the ingredients list from table 1?

The product name Mango-pineapple cookies is allowed. Pineapple and mango are both present in the product, even if the pineapple is present as a natural flavoring. According to the legislation it does not matter what quantity of an ingredient is added. The percentage of ingredients should be mentioned in the declaration and label (EU Nr. 1169/2011 Directive).

2. Are the suggested picture of mango and a slice of pineapple allowed on this packaging?

The picture shall not be misleading for the consumer. The picture of the mango may be used; provided that this ingredient is mentioned in the ingredient list with % is on the end product. A picture of the pineapple is NOT possible, because only the pineapple flavor has been used (EU Nr. 1169/2011 Directive).

3. Can we state that the product is without artificial flavors?

This claim is only allowed if the product contains only natural flavors. In this case a natural pineapple flavor is used, so yes, we can state that the product does not have any artificial flavors (EU Nr. 1169/2011 Directive).

CONCLUSIONS

The directive concerns the display of product information on product packaging and online stores; pertaining to food and beverages sold in the EU. The objective of which, is to standardize food labeling and provide greater clarity to consumers on ingredients, nutrition and allergens (Popa M., 2014).

The directive came into effect in December 2014. Food and beverage manufacturers will have to update their packaging to comply with this new legislation. Online retailers must ensure that the information they provide conforms to the regulation and it must be identical to the product information provided on the manufacturers physical packaging. Compliance with the new legislation will ensure that product information is consistent throughout the supply chain at every consumer touch-point, both in-store and online.

The EU Regulation 1169/2011 changes existing legislation on food labeling in order to help the consumer understand better what they eat. The most important thing that has changed is the font size, increasing it to a minimum 1.2 mm

makes the label much easier to read. Mandatory nutrition information on processed foods is now mandatory and the origin labeling of unprocessed meats from pigs, sheep, goat and poultry is too (Ellis van Diermen, 2014).

Food and beverage manufacturers will be responsible for:

- Displaying EU 1169/2011 compliant information on all product packaging. The directive specifies a list of mandatory information and details on how the information should be formatted – covering ingredients, allergens and nutritional values.
- Where product information is requested electronically, that information must also be EU 1169/2011 compliant. The directive specifies exactly what information needs to be provided.

Food and beverage products that do not adhere to the legislation cannot be sold in the EU.

REFERENCES

Ellis van Diermen., 2014. Ways to make a clean label under the EU Regulation 1169/2011, Course Notes, Wageningen, NL.

Popa Mona Elena, 2014. Modern technologies for food packaging, Course Notes for Master Programme,Bucharest.

*http://www.anpc.gov.ro/anpcftp/legislatie/140731/proiect_hg_informare_produse_alim_140731.pdf

*http://www.upg-bulletin-se.ro/archive/2014-1/9.Ene.pdf

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PRETREATMENT BEHAVIOR OF FROZEN STRAWBERRIES AND STRAWBERRY PUREES FOR SMOOTHIE PRODUCTION

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Abstract

Recently, for increasing fruits shelf life, food industries used thermal processing, which has been shown in some studies to affect the sensorial and physic-chemical properties of these products. During smoothie production is mandatory to have raw materials for the whole year, so it is necessary to storage it after different pretreatments such as freezing.
Color, flavor, texture and physic-chemical properties of food products have an important role in correlation with taste, sensory perception and consumer acceptance. These are critical quality attributes affecting the acceptability of fruits, fresh, frozen or processed (concentrates, jam, juice, nectar, syrup, dairy products), thus being of major concern in product design.
Strawberries are popular fruit with high visual appeal and desirable flavor, but are highly perishable, being susceptible to mechanical injury, water loss, decay and physiological deterioration. The natural color of strawberries and their products easily fades or deteriorates during processing and storage.
The aim of this study is to observe the influence of freezing storage on color, texture, antioxidant activity, vitamin C and physic-chemical properties of strawberry and strawberries puree.
For this purpose, were realized and analysed 4 different samples starting with just harvested strawberries, pressed strawberries, strawberries immersed in ascorbic acid 1% for 5 minutes and strawberries blanched at 95^0C for 5 minutes, before and after freezing. These purees were stored for 6 months in the freezer. During frozen storage the lightness index, L, yellowness index, b and the redness index, a, were also measured and it was observed colour changes for all of strawberry samples. pH and acidity values, showed that the strawberry samples registered insignificant changes compared to standard (control) samples.

Key words*: color, strawberry, freezing, shelf life.*

INTRODUCTION

Previous studies have shown that the fruits and vegetables contain high levels of antioxidant compounds that have proven capacity of reducing oxidative stress responsible for DNA, protein and membrane damage (Mandavea et al., 2014). It therefore lower the incidence and mortality rates of various cancers and heart diseases (Cao et al., 1996; Bagchi and Puri, 1998; Lobo et al., 2010; Sen and Chakraborty, 2011). Strawberry is one of the most popular summer fruit worldwide that are characterized with unique and highly desirable taste and flavour (organic acids and soluble sugars content), color (Campaniello, Bevilacqua, Sinigaglia, & Corbo, 2008; Koyuncu & Dilmaçünal, 2010) and texture (Velickova et al., 2013). Strawberries are a good source of vitamin C (Patras, Brunton, Da Pieve, & Butler, 2009), being available worldwide as a whole fruit and also as a puree/juice/concentrate to be incorporated in nectar, ice cream, yoghurt, baby food and confectionary (Sulaiman and Silva, 2013). Is a widely researched fruit for nutritional and health benefits and organoleptic properties. This fruit is rich in vitamins, minerals, fibre and phytochemicals. In addition, strawberries contain potentially bioactive compounds and are a great source of phenolic compounds such as flavonoids and phenolic acids (Aaby, Skrede, & Wrolstad, 2005; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004; Seeram, Lee,

Scheuller, & Heber, 2006). These compounds make strawberries a highly antioxidant fruit (Aaby et al., 2005; Wolfe et al., 2008) with potential health benefits. Among the numerous healthy properties described in the literature are anti-proliferative effects on cancer cells (Meyers, Watkins, Pritts, & Liu, 2003; Olsson, Andersson, Oredsson, Berglund, & Gustavsson, 2006) and the antioxidant and anti-inflammatory effects that have been shown to reduce cardiovascular disease risk factors in several prospective cohort studies (Hannum, 2004). Also, strawberries may exert protection against inflammation, type 2 diabetes, cardiovascular disease, hypertension, oesophageal cancer, obesity in humans (Cassidy et al., 2011; Chen et al., 2012; Giampieri et al., 2012) and oxidative stress (Kanter et al., 2012). Cheel et al. (2007) demonstrated that strawberry extracts had significantly higher antioxidant activity compared to 11 other fruits. Qualitative and quantitative variations in the antioxidant activity have been observed among strawberry cultivars as well as within the same variety, depending on the genetic background, degree of ripening, postharvest storage of the fruits, and climatic factors (Maatta et al., 2004; Lopes-da-Silva et al., 2007). However strawberries are very problematic for industrial processing as they are seasonal, and have a high water content which makes them very perishable (Oey et al., 2008; Peinado et al., 2013; Taiwo et al., 2003). Furthermore, color plays a major role in quality assessment of food significantly determining consumers' choice (Stintzing & Carle, 2004). Accordingly, strawberries are commonly frozen, thus allowing a year-round production of jams, juices, fruit preparations, purées, concentrates (Oszmiański, Wojdylo, & Kolniak, 2009; Skrede, 1996) and smoothies. Although freezing is an efficient preservation measure (Holzwarth et al., 2012, Singh & Wang, 1977), it is inevitably accompanied by irreversible structural damage of the cell wall, middle lamella, and protoplast, resulting in textural quality losses (Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrick, 2009). Therefore, the aim of this study is to observe the influence of freezing storage on color, texture, antioxidant activity, vitamin C and physic-chemical properties of strawberry and strawberries puree used for smoothie production.

MATERIALS AND METHODS

Samples

Strawberries were purchased from a local market from Romania, Bucharest. All fruits were selected based on the same ripening stage (>90% red surface color), uniform size, absence of any physical damage and fungal infection. They were either processed immediately and packed for freezing within 2 h, or analyzed. Strawberries were packed in 200 g plastic bags and stored for 6 months at -20^{0}C. Were realized 4 different samples starting with just harvested strawberries, pressed strawberries, strawberries immersed in ascorbic acid 1% for 5 minutes and strawberries blanched at 95^{0}C for 5 minutes. These were analysed before and after freezing. Frozen strawberries were kept for 24 h at 4^{0}C before they were analyzed.

Physico-chemical analysis

pH determination

pH was determined with a pH meter WTW INOLAB 720 series type with automatic temperature compensator, whose pH domain is between 0,00-14.00, with a precision of ± 0,01.

Titratable acidity (TA)

Titratable acidity was determined by titrating 10 g of homogenized sample with 0.1 N NaOH to an end point of pH 7.3 using Schott automatic titrator type Titronic basic. TA was analyzed in duplicates and expressed as citric acid/100 g product (factor 0.64).

Brix degree, dry matter content (S.U. %) and water activity (a_w) determination

The level of sugars was measured as Brix by a Krüss Refractometer and correlated with the amount of soluble solids (expressed as sucrose concentration) using the conversion table or read directly on the scale Refractometer. The

dry matter content was determined after drying approximately 5 g of pulp at 140^0C till a stable weight, with Precisa XM 60 thermobalance. Water activity was conducted with special system Novasina.

Color

Color assessment of the samples was conducted at room temperature using a HunterLab colorimeter, Miniscan XE Plus. This instrument was calibrated using the black and white tiles provided. Instrumental color was measured using Illuminant D65 and 10^0 observer angle. Samples were filled into a low reflectance sample container and placed over the colorimeter chamber. For each sample, measurements were made in ten different points and results were averaged. Therefore the total color change (ΔE) was calculated with the following equation (Hunter Lab, (1996)):

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}.$$

Antioxidant activity

The effect of antioxidant activity on DPPH was estimated according to the procedure described by Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007, with some modification. To obtain DPPH solution (60 μM), 2.36 mg DPPH were diluted in 100 ml ethanol. Samples were diluted appropriately in ethanol. Sample preparation was done by maceration in ethanol (75%) for 2-3 days in the dark at room temperature. All measurements were performed in triplicate. For each measurement, 0.05 ml sample in ethanol was added to 1.95 ml DPPH ethanolic solution (60 μM). These solutions were vortexed thoroughly, and incubated in dark at room temperature for 30 min (Gülçin, 2010). After 30 min, sample absorbance was measured at 515 nm (t=30min) against DPPH ethanolic solution alone (t=0 min). For calibration curve were used six different concentrations of Quercitin (100-3.125 μM). Absorbance measurements were recorded on a UV/Vis spectrophotometer Unicam Helios Gamma. Results were expressed as quercitin equivalents using the following equations:

A_{AR} (QE) = (%ΔA_{515} –3.4954) / 0.0811

where:

A_{AR} (QE) - antiradical activity expressed in quercitin equivalents

%$\Delta A_{515} = [(A_{515\ (t=0)} - A_{515\ (t=30)})/A_{515\ (t=0)}] \times 100$)

Ascorbic acid spectrophotometric determination

The content of ascorbic acid from strawberry samples was determined by using a UV/VIS spectrophotometer Unicam Helios Gamma at 500 nm. 10 g of fruit pulp was extracted with 30 ml of 1% oxalic acid in a homogenizer for 1 min. The extract was filtered through a filter paper. After filtration, 2 ml from extract solution, 1 ml oxalic acid 1%, 5 ml tampon solution, 2 ml indophenol (2, 6-Dichlorophenol Indophenol) and 20 ml xylene, were placed in a centrifuge tube and centrifuged 20 min at 4^0C and 9000 rpm. After absorbance measurements, ascorbic acid content was expressed in milligrams at 100 g product, and is calculated with following equation:

$$Vit.C_{(mg/100g)} = [(V_0 - V_1) \times V_3 \times C/(V_4 \times V_2)] \times 100$$

where:

V_0 - indophenol solution volume added for reduction,

V_1 - indophenol solution excess volume read on the standard curve,

V_2 - sample volume for analysis,

V_3 - sample volume brought for analysis,

V_4 – acid extract volume used for analysis,

C - ascorbic acid corresponding quantity for 1 ml indophenol solution.

RESULTS AND DISCUSSIONS

Evolution of physical chemical properties

The obtained results showed that the pH value of the samples registred insignificantly changes. The highest difference was recorded at just harvest strawberry samples (fresh: 3.6 (±0.01) and frozen: 3.9 (±0.02)). In this case, pH value is apparently not responsible for color changes during on freezing storage as it also observed by Gössinger et all. (2009). Acidity, expressed as citric acid content, which is the main acid of strawberries (Kamperidou & Vasilakakis, 2006), ranged between 0.59 (±0.1) (P1 before freezing) and 0.9 (±0.01) (P2 after freezing). Samples of frozen stored

strawberry had higher acidity than those fresh, and is high correlated with Brix values, which decrease (Galoburda et all., 2014) after freezing storage for 6 months (Table 1, Figure 2 and 3). It means that acidity and Brix are responsible for color changes during on freezing storage. For dry matter content, three strawberry samples (P2, P3 and P4) recorded significant decreases from 56.07% to 18.48% for blanched strawberries before and after freezing (P4) (Tabel 1, Figure 4). Trough processing method, first samples (P1 – just harvested strawberry) recorded insignificant changes for dry S.U. and aw (Table 1, Figure 4 and 5).

Table 1. Physico-chemical results of strawberries before and after freezing (P1 – just harvested strawberries, P2 – pressed strawberries, P3 – strawberries immersed in ascorbic acid 1% for 5 minutes, P4 – strawberries blanched at 95^{0}C for 5 minutes)

	pH		Titratable acidity (g citric acid/100g product)		Brix		aw		S.U. %	
	Before	After	Before	After	Before	After	Before	After	Before	After
P1	3.6	3.9	0.59	0.74	11.1	10.3	0.932	0.928	69.33	60.93
P2	3.5	3.6	0.81	0.9	9.8	9.2	0.932	0.931	53.36	18.52
P3	3.4	3.5	0.75	0.83	7.8	7.4	0.932	0.923	52.59	18.46
P4	3.6	3.7	0.63	0.64	8.5	8.3	0.948	0.933	56.07	18.48

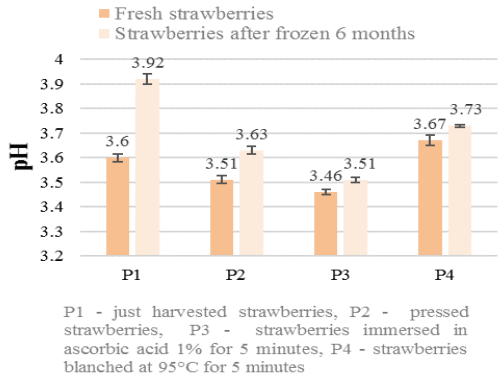


Figure 1. Variation of the strawberries pH values before and after freezing (for 6 months)

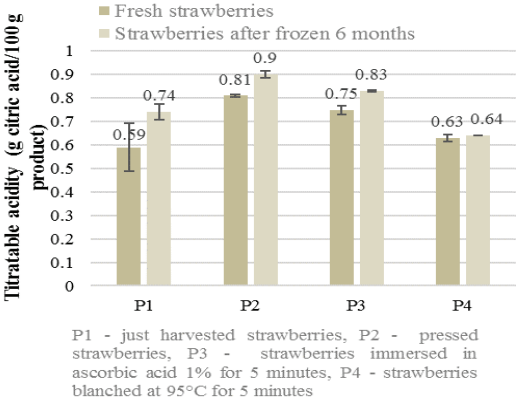


Figure 3. Variation of the strawberries titratable acidity values before and after freezing (for 6 months)

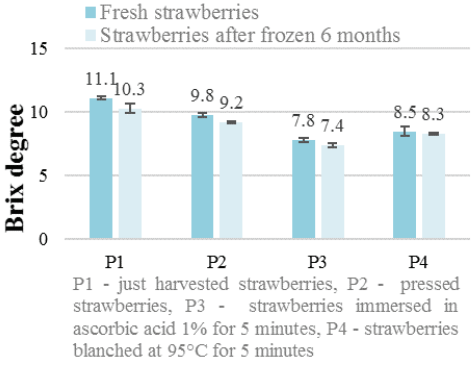


Figure 2. Variation of the strawberries Brix degree values before and after freezing (for 6 months)

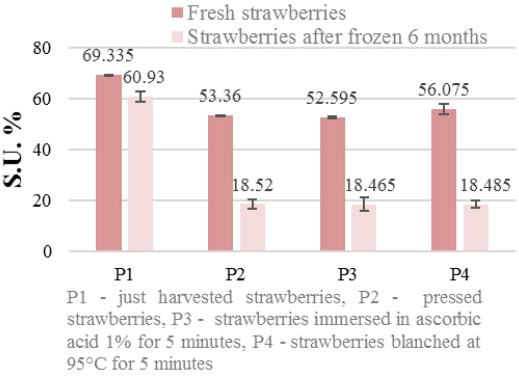


Figure 4. Variation of the strawberries dry matter content before and after freezing (for 6 months)

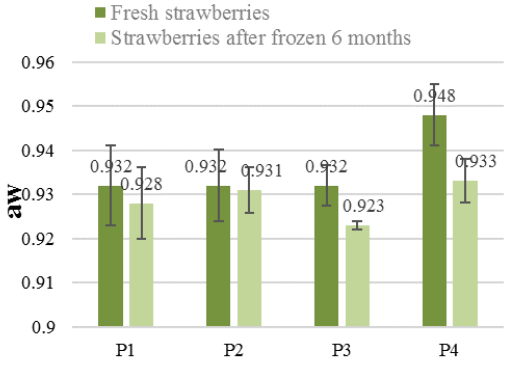


Figure 5. Variation of the strawberries water activity (aw) values before and after freezing (6 months)

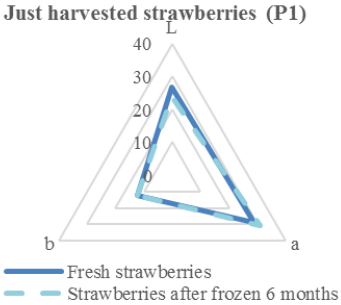
Color changes during freezing storage period

Besides texture and economic considerations, color is one of the most important factor in the perception of strawberry fruit quality, affecting consumer acceptance (Abd-Elhady, 2014). Color stability of strawberry products, particularly after heat or cold and light exposure, remains a challenge (Carle et al., 2001). In general, several factors are believed to affect the color and stability of strawberry anthocyanins include structure and concentration, pH, temperature, light, presence of co pigments, self-association metallic ions, enzymes, oxygen, ascorbic acid, sugar and their degradation products, proteins and sulfur dioxide (Rhim, 2002). During the 6 months of freezing storage period were not observed visually detectable color changes for any of the analysed strawberry samples. Changes were observed when the color characteristics were analysed with colorimeter Hunter Lab according to Universal Software V4.01 MiniScan^{TM} XE Plus program. During freezing storage period, the L (lightness), a (redness) and b (yellowness) values of just harvested strawberries (P1), pressed strawberries (P2), strawberries immersed in ascorbic acid 1% for 5 minutes (P3) and strawberries blanched at 95^{0}C for 5 minutes (P4) tended to decrease, indicating color changes, as can be observed in figures 6,7,8 and 9. For all strawberry samples (P1, P2, P3 and), the L (lightness), a (redness) and b (yellowness) values tended to decrease during freezing period, indicating a discoloration of the samples (figures 6, 7, 8 and 9). This are in correlation with increasing acidity and decreasing Brix for freezing storage period.

The ΔE values, which are an indicator of total color difference (table 2), showed that freezing storage for 6 months affected color attributes for all strawberry samples.

Table 2. Instrumental color variables of strawberry samples before and after freezing

	L		a		b		ΔE
	Before	After	Before	After	Before	After	
P1	26.7	24	30.8	28.65	12.31	12.08	3.45
P2	21.83	19.84	29.83	24.06	10.87	8.21	6.65
P3	22.67	20.85	30.62	25.65	11.47	8.81	5.59
P4	31.62	31.55	28.11	25.91	11.27	10.49	2.33



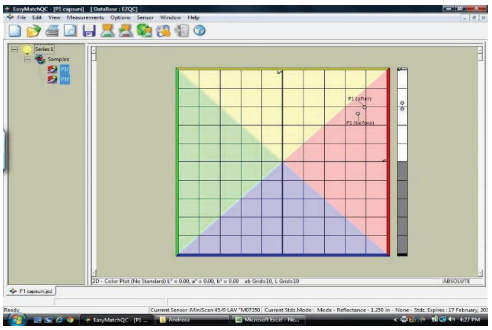


Figure 6. Graphical representation of the values of L, a, b, according to Universal Software V4.01 MiniScan^{TM} XE Plus program for just harvested strawberry samples before and after frozen 6 months

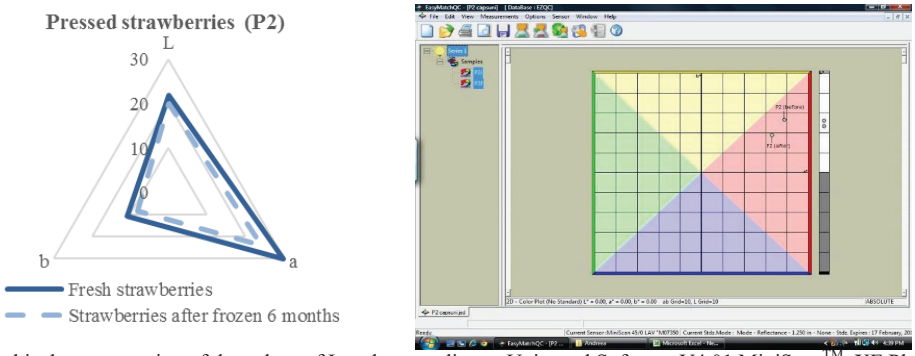


Figure 7. Graphical representation of the values of L, a, b, according to Universal Software V4.01 MiniScan^{TM} XE Plus program for pressed strawberry samples before and after frozen 6 months

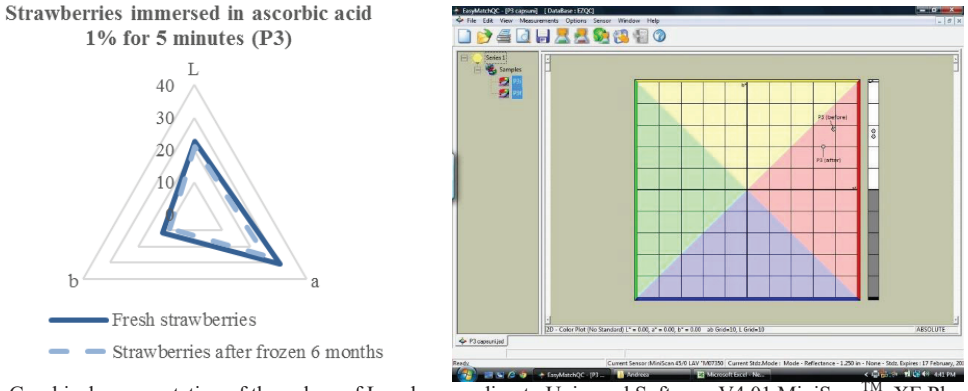


Figure 8. Graphical representation of the values of L, a, b, according to Universal Software V4.01 MiniScan^{TM} XE Plus program for strawberry immersed samples before and after frozen 6 months

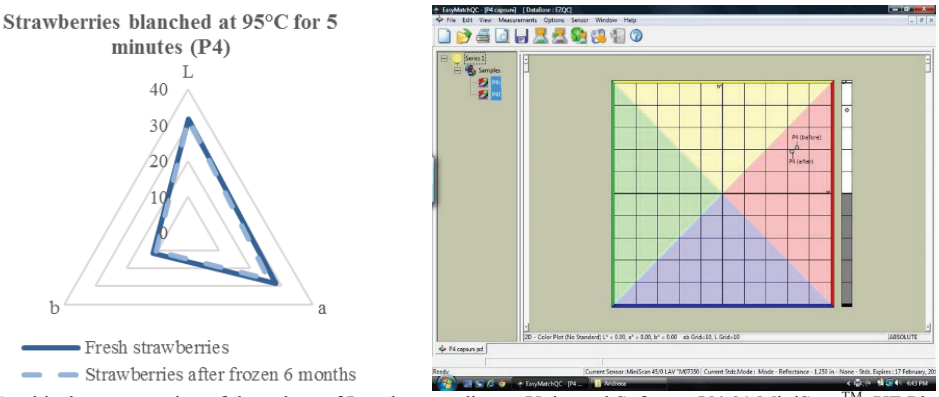


Figure 9. Graphical representation of the values of L, a, b, according to Universal Software V4.01 MiniScan^{TM} XE Plus program for strawberry blanched samples before and after frozen 6 months

Antioxidant activity

DPPH assay is one of the best known, frequently employed and accurate method of assessing free radical scavenging activity. DPPH is a stable free radical because of its spare electron delocalization over the whole molecule. Decolourization causes deep violet color with maximum around 520 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen ion donor, a stable non-radical form of DPPH is obtained with simultaneous change from violet to pale yellow (Mandave et all., 2014). Antioxidant activity content in just harvest strawberry samples (P1)

decreased from 1069.26 µM quercitin equivalents to 1045.08 µM quercitin equivalents for frozen sample (P1). Highest value for DPPH content it was seen at fresh strawberries immersed in ascorbic acid 1% for 5 minutes (1128.31 µM quercitin equivalents) but also here it was recorded the most significant decrease like 1011.96 µM quercitin equivalents. For blanched strawberry samples, DPPH values recorded insignificant changes like from 1085.48 µM quercitin equivalents to 1082.74 µM quercitin equivalents after freezing sample. In conclusion, antioxidant capacity of blanched strawberry samples (95^0C fir 5 min) is more stable (fresh: 1085.48µM quercitin equivalents and frozen: 1082.74 µM quercitin equivalents) than samples immersed in ascorbic acid 1% for 5 minutes (fresh: 1128.31 µM quercitin equivalents and frozen: 1011.96 µM quercitin equivalents).

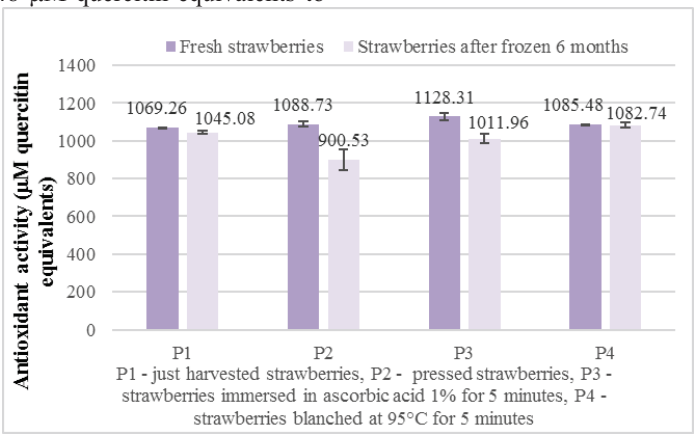


Figure 10. Variation of antioxidant activity for strawberry samples before and after freezing storage

V*itamin C (Ascorbic Acid spectrophotometric determination)*

Generally, fruits and vegetables show a gradual decrease in vitamin C content as the storage temperature or duration increases (Koyuncu & Dilmaçünal, 2010). Vitamin C content in just harvest strawberry samples (P1) decreased from 26.21 (±0.02) mg Vit C/ 100 g product up to 26.06 (±0.02) mg Vit C/ 100 g product for frozen sample (P1). Lowest values for vitamin C content was recorded from pressed strawberry samples (P2) from 20.62 (±0.07) mg Vit C/ 100 g product to 20.19 (±0.03) mg Vit C/ 100 g product. All changes in vitamin C content of strawberries during freezing storage is shown in Figure 11. Similar results were found by Koyuncu & Dilmaçünal, 2010, and Galoburda, 2014. As a result strawberry purees contains smaller amount of vitamin C compared to whole strawberry fruits.

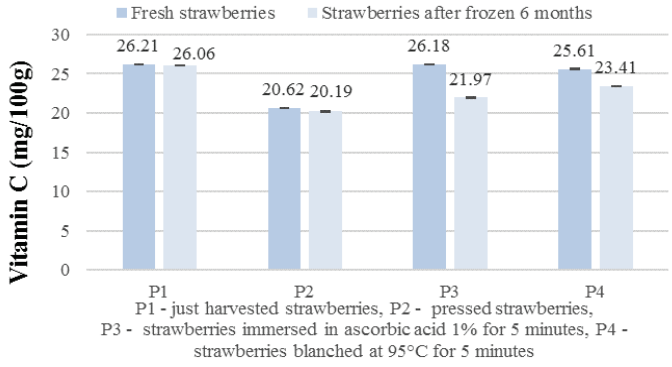


Figure 11. Variation of vitamin C content of strawberry samples before and after freezing storage

CONCLUSIONS

During smoothie production is mandatory to have raw materials for the whole year, so it is necessary to storage it after different pre-treatments such as freezing.

Color is one of the most important factor in the perception of strawberry fruit quality, affecting consumer acceptance and preference, but only after texture and economic considerations.

During the 6 months of freezing storage period were not observed visually detectable color changes for any of the analysed strawberry samples. No significant changes were observed when the color characteristics were analysed with colorimeter.

The obtained results showed that the pH value of the strawberry samples registered insignificantly changes, during the freezing period.

ACKNOWLEDGEMENTS

This paper was *published under the frame of* European Social Fund, *Human Resources Development Operational Programme 2007-2013, project no.* POSDRU/159/1.5/S/132765.

REFERENCES

Sen, S., Chakraborty, R., 2011. The role of antioxidants in human health. ACS Symp.Ser. Chapter 1 1083, 1–37.

Lobo, V., Patil, A., Phatak, A., Chandra, N., 2010. Free radicals, antioxidants and func-tional foods: impact on human health. Pharmacogn. Rev. 4 (8), 118–126.

Cheel, J., Theoduloz, C., Rodriguez, J.A., Caligari, P.D.S., Hirscmann, G.S., 2007, Freeradical scavenging activity and phenolic content in achenes and thalamus fromFragaria chiloensis ssp. chiloensis, F. vesca and F. × ananassa cv. Chandler. FoodChem. 102, 36–44.

Määttä-Riihinen, K. R., Kamal-Eldin, A., & Törrönen, A. R., 2004, Identification and quantification of phenolic compounds in berries of Fragaria and Rubus species (family Rosaceae). Journal of Agricultural and Food Chemistry, 52, 6178 – 6187.

Gülçin İ., 2010, Antioxidant properties of resveratrol: A structure-activity insight, Innov. Food Sci. Emerg. 11, 210-218.

Seeram, N. P., Lee, R., Scheuller, H. S., & Heber, D., 2006, Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionization mass spectroscopy. Food Chemistry, 1 – 11.

Gössinger M., Moritz S., Hermes M., Wendelin, Hannes Scherbichler S., Halbwirth H., Stich K., Berghofer E., 2009, Effects of processing parameters on colour stability of strawberry nectar from puree, Journal of Food Engineering 90, 171–178.

Kamperidou I., M. Vasilakakis M., 2006, Effect of propagation material on some quality attributes of strawberry fruit (Fragaria x ananassa, var. Selva), Scientia Horticulturae 107, 137–142.

Koyuncu M. A., Dilmaçünal T., 2010, Determination of Vitamin C and Organic Acid Changes in Strawberry by HPLC During Cold Storage, Not. Bot. Hort. Agrobot. Cluj 38 (3), 95-98.

Galoburda R., Boca S., Skrupskis I., Seglina D., 2014, Physical and chemical parameters of strawberry puree, FoodBalt, 172-177.

Patras, A., Brunton, N. P., Da Pieve, S., & Butler, F., 2009, Impact of high pressure processing on total antioxidant activity, phenolic, ascorbic acid, anthocyanin content and colour of strawberry and blackberry purees. Innovative Food Science & Emerging Technologies, 10(3), 308-313.

Aaby, K., Skrede, G., & Wrolstad, R. E. (2005). Phenolic composition and antioxidant activities in flesh and achenes of strawberries (Fragaria ananassa). Journal of Agricultural and Food Chemistry, 53, 4032 – 4040.

Wolfe, K. L., Kang, X., He, X., Dong, M., Zhang, Q., & Liu, R. H., 2008, Cellular antioxidant activity of common fruits. Journal of Agricultural and Food Chemistry, 56, 8418 – 8426.

Meyers, K. J., Watkins, C. B., Pritts, M. P., & Liu, R. H. 2003, Antioxidant and antiproliferative activities of strawberries. Journal of Agricultural and Food Chemistry, 51, 6887 - 6892.

Olsson, M. E., Andersson, C. S., Oredsson, S., Berglund, R. H., & Gustavsson, K., 2006, Antioxidant levels and inhibition of cancer cell proliferation in vitro by extracts from organically and conventionally cultivated strawberries. Journal of Agricultural and Food Chemistry, 54, 1248 - 1255.

Hannum, S. M. 2004, Potential impact of strawberries on human health: a review of the science. Critical Reviews in Food Science and Nutrition, 44, 1 - 17.

Lopes-da-Silva, F., Escribano-Bailon, M.T., Perez, A.J.J., Rivas-Gonzalo, J.C., Santos-Buelga, C., 2007. Anthocyanin pigments in strawberry. LWT-Food Sci. Technol.40, 374–382.

Kanter, M., Aktas, C., Erboga, M., 2012, Protective effects of quercetin against apo-ptosis and oxidative stress in streptozotocin-induced diabetic rat testis. FoodChem. Toxicol. 50 (3–4), 719–725.

Cassidy, A., O'Reilly, E. J., Kay, C., Sampson, L., Franz, M., Forman, J. P., et al. 2011, Habitual intake of flavonoid subclasses and incident hypertension in adults. American Journal of Clinical Nutrition, 93(2), 338–347.

Chen, T., Yan, F., Qian, J., Guo, M., Zhang, H., Tang, X., et al. 2012, Randomized phase II trial of lyophilized strawberries in patients with dysplastic precancerous lesions of the esophagus. Cancer Prevention Research, 5(1), 41–50.

Giampieri, F., Tulipani, S., Alvarez-Suarez, J. M., Quiles, J. L., Mezzetti, B., & Battino, M., 2012, The

strawberry: Composition, nutritional quality, and impact on human health. Nutrition, 28 (1), 9–19.

Stintzing, F. C., & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. Trends in Food Science and Technology, 15, 19–38.

Singh, R. P., & Wang, C. Y. (1977). Quality of frozen foods — A review. Journal of Food Process Engineering, 1, 97–127.

Holzwarth, M., Korhummel, S., Carle, R., & Kammerer, D. R. (2012). Impact of enzymatic mash maceration and storage on anthocyanin and color retention of pasteurized strawberry purées. European Food Research and Technology, 234, 207–222.

Oszmiański, J., Wojdylo, A., & Kolniak, J. (2009). Effect of L-ascorbic acid, sugar, pectin and freeze–thaw treatment on polyphenol content of frozen strawberries. Food Science and Technology, 42, 581–586.

Skrede, G. (1996). Effect of freezing on fruits. Food Science and Technology, 72, 183–245.

Carle, R., Borzych, P., Dubb, P., Siliha, H., & Maier, O. (2001). A new process for firmer canned cherries and strawberries. Food Australia, 53, 343–348.

Van Buggenhout, S., Sila, D. N., Duvetter, T., Van Loey, A., & Hendrickx, M. (2009). Pectins in processed fruits and vegetables: Part III — Texture engineering comprehensive Reviews in Food Science and Food Safety, 8, 105–117.

Oey, I., Lille, M., Van Loey, A., Hendrickx, M., 2008. Effect of high-pressure processing on colour, texture and flavour of fruit-and vegetable-based food products: a review. Trends Food Sci. Technol. 19 (6), 320–328.

Taiwo, K.A., Eshtiaghi, M.N., Ade-Omowaye, B.I., Knorr, D., 2003. Osmotic dehydration of strawberry halves: influence of osmotic agents and pretreatment methods on mass transfer and product characteristics. Int. J. Food Sci. Technol. 38 (6), 693–707.

Koyuncu, M. A., & Dilmaçünal, T. (2010). Determination of vitamin C and organic acid changes in strawberry by HPLC during cold storage. Notulae Botanica Horti Agrobotanici, 38, 95 - 98.

Campaniello, D., Bevilacqua, A., Sinigaglia, M., & Corbo, M. R. (2008). Chitosan: antimicrobial activity and potential applications for preserving minimally processed strawberries. Food Microbiology, 25, 992-1000.

Velickova, E., Tylewicz, U., Dalla Rosa, M., Winkelhausen, E., Kuzmanova, S., & Gomez Galindo, F. (2011). Effect of vacuum infused cryoprotectants on the freezing tolerance of strawberry tissues. LWT-Food Science and Technology, http://dx.doi.org/10.1016/j.lwt.2011.09.013

Mandave P. C., Pawar P. K., Ranjekar P. K., Mantri N., Kuvalekar A.A., 2014, Comprehensive evaluation of in vitro antioxidant activity,total phenols and chemical profiles of two commerciallyimportant strawberry varieties, Scientia Horticulturae 172, 124–134.

Sulaiman A., Silva F. V.M, 2013, High pressure processing, thermal processing and freezing of 'Camarosa' strawberry for the inactivation of polyphenoloxidase and control of browning, Food Control 33, 424-428.

Abd-Elhady M.,2014, Effect of citric acid, calcium lactate and low temperature prefreezing treatment on the quality of frozen strawberry, Annals of Agricultural Science 59(1), 69–75.

Rhim, J.W., 2002. Kinetics of thermal degradation of anthocyanin pigment solutions driven from red flower cabbage. Food Sci. Biotechnol. 11, 361–364.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

UNCONVENTIONAL ANTIMICROBIAL TREATMENTS FOR FOOD SAFETY AND PRESERVATION

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Abstract

Despite intensified prevention efforts, foodborne illness remains a serious health problem worldwide. Food spoilage is caused by both biologically and chemically agents. The growth of microorganisms is the major route for food spoilage, leading to low quality, shortened shelf-life, and changes in natural micro-flora that could induce pathogenic problems. Microbial spoilage of food products is caused by many bacteria, yeast, and moulds. For the food industries, the prevention of food spoilage is a very important issue in determining profit. Furthermore, reducing food spoilage can prolong the shelf-life of food products and accordingly extend market boundary, resulting in increased profit.
The objective of this work is to make a short review in respect to unconventional antimicrobial treatments of food, which are used nowadays in industry or are in the research and development phase. The paper presents an inventory of novel techniques such as: ohmic treatment, PEF, microwave treatment, IR, UV, UHP, ozone treatment, light pulses treatment, plasma treatment, active packaging, encapsulation of antimicrobial compounds, edible films, radio frequency treatment.

Key words*: food spoilage, antimicrobials, food safety and preservation.*

INTRODUCTION

In recent years, there has been a significant increase in consumer interest in the quality and safety of food products (Marszałek et al., 2015). Food safety and food quality are the major concerns for food producers, food industries, governments, and consumers. Spoilage of food products is caused by physical, chemical, and biological factors in the detriment of the organoleptic characteristics and consumer safety. Microbial growth damages the overall quality and safety of a product. As a result of microbial growth, off-odours and changes in the aroma, colour, and texture can be accelerated. Additionally, some microorganisms and their toxins may cause food recalls and serious foodborne outbreaks. Effective preventive measures and intelligent preservation methods have been put into place to reduce food spoilage and to prolong food shelf life (Corrales et al., 2014). The food industry is interested in developing alternative process technologies to accomplish a microbiological reduction in various foods without compromising fresh-like product characteristics (Gupta and Balasubramaniam, 2012).

Innovative nonthermal processes for food preservation have attracted the attention of many food manufacturers (Tao et al. 2014). For example, the conventional method of heat sterilization often leads to overcooking the food material causing unwanted loss of nutrients and organoleptic changes but the electric heating methods offer novel possibilities for sterilization providing better retention of quality attributes (Deak, 2014). Two types of electrical heating methods are known and have been practically explored: direct and indirect. In the case of the direct method electrical current is passed directly into the food (called ohmic heating, OH, or electrical resistance heating). With indirect electroheating the electric energy is first converted to electromagnetic radiation which subsequently generates heat within a product (microwave (MW) and radiofrequency (RF) heating) (Deak,

2014). This study presents some unconventional antimicrobial treatments of food which are used nowadays in industry or are in the research and development phase.

OHMIC TREATMENT (OH)

Ohmic heating is a thermal processing method in which an alternating electrical current is passed through food products to generate heat internally (Darvishi et al., 2012). This treatment appears as a solution to reduce thermal damage because it heats materials in a rapid and homogeneous manner and may allow improved retention of vitamins, pigments and nutrients, resulting in less thermal damage to labile substances (Sarkis et al., 2012). The obvious advantage of ohmic treatments over conventional methods is the lack of high wall temperatures and limiting heat transfer coefficients requirements (Icier, 2012). To our knowledge, this is the only electric resistance heating technology exhibiting a wall temperature that can be cooler than the heated medium, a fouling that can be kept to a minimum, and high energy efficiency (Goullieux and Pain, 2014).

Ohmic treatment is used in a wide range of applications such as preheating, cooking, blanching, pasteurization, sterilization and extraction of food products (Yildiz-Turp et al., 2013). Other examples for applications are the following: it's potential to increase dye diffusion in beet, its capability to extract sucrose from sugar beet, and its possibility to enhance the diffusion of soy milk from soybeans. Several past studies have shown an additional effect of electricity during the ohmic heating of plant tissues, vegetative microorganisms and bacterial spores (Somavat et al., 2013). Park reviewed the effective mechanisms of electrical current on microorganisms and he observed that the mechanism may include disruption of bacterial membrane integrity or electrolysis of molecules on the cell surface. When a voltage is applied, it increases the energy of the membrane such that an increase in membrane pore size takes place up to a transition to hydrophilic pores, where free diffusion may occur (Icier, 2012). That explains how many microorganisms such as *Escherichia coli, Bacillus subtilis, Bacillus licheniformis, Streptococcus thermophilus Geobacillus stearothermophilus, V. parahaemolyticus* are destroyed.

Ohmic heating is not only a useful thermal process in food stabilization, but also a pretreatment to prepare vegetal tissues before a mass transfer operation (e.g., diffusion, extraction, or dehydration).

PULSED ELECTRIC FIELD (PEF)

Pulsed electric field (PEF) processing is a non-thermal food-processing technology, which uses short bursts of electricity, providing fresh-like, safe foods and reduces loss of quality (Wang et al., 2014).

In general, PEF treatment systems are composed of PEF treatment chambers, a pulse generator, a fluid-handling system, and monitoring systems. The treatment chamber is used to house electrodes and deliver a high voltage to the food material. It is generally composed of two electrodes held in position by insulating material, thus forming an enclosure containing the food material. Therefore, the proper design of the treatment chamber is an essential component for the efficiency of the PEF technology (Elez- Martinez et al., 2012). Regarding microorganisms a uniform distribution of electric field strength in the PEF treatment chamber is necessary to ensure that each microbial cell within a population receives the same PEF treatment and, thus, to develop mathematical kinetic models for the prediction of microbial inactivation and quality control (Min et al., 2014). Compared to traditional thermal pasteurization, PEF technology is a non-thermal food preservation method, which kills most pathogenic or spoilage microorganisms and inactivates enzymes, and minimizes the loss of taste, colour, texture, nutrients, and heat labile functional components of foods (Han et al., 2009). Most of the studies in PEF have concentrated on aspects of food science (Wang et al., 2014) such as: extracting bioactive compounds from raw material (Zhao et al., 2011); extension of food storage shelf life by food sterilization and enzyme inactivation (Salvia-Trujillo et al., 2011); maintaining physical–chemical property and nutritional values of foods (Zhao et al., 2008); and degrading the behaviour of two

pesticides, methamidophos and chlorpyrifos in apple juice (Chen et al., 2009).

The effects of PEF on the microorganisms in foods have mainly been studied in juices and milk, although there have been attempts to use PEF technology to process other products (Martin- Belloso et al, 2014). Antimicrobial effect of PEF is depending on the electric field strength, number of pulses applied, temperature and added antimicrobials (Nguyen and Mittal, 2006). The use of this technology in combination with other nonthermal technologies or mild heating would increase the microbial destruction (Martin-Belloso et al., 2014; Pool et al., 2001).

In the last years, there has been considerable interest in the adoption of PEF processing, and research into process scale-up. In other applications than foods, PEF processing can also improve the performance of industrial processes such as the removal of water from sludge, or the extraction of sugars and starches from plants, because the ruptured cells release their intracellular liquids more easily into their surroundings. (Kempkes, 2010).

MICROWAVE TREATMENT (MW)

Microwaves are electromagnetic radiation with wavelengths from 1 mm to 1 m in length and with frequencies from about 300 MHz to 300 GHz (Scaman et al., 2014). Since its first application for cooking, microwaves have been used for many purposes, including chemical synthesis of organic and inorganic substances, industrial processes, biosciences, food industry, and environmental treatments, among others. Dielectric heating, which uses electromagnetic radiations such as microwave (MW) is gaining popularity in food processing (Hebbar and Rastogi, 2012) and it provides volumetric heating, which means that heat is generated inside the material via absorption of electromagnetic energy from the applied field. Microwaves from household ovens and many industrial applications are produced efficiently by continuous wave magnetrons. A magnetron is a vacuum diode in which the cathode is surrounded by a coaxial anode (Scaman et al., 2014). The anode has an even number of vanes that extend toward the cathode. The open areas between each of the vanes are resonant cavities that determine the output frequency of the magnetron. The intensity and frequency of the field as well as dielectric properties of the material determine the degree of volumetric power absorption and the rate of heat generation (Ozkoc, 2014). Microwave heating is most efficient on liquid water, but much less efficient on fats and sugars (which have less molecular dipole movement), and frozen water (where the molecules are not free to rotate). MW heating sometimes occurs due to rotational resonance of water molecules, which happens only at much higher frequencies, in the tens of Gigahertz (Dev et al., 2012).

The mechanism of inactivation of microorganisms in volumetric heating processes is mainly due to thermal effects. Electromagnetic energy in MW inactivates microbes via conventional thermal mechanisms, including thermal irreversible denaturation of enzymes, proteins, and nucleic acids (Dev et al., 2012). The future of MW heating in food processing applications is promising, but successful exploration of MW heating applications relies on a thorough understanding of the interaction between MW and foods, and on the ability to predict and provide a desired heating pattern in foods for specific applications. These facts together with the possibility of offering continuous systems are seen as advantages in the food processing industry, although the issue of non-uniformity remains unresolved (Dev et al., 2012).

INFRARED TREATMENT (IR)

IR radiation is part of the electromagnetic spectrum in the wavelength range between 0.5 and 1000 mm, which is mainly utilized for food processing because of the several advantages such as higher heat-transfer capacity, instant heating because of direct heat penetration, high energy efficiency, faster heat treatment, fast regulation response, better process control, no heating of surrounding air, equipment compactness, uniform heating, preservation of vitamins, and less chance of flavor losses from burning of food products (Rastogi, 2012). All matter above the temperature of absolute zero possesses electromagnetic energy and emits radiation, in a wide range of electromagnetic spectral frequencies (Susek, 2010). These

frequencies are produced by the oscillation of individual atoms or molecules with electric charges. The temperature of the emitting surface has a direct impact on these frequencies and the total amount of energy radiated. Since the maximum radiated power at room temperature occurs in the IR region (0.78–1000 mm) of the electromagnetic spectrum, using this frequency of radiation holds special significance, especially in food applications (Ramaswamy et al., 2012).

IR technology is generally applied to: the dehydration of vegetables, fish, pasta and rice; heating flour; frying meat; roasting cereals; roasting coffee and cocoa; and baking biscuits and bread. The technique has also been used for thawing, surface pasteurization of bread and packaging materials (Rastogi, 2015). However, the use of IR irradiation for food decontamination applications has not been widely researched and reported; the literature available is limited to a very few microorganisms and some raw foods. It is clear that the equipment (lamp, waveguide, power, etc.) and process parameters (time, power of exposure, distance of application, etc.) need to be optimized for specific applications (Ramaswamy, et al., 2012).

ULTRAVIOLET TREATMENT (UV)

Ultraviolet (UV) light is the part of the electromagnetic spectrum with wavelengths between 100 and 400 nm (Gomez- Lopez et al., 2012). Ultraviolet (UV) light is an economical intervention toward improved hygiene control measures in the food industry. Sanitation, decontamination, disinfection, and oxidation with UV light is a versatile, environmental-friendly technology, which can be used in the food production and storage facilities to reduce microbial contamination and consequently to improve safety of finished products (Koutchma, 2014). UV light is emitted by the source that consists of an inert-gas flash lamp that converts high-power electricity to high-power radiation. UV is categorized in long-wave (UV-A; 315-400 nm), medium-wave (UV-B; 280-315 nm), and short-wave (UV-C; 200-280 nm) diapasons. A few types of continuous light UV sources are commercially available that include LPM and MPM lamps, low-pressure amalgam (LPA), and ELs. LPM and MPM lamps are the dominant sources for UV light treatment of fluid foods, drinks and beverages including water processing. However, only LPM lamps that emit UV light at 253.7 nm are currently approved by the US FDA for food applications (Keklik et al., 2012; Koutchma, 2014). The use of UV has been proposed for the pasteurization and sterilization of food and contact surfaces (Fredericks et al., 2011; Molina et al., 2014) being the potential of the UV light on the destruction of bacteria, viruses and parasites widely documented (Mukhopadhyay and Ramaswamy, 2012). The inactivation mechanism of UV is the formation of photoproducts in the DNA. UV light inactivates microorganisms by disrupting their nucleic acid (DNA) through the formation of pyrimidine dimers between adjacent pyrimidine molecules on the same strand of DNA. (Franz et al., 2009; Lacroix, 2014). Microorganisms can find protective sites in some product surfaces (e.g. lettuce, carrots) and can migrate to these sites when UV radiation is applied. The DNA damage inflicted by UV-C radiation leads to lethality by directly altering microbial DNA through dimer formation between neighbouring pyrimidine nucleoside bases in the same DNA strand (Birmpa et al., 2013). UV systems demonstrated capability to deliver the performance that is equivalent to existing industrial practices using thermal processing and achieves required food safety objective. The examples of existing and potential applications of UV light include juice products, raw milk, cheese milk, sugar syrups, liquid eggs and egg components, and wine and whey protein ingredients. Additionally, the unique advantages of UV processing and added value products have been produced in commercial scale (Koutchma, 2014).

ULTRA HIGH PRESSURE (UHP)

High-pressure processing (HPP) is a method of food processing where food is subjected to elevated pressures (up to 87,000 pounds per square inch or approximately 600 MPa), with or without the addition of heat, to achieve microbial inactivation or to alter the food attributes in order to achieve consumer-desired qualities. The technology is also referred as

High Hydrostatic Pressure Processing (HHP) and Ultra High Pressure Processing (UHP) in the literature. HPP retains food quality, maintains natural freshness, and extends the microbiological shelf-life of the food (Gupta and Balasubramaniam, 2012; Ramaswamy et al., 2013). The history of the use of high pressure to inactivate microorganisms in food dates back to 1899, when Hite demonstrated the application of high pressure in preserving milk and later to preserve fruits and vegetables (Gupta and Balasubramaniam, 2012). Earlier studies have shown that by subjecting foods to high pressures in the range of 300–400 MPa, vegetative cells of microorganisms and certain enzymes can be inactivated at ambient temperature without degradation of flavour and nutrients. However, bacterial spores can only be killed by high pressures (600–700 MPa) in combination with heat (>70°C) (Daryaei and Balasubramaniam, 2012).

Today, a wide range of value-added pressure-treated foods such as fruit smoothies, guacamole, ready meals with meat and vegetables, oysters, ham, chicken strips, fruit juices, and salsa (Ramaswamy et al., 2013) are available to consumers. To carry out HP processing cyclically on a production line, it is necessary to design HP equipment with sufficient capacity and durability. Well-designed HP equipment should be composed of a pressure chamber, closures to seal the chamber, a device to hold the closures during processing, HP intensifier pumps, systems to monitor and control the pressure and temperature, a temperature control device, and a product-handling system to transfer product to and from the pressure chamber (Tao et al., 2014). After treatment, the pumpable product (e.g. juices) can be pumped to an aseptic filling line, similar to that used for ultra-high-temperature (UHT) processed liquids to be packaged in glass bottles or gable cartons. The realization of HPP sterilization concepts for low-acid foods could represent a breakthrough in ambient distribution as it will result in higher nutritional and sensory standards of preserved food (Daryaei and Balasubramaniam, 2012).

OZONE TREATMENT

Ozone is a triatomic form of oxygen and is characterized by a high oxidation potential that conveys bactericidal and virucidal properties. It is a powerful broad-spectrum antimicrobial agent active against bacteria, fungi, viruses, protozoa, and also against bacterial and fungal spores. Ozone inactivates microorganisms through oxidization, and residual ozone spontaneously decomposes to non-toxic products (oxygen), making it an environmentally friendly antimicrobial agent for use in the food industry (Patil and Bourke, 2012). The rising interest in novel food processing and preservation systems is driven by a number of factors including consumer preference for minimally processed food free of chemical preservatives; recent highly-publicized outbreaks of foodborne diseases caused by pathogens such as *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes*; and the passage of new food safety legislations in the US and other countries (e.g., FDA, 2011) (Chawla et al., 2012). One of the most important factors in the efficacy of the ozone application is the treatment temperature since it affects the solubility, stability and reactivity of gas. As the temperature increases, reaction rate also increases but ozone becomes less soluble and less stable. Therefore as the treatment temperature increases, the increase in ozone reactivity is negated by the decrease in its stability, without causing significant changes in the efficacy of ozone (Cárdenas et al., 2011). Ozone is now an accepted commercial technology in many aspects of the agri- foods industry, ranging from irrigation and soil treatment, to spraying crops, odour control in animal housing and for uses in food processing plants (water and air treatment, food processing, packaging and storage) (Rice, 2010). Meanwhile, relevant literature has indicated ozone treatment to be a good candidate for the seafood industry (Okpala, 2014). In recent years it has been recognized that the combination of ozone with other acceptable food processing technologies (electrolyzed water, ultrasound, modified air packaging, ultraviolet radiation) can overcome the deficiencies of employing ozone by itself to solve a particular food disinfection problem

(Rice, 2010). Bermúdez-Aguirre and Barbosa-Cánovas (2012) demonstrated that the effectiveness of the ozone disinfection treatment is influenced by the dose of the agent, the exposure time and the surface of the food product. Smooth surface of vegetables such as tomatoes represents an easy product to allow direct contact of the sanitizer with the bacteria. When the surface becomes more complex in terms of porosity and roughness, the inactivation seems to be more complicated and reduced. Some changes in the color of produce can be controlled if the exposure time and/or concentration of the disinfection agent are kept as low as possible to inactivate the microorganism but still preserving the quality of the product.

PULSED LIGHT TREATMENT (PLT)

Pulsed light technology (PLT) involves the use of inert-gas flash lamps that convert short-duration and high-power electric pulses. Electromagnetic radiation is emitted and propagated by means of waves that differ in wavelength, frequency, and energy. The term "light" is generally used to mean radiation in which ranges from about 100 to 1100 nm, which includes ultraviolet rays (UV, λ=100-400 nm, roughly subdivided into UV-A, λ= 315-400 nm, UV-B, λ= 280-315 nm, UV-C, λ= 200-280 nm, and vacuum UV, λ=100-200 nm), visible light (VL, λ= 400-700 nm), and IR rays (IR, λ= 700- 1100 nm) (Cacace and Palmieri, 2014). Pulsed light results in very few residual compounds and does not involve the use of chemicals that cause environmental pollution or harm humans. Moreover, since a xenon lamp does not contain mercury, it is also more eco-friendly than a UV lamp. Each flash has an intensity almost 20,000x that of sunlight at sea level, and contains UV wavelengths that do not reach the earth's surface since they are filtered by the atmosphere (Choi et al., 2009). Microbial inactivation is mainly attributed to photochemical damage caused by the UV-C component, although photothermal damage has also been proposed (Hierro et al., 2012). Various studies have demonstrated the positive effect of pulsed light on inactivation of microbial populations on food surfaces. Reductions in counts of *Escherichia coli* O157:H7 on alfalfa seeds, *Aspergillus niger* spores on corn meal, *Listeria monocytogenes* and *E. coli* O157:H7 on raw salmon fillets, *Salmonella enterica* and *E. coli* O157:H7 on raspberries and strawberries and *L. monocytogenes* on infant foods have been reported, indicating this technology could be a powerful nonchemical (residue-free) option for decontaminating foods (Gómez et al., 2012).

PLASMA TREATMENT

Cold plasma is promising as a nonthermal food processing technology. Plasma plumes have been used to treat glass, electronics, textiles, paper, and other products. More recently it became a subject of research as an intervention to improve the safety of foods. However, the technical aspects of cold plasma are as yet largely unfamiliar to food producers, processors, and researchers (Niemira, 2014).
Cold plasma technology is offering many potential applications for food packaging. While it was originally developed to increase the surface energy of polymers, enhancing adhesion and printability, it has recently emerged as a powerful tool for surface decontamination of both foodstuffs and food packaging materials (Pankaj et al., 2014). Plasma source may offer significantly different modes of application. A plasma-jet for instance, can be applied to a sample directly, if the treatment distance is held short enough that the plasma filaments are touching the sample surface. This mode allows for interactions of the complete composition of plasma species with the sample surface (Baier et al., 2014). There are three primary mechanisms by which cold plasma inactivates microbes: (1) direct chemical interaction of cells with reactive species and charged particles; (2) UV damage of cellular components and membranes; (3) UV-mediated DNA strand breakage. While one mode of action may be more predominant than another in any given cold plasma system, the greatest sanitizing efficacy will result from multiple antimicrobial mechanisms (Niemira, 2014). Special attention needs to be given to food products, which do not undergo further heat treatment (pasteurization and sterilization processes), like ready-to-eat dishes. For example, the decontamination of dried

products, like herbs and spices is difficult, because the resistance of microorganisms, especially sporulated ones, in a medium with a low a_w is higher when compared to the resistance of the same microorganisms in a water rich medium. Thus, the remote plasma system was able to inactivate bacterial spores, vegetative bacteria, molds and yeast under ambient conditions on different types of herbs and spices with various surface- to-volume ratios (Hertwig et al., 2014).

ACTIVE PACKAGING

Developments in food packaging have evolved in response to the need for protection of the food product from both external and internal environments and in response to consumer expectations for convenience and product safety (Singh and Heldman, 2014).

Active packaging can be looked at from different perspectives. For example, a food technologist will be interested in studying the effects of active packaging solutions on food quality; a polymer engineer will focus his attention on the modification of traditional polymers to modulate the absorption or release of active substances; a chemical scientist will focus his research on the interactions between active substances and foods, and so on (Limbo and Khaneghah, 2015).

Active packaging is an innovative approach to enhance the shelf life of food stuffs while improving their quality, safety and integrity. Active packaging can be defined as a packaging system that interacts with the package components and the food to extend the shelf life or to improve the safety or sensory properties of the food, while maintaining the quality of the packaged product. Active packaging systems can be classified into active-releasing systems (emitters) which add compounds to the packaged food or into the headspace, or active scavenging systems (absorbers), which remove undesired compounds from the food or its environment (Yildirim, 2011).

One of the earliest active packaging systems was Modified Atmosphere Packaging (MAP) (Singh and Heldman, 2014). Modified atmosphere packaging (MAP) is a packaging system that involves changing the gaseous atmosphere surrounding a food product inside a pack, and employing packaging materials and formats with an appropriate level of gas barrier to maintain the changed atmosphere at an acceptable level for preservation of the food. Changing the gaseous atmosphere may mean removing air completely, that is by vacuum packaging, or replacing air with other gases (Emblem, 2013). Its use has been extended to fish, fresh produce, pasta, pizza, other baked goods, and dry products such as nuts and snacks, and it is believed that MAP is the fastest growing method of food preservation at the expense of more traditional methods such as retorting and freezing (Emblem, 2013). Another interesting development is incurporation of antimicrobial agents directly into packaging, which allows the industry to combine the preservative function of antimicrobials with the protective function of packaging (Yildirim, 2011).

Antimicrobial food packaging materials extend the lag phase and reduce the growth phase of microorganisms in order to extend shelf life and to maintain food quality and safety. (Realini and Marcos, 2014). It has been considered as a complementary method to the existing preservation methods to control undesirable microorganisms on foods by means of the incorporation of antimicrobial substances in the packaging films or application as a coating onto the packaging materials (Yildirim, 2011).

ENCAPSULATION OF ANTIMICROBIAL COMPOUNDS

In the last years, natural antimicrobials have attracted considerable attention due to the increased consumer awareness on the aspects of food quality and safety (Donsi et al., 2011).

Nanoencapsulation of bioactive compounds represents a viable and efficient approach to increase the physical stability of the active substances, protect them from the interactions with the food ingredients and, because of the subcellular size, increasing their bioactivity. In the case of antimicrobials, encapsulation can increase the concentration of the bioactive compounds in food areas where microorganisms are preferably located, for example water-rich phases or liquid-solid interfaces (Donsi et al., 2011).

Encapsulation technologies that effectively reduce antimicrobial interaction with food components or protect antimicrobial compounds from food processing measures have the potential to improve the microbiological safety of ready-to-eat foods (Taylor et al., 2008). Many compounds have been encapsulated; some of them are antioxidants, flavours, and antimicrobial compounds. Each of the different encapsulation systems has advantages and disadvantages. In general, the nanoencapsulation systems have excellent sustained-release properties, subcellular size, and biocompatibility with tissue and cells, allow alterations in the bioavailability of drugs, and improve the pharmacokinetic profile of numerous actives. Additionally, the encapsulation of antimicrobial compounds reduced their toxicity, the resistance is overcome, and the cost of using them is decreased because a less amount of the active is required. Limitations of all nanoencapsulation systems for their use in food industry are related to their high production costs and lack of allowed materials (Blanco-Padilla et al., 2014).

EDIBLE FILMS

An edible film is defined as a thin layer, which can be consumed, coated on a food or placed as barrier between the food and the surrounding environment (Skurtys et al., 2010). The use of edible films in food protection and preservation has recently increased since they offer several advantages over synthetic materials, such as being biodegradable and environmentally friendly. When those films take contact with food, moisture from food induce liposome membrane to slowly release antimicrobial extracts which will be trapped between food surface and liposome membrane. This fact is more efficient for inhibition food spoilage and food pathogen microorganisms because it maintain a high concentration. Moreover, edible film has abilities to retard moisture, oxygen, aromas and solute transportation (Mekkerdchoo et al., 2010).

The development of new natural edible films with the addition of antimicrobial compounds to preserve fresh and minimally processed fruits and vegetables is a technological challenge for the industry and a very active research field worldwide. Antimicrobial agents have been successfully added to edible composite films based on polysaccharides or proteins such as starch, cellulose derivatives, chitosan, alginate, and fruit puree, isolated whey protein, soy protein, egg albumen, wheat gluten, or sodium caseinate (Chamorro et al., 2011).

The main disadvantage of these techniques is the loss of quality of the edible coatings and films since there is no control over the shape, size and size distribution of the dispersed elements (e.g. additives, ingredients, etc.) and the support structure matrix is poor. Another disadvantage

of these techniques is that the thickness of the films is generally not constant or controlled (Skurtys et al., 2010).

RADIOFREQUENCY TREATMENT

Radio frequency (RF) heating forms part of a group of innovative techniques based on electromagnetic heating (example: infrared, and microwave), and, non-thermal methods (such as high pressure, pulsed electric and ultrasonic waves) that have been touted to have the potential of providing high quality foods from an economically point of view (Awuah et al., 2005). The use of radio frequency electric fields (RFEF) as a pasteurization method has been studied for more than 60 years. There has been a long debate for over 50 years over whether there are nonthermal effects associated with electromagnetic fields (Trujillo and Geveke, 2014). In a RF heating system, the RF generator creates an alternating electric field between two electrodes. The material to be heated is placed between the electrodes, where the alternating energy causes polarization, in which the molecules in the material continuously reorient themselves to face opposite poles. At radio frequencies (e.g. 27.12 MHz), the electric field alternates 27,120,000 times per second. The friction resulting from the rotational movement of the molecules and from the space charge displacement causes the material to rapidly dissipate energy as heat throughout its mass (Orsat and Raghavan, 2014). Although identical to the microwave in terms of its heating characteristics, radio frequency has the additional advantage of uniform heating in homogeneous foods, and

most important of all, high penetration depth that could be used to pasteurize or sterilize liquid products. For RF heating, penetration depth is generally greater than 1 m, and can be determined from a relationship that embodies the dielectric constant, the loss factor, the speed of wave propagation in vacuum and, operating frequency. Depending on concentration and temperature, the penetration depth of starch solutions ranged from 0.2 to 2.1 m in the radio frequency range, while salt enriched starch solutions had comparatively low penetration depths (Awuah et al., 2005).
Cathcart and Park first studied the use of RF heating to thaw frozen eggs, fruits, vegetables, and fish. Radio frequency dielectric heating is now widely used in industrial applications such as drying textile products (spools, rovings, skeins), final drying of paper, final dehydration of biscuits at outlets of baking ovens, and melting honey (Wang et al., 2003). As a rapid heating method, RF heating offers a considerable speed advantage over conventional heating methods, particularly in solid foods in which heat transfer is predominantly governed by heat conduction. However, even with this major advantage and the fact that this technology has been available for many years, its uptake by industry have been relatively slow (Marra et al., 2008).

In Table 1 is presented an overview of the present work, where for each analyzed new technology there are described the parameters that were used by researchers in the treatment of food products. These parameters were presented as effective for food decontamination and shelf life prolongation.

Table 1. Parameters used by researchers in the treatment of food products

Unconventional treatment	Product	Parameters	Author, year
Ohmic treatment	Pomegranate juice	Voltage gradient=30- 35 V/cm	Darvishi et al., 2012
	Blueberry pulp	Voltage=160V, 200 V and 240 V	Sarkis et al., 2012
	Tomato juice	Frequency=10 and 60kHz	Somavat et al., 2013
PEF	Green tea beverage	Electric field strength= 18.1 kV/cm; 27.4 kV/cm and 38.4kV/cm	Zhao et al., 2008
	Corn- starch	Electric field strength=30 kV/cm, 40 kV/cm and 50 kV/cm Temperature= 50°C	Han et al., 2009
	Glutathione from different products	Electric field intensity= 9.74 kV/cm Frequency=2549.08Hz	Wang et al., 2014
MW	Apple cylinders	Temperature=40°C Incident microwave power=3 and 10 W/g Air velocity=1m/s	Bilbao- Sainz et al., 2006
	Strawberry halves	Temperature= 40°C Incident microwave power=0.2 W/g Air velocity=2.6m/s	Contreras et al., 2008
	Potato omelet	P= 300W, 450W, 600W and 800W τ= 30s and 40s	Valero et al., 2014
	Hazelnut	Temperature=100-160°C Activation energy= 1891.6kJ/kg	Ozdemir and Devres, 2000

IR	Banana slices	Intensity= 3000 W/m^2, 4000 W/m^2 and 5000W/m^2,	Zhongli et al., 2008
UV	Porcine and fish gelatine	Radiation absorbed dose=2-10 kGy	Sung and Chen, 2013
	Sea bass fillets	λ= 250mm	Molina et al., 2014
UHP	Lychee (*Litchi chimensis* Sonn.	Pressure= 200- 600 MPa Temperature= 20-60°C τ= 10min and 20min	Phunchaisri and Apichartsrangkoon, 2005
	Corn starch	Pressure= 0.1-400 MPa	Choi et al., 2009
Ozone treatment	Dried oregano	Ozone concentration= 2.8 mg/L and 5.3 mg/L τ=120min	Torlak et al., 2013
	Wheat grains	40 and 60 µmol/mol τ=30min, 60min, 120min and 180 min	Savi et al., 2014
PLT	Beef and tuna slices	Fluences dose= 0.7J/cm^2, 2.1J/cm^2, 4.2 J/cm^2, 8.4 J/cm^2 and 11.9 J/cm^2	Hierro et al., 2012
	Fresh cut apples	Fluences dose= 71.6J/cm^2	Gomez et al., 2012
Plasma treatment	Sour cherry Marasca juice	Volume of juice= 3ml τ=3min	Garofulic et al., 2014
Atmospheric cold plasma	Strawberry	DBD= 60kV Frequency= 50Hz	Misra et al., 2014
Active packaging	Meat	60-70%CO_2 and 30-40%N_2	Cooksey, 2014
	Fatty fish	40%CO_2 and 60%N_2	Cooksey, 2014
	Non- fatty fish	30%O_2, 40%CO_2 and 30%N_2	Cooksey, 2014
RF	Milk	Frequency=27.12MHz	Awuah at al., 2005
	Soybean milk	Frequency=28MHz	Uemura et al., 2010
	Black and red pepper spice	Frequency=27.12MHz	Kim et al., 2011

CONCLUSIONS

Research in novel heating of foods, for applications such as cooking, pasteurisation/sterilisation, defrosting, thawing and drying, often focuses on areas such as the assessment of processing time, the evaluation of heating uniformity, the appraisal of the impact on quality attributes of the final product as well as the prediction of the energy efficiency of these heating processes (Marra et al., 2008).

The aim of the current review is to establish the unconventional antimicrobial treatments for foods, as evidenced by the refereed publications which have appeared in this area in the last years. In addition future trends for research in this field were also discussed. To ensure a comprehensive overview is provided, this paper included a description of the mechanism of unconventional treatments; an overview of typical equipment used for non- thermal treatments; examples of the wide range of this methods applications in food processing which have been proposed in the scientific literature in recent years, with related description of the effects of unconventional treatments on quality attributes of products.

ACKNOWLEDGEMENTS

This paper was published under the frame of Partnerships in priority areas Programme, PCCA Contract no. 164 / 2014, RAFSIG.

REFERENCES

Awuah G.B., Ramaswamy H.S., Economides A., Mallikarjunan K., 2005. Inactivation of Escherichia coli K-12 and Listeria innocua in milk using radio frequency (RF) heating. Innovative Food Science and Emerging Technologies, 6, 396 – 402.

Baier M., Görgena M., Ehlbeck J., Knorr D., Herppicha W. B., Schlüter O., 2014. Non thermal atmospheric pressure plasma: Screening for gentle process conditions and antibacterial efficiency on perishable fresh produce. Innovative Food Science and Emerging Technologies, 22, 147–157.

Bermúdez-Aguirre D., Barbosa-Cánovas G. V., 2012. Disinfection of selected vegetables under nonthermal treatments: Chlorine, acid citric, ultraviolet light and ozone. Food Control, 29, 1, 82–90.

Bilbao-Sainz C., Andres A., Chiralt A., Fito P., 2006. Microwaves phenomena during drying of apple cylinders. Journal of Food Engineering, 74, 160- 167.

Birmpa A., Sfika V., Vantarakis A., 2013. Ultraviolet light and Ultrasound as non-thermal treatments for the inactivation of microorganisms in fresh ready-to-eat foods. International Journal of Food Microbiology, 167, 96–102.

Blanco-Padilla A., Soto K.M., Iturriaga M. H., Mendoza S., 2014. Food Antimicrobials Nanocarriers. The Scientific World Journal, 2014, Article ID 837215, 11 pages.

Cacace D., Palmieri L., 2014. High-intensity Pulsed Light Technology. Emerging Technologies for Food Processing (Second Edition), 239–258.

Cárdenas F.C., Andrés S., Giannuzzi L., Zaritzky N. Antimicrobial action and effects on beef quality attributes of a gaseous ozone treatment at refrigeration temperatures. Food Control, 22, 8, 1442–1447.

Chamorro S. A. V., Paloua L., Ríoa M. A. Pérez-Gagoa M. B., 2011. Antimicrobial Edible Films and Coatings for Fresh and Minimally Processed Fruits and Vegetables: A Review. Critical Reviews in Food Science and Nutrition, 51, 9, 872-900.

Chawla A. S., Kasler D. R., Sastry S. K., Yousef A. E., 2012. Microbial decontamination of food using ozone. Woodhead Publishing Limited, 495- 532.

Chen F., Zeng L. Q., Zhang Y. Y., Liao X. J., Ge Y. Q., Hu X. S., Jiang L., 2009. Degradation behaviour of methamidophos and chlorpyrifos in apple juice treated with pulsed electric fields. Food Chemistry, 112, 956–961.

Choi M.S., Cheigh C.I., Jeong E.A., Shin J.K., Chung M.S., 2009. Nonthermal sterilization of Listeria monocytogenes in infant foods by intense pulsed-light treatment. Journal of Food Engineering, 97, 504–509.

Contreras C., Martin-Esparza M.E., Chiralt A., Martinez-Navarrete N., 2008. Influence of microwave application on convective drying: effects on drying kinetics, and optical and mechanical properties of apple and strawberry. Journal of Food Engineering, 88, 55-64.

Cooksey K., 2014. Modified Atmosphere Packaging of Meat, Poultry and Fish. Innovations in Food Packaging, 475- 493.

Corrales M., Fernandez A., Han J.H., 2014. Antimicrobial Packaging Systems. Innovations in Food Packaging (Second Edition), 133-170.

Darvishi H., Khostaghaza M. H., Najafi G., 2012. Ohmic heating of pomegranate juice: Electrical conductivity and pH change. Journal of the Saudi Society of Agricultural Sciences 12, 101–108.

Daryaei H., Balasubramaniam V. M., 2012. Microbial decontamination of food by high pressure processing. Woodhead Publishing Limited, 370- 406.

Deak T., 2014. Chapter 17 – Thermal Treatment. Food Safety Management A Practical Guide for the Food Industry, 423–442.

Dev S. R. S., Birla S. L., Raghavan G. S. V., Subbiah J., 2012. Microbial decontamination of food by microwave (MW) and radio frequency (RF). Woodhead Publishing Limited, 274- 299.

Donsi F., Annunziata M., Sessa M., Ferrari G., 2011. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. LWT - Food Science and Technology, 44, 1908-1914.

Elez-Martinez P., Sobrino-Lopez A., Soliva-Fortuny R., Martin-Belloso O., 2012. Pulsed Electric Field. Processing of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods 63–108.

Emblem A., 2013. Modifi ed atmosphere packaging and other active packaging systems for food, beverages and other fast- moving consumer goods. Woodhead Publishing Limited, 22- 34.

Franz C.M.A.P., Specht I., CHO G.S., Graef V., Stahl M.R., 2009. UV-C inactivation of microorganisms in naturally cloudy apple juice using novel inactivation equipment based on Dean vortex technology. Food Control 20, 1103-1107.

Fredericks I. N., du Toit M., Krügel M., 2011. Efficacy of ultraviolet radiation as an alternative technology to inactivate microorganisms in grape juices and wines. Food Microbiology, 28, 510–517.

Garofulić I. E., Jambrak A. R., Milošević S., Dragović-Uzelac V., Zorić Z., Herceg Z., 2014. The effect of gas phase plasma treatment on the anthocyanin and phenolic acid content of sour cherry Marasca (Prunus cerasus var. Marasca) juice. LWT - Food Science and Technology, 1-7.

Gómez P.L, García-Loredo A., Nieto A., Salvatori D.M., Guerrero S., Alzamora S.M., 2012. Effect of pulsed light combined with an antibrowning pretreatment on quality of fresh cut apple. Innovative Food Science and Emerging Technologies, 16, 102–112.

Gomez-Lopez V.M., Koutchma T., Linden K., 2012. Ultraviolet and Pulsed Light Processing of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods, 185–223.

Goullieux A., Pain J.P., 2014. Ohmic Heating. Emerging Technologies for Food Processing (Second Edition) 399–426.

Gupta R., Balasubramaniam V.M., 2012. High-Pressure Processing of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods, 109-133.

Gupta R., Balasubramaniam V.M., 2012. High-Pressure Processing of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods, 109–133.

Han Z., Zeng X., Zhang B., Yu S., 2009. Effects of pulsed electric fields (PEF) treatment on the properties of corn starch. Journal of Food Engineering, 93, 318–323.

Hebbar H. U., Rastogi N. K., 2012. Microwave Heating of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods, 369–409.

Hertwig C., Reineke K., Ehlbeck J., Erdogdu B., Rauh C., Schlüter O., 2014. Impact of remote plasma treatment on natural microbial load and quality parameters of selected herbs and spices. Journal of Food Engineering.

Hierro E., Ganan M., Barroso E., Fernández M., 2012. Pulsed light treatment for the inactivation of selected pathogens and the shelf-life extension of beef and tuna carpaccio. International Journal of Food Microbiology 158, 42–48.

Icier F., 2012. Ohmic Heating of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods, 305–367.

Keklik N. M., Krishnamurthy K., Demirci A., 2012. Microbial decontamination of food by ultraviolet (UV) and pulsed UV light. Woodhead Publishing Limited, 344- 369.

Kempkes M. A., 2010. Pulsed electric field (PEF) systems for commercial food and juice processing. Woodhead Publishing Limited, 73-102.

Kim S.Y., Sagong H.G., Choi S. H., Ryu S., Kang D.H., 2012. Radio-frequency heating to inactivate Salmonella typhimurium and Escherichia coli O157:H7 on black and red pepper spice. International Journal of Food Microbiology, 153, 171–175.

Koutchma T., 2014. Chapter 1 – Introduction, Food Plant Safety. UV Applications for Food and Non-Food Surfaces, 1.

Koutchma T., 2014. Chapter 3 – UV Light Sources, Preservation and Shelf Life Extension. UV Applications for Food, 13–15.

Lacroix M., 2014. Irradiation. Emerging Technologies for Food Processing (Second Edition), 293–312.

Limbo S., Khaneghah A.M., 2015. Active packaging of foods and its combination with electron beam processing. Electron Beam Pasteurization and Complementary Food Processing Technologies, Woodhead Publishing Series in Food Science, Technology and Nutrition, 195–217.

Marra F., Zhang L., Lyng J.G., 2008. Radio frequency treatment of foods: Review of recent advances. Journal of Food Engineering 91, 497–508.

Marszałek K., Mitek M., Skąpska S.,2015. The effect of thermal pasteurization and high pressure processing at cold and mild temperatures on the chemical composition, microbial and enzyme activity in strawberry purée. Innovative Food Science and Emerging Technologies, 27, 48-56.

Martin-Belloso O., Marsellés-Fontanet R., Elez-Martinez P., 2014. Food Safety Aspects of Pulsed Electric Fields Emerging Technologies for Food Processing (Second Edition), 155–168

Mekkerdchoo O., Patipasena P., Borompichaichartkul C., 2010. Liposome encapsulation of antimicrobial extracts in pectin film for inhibition of food spoilage microorganisms. Asian Journal of Food and Agro-Industry, 2(04), 817-838.

Min S. C., Zhang H. Q., Han J. H., 2014. Packaging for Nonthermal Food Processing. Innovations in Food Packaging (Second Edition) 515–535.

Misra N.N., Patil S., Moiseev T., Bourke P., Mosnier J.P., Keener K.M., Cullen P.J., 2014. In-package atmospheric pressure cold plasma treatment of strawberries Journal of Food Engineering, 125, 131–138.

Molina B., Sáez M.I., Martínez T.F., Guil-Guerrero J.L., Suárez M.D., 2014. Effect of ultraviolet light treatment on microbial contamination, some textural and organoleptic parameters of cultured sea bass fillets (Dicentrarchus labrax). Innovative Food Science and Emerging Technologies, 26, 205–213.

Mukhopadhyay S., Ramaswamy R., 2012). Application of emerging technologies to control Salmonella in foods: A review. Food Research International, 45, 666–677.

Nguyen P., Mittal G.S., 2006. Inactivation of naturally occurring microorganisms in tomato juice using pulsed electric field (PEF) with and without antimicrobials. Chemical Engineering and Processing, 46, 360–365.

Niemira B. A., 2014. Decontamination of Foods by Cold Plasma. Emerging Technologies for Food Processing (Second Edition), 327–333.

Okpala C.O. R., Investigation of quality attributes of ice-stored Pacific white shrimp (Litopenaeus vannamei) as affected by sequential minimal ozone treatment. LWT - Food Science and Technology, 57, Issue 2, 538–547.

Orsat V., Raghavan G.S.V., 2014. Radio-Frequency Processing. Emerging Technologies for Food Processing (Second Edition), 385–398.

Ozdemir, M., Devres, O., 2000. Analysis of color development during roasting of hazelnuts using response surface methodology. Journal of Food Engineering, 45, 17-24.

Ozkoc S. O., Sumnu G., Sahin S., 2014. Recent Developments in Microwave Heating. Emerging Technologies for Food Processing (Second Edition), 361–383.

Pankaj S.K., Bueno-Ferrera C., Misraa N.N., Milosavljevi V., O'Donnell C.P., Bourkea P., Keenera K.M., Cullen P.J., 2014. Trends in Food. Science & Technology, 35, 5-17.

Patil S., Bourke P., 2012. Ozone Processing of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods, 225–261.

Phunchaisri C., Apichartsrangkoon A., 2005. Effects of ultra-high pressure on biochemical and physical modification of lychee (Litchi chinensis Sonn.). Food Chemistry, 93, 57–64.

Pool, I.,E., Hennie, C.,M., Slump, R.,A., Popa, M.E., Smid, E.,J., 2001.Influence of food matrix on inactivation of Bacillus cereus by combinations of nisin, pulsed electric field treatment and carvacrol, in Journal of Food Protection, 64, 7, 1012-1018.

Ramaswamy R., Ahn J., Balasubramaniam V.M., L.R. Saona, Yousef A. E., 2013. Food Safety Engineering. Handbook of Farm, Dairy and Food Machinery Engineering, 43–66.

Ramaswamy R., Krishnamurthy K., Jun S., 2012. Microbial decontamination of food by infrared (IR) heating. Woodhead Publishing Limited, 450- 471.

Rastogi N. K., 2012. Infrared Heating of Fluid Foods. Novel Thermal and Non-Thermal Technologies for Fluid Foods, 411–432.

Rastogi N.K., 2015. Infrared heating of foods and its combination with electron beam processing. Electron Beam Pasteurization and Complementary Food Processing Technologies, Woodhead Publishing Series in Food Science, Technology and Nutrition, 61–82.

Realini C.E., Marcos B., 2014. Active and intelligent packaging systems for a modern society. Meat Science, 98, 404–419.

Rice R. G., 2010. Commercial applications of ozone in food processing. Woodhead Publishing Limited, 258-282.

Salvia-Trujillo L., Morales P. M., Rojas-Graü M. A., Martín-Belloso O., 2011. Changes on phenolic and carotenoid composition of high intensity pulsed electric field and thermally treated fruit juice–soymilk beverages during refrigerated storage. Food Chemistry, 129, 982–990.

Sarkis J. R., Jaeschke D.P., Tessaro I.C., Marczak L.D.F., 2012. Effects of ohmic and conventional heating on anthocyanin degradation during the processing of blueberry pulp. Food Science and Technology 51, 79-85.

Savi G. D., Piacentini K. C., Bittencourt K. O., Scussel V. M., 2014. Ozone treatment efficiency on Fusarium graminearum and deoxynivalenol degradation and its effects on whole wheat grains (Triticum aestivum L.) quality and germination. Journal of Stored Products Research, 59, 245-253.

Scaman C.H., Durance T. D., Drummond L., Sun D.W., 2014. Combined Microwave Vacuum Drying. Emerging Technologies for Food Processing (Second Edition), 427–445.

Singh R. P., Heldman D. R., 2014. Packaging Concepts. Introduction to Food Engineering (Fifth Edition), Food Science and Technology, 767–791.

Skurtys O., Acevedo C., Pedreschi F., Enrione J., Osorio F., Aguilera J. M., 2010. Food Hydrocolloid Edible Films and Coatings

Somavat R., Mohamed H.M.H., Sastry S.K., 2013. Inactivation kinetics of Bacillus coagulans spores under ohmic and conventional heating. LWT - Food Science and Technology 54 (2013) 194-198.

Sung W.C., Chen Z.Y., 2014. UV treatmentand γ irradiation processing on improving porcine and fish gelatin and qualities of their premix mousse. Radiation Physics and Chemistry, 97, 208–211.

Susek W., 2010. Thermal microwave radiation for subsurface absolute temperature measurement. Acta Physica Polonica A, 118, 1246–1249.

Tao Y., Sun D.W., Hogan E, Kelly A. L., 2014. High-Pressure Processing of Foods: An Overview. Emerging Technologies for Food Processing (Second Edition), 3-24.

Taylor T. M., Bruce B. D., Weiss J., Davidson M., 2008. Listeria monocytogenes and Escherichia coli O157:H7 inhibition in vitro by liposome encapsulated nisin and ethylene diaminetetraacetic acid. Journal of Food Safety 28, 2, 183–197.

Torlak E., Sert D., Ulca P., 2013. Efficacy of gaseous ozone against Salmonella and microbial population on dried oregano. International Journal of Food Microbiology, 165, 276–280.

Trujillo F. J., Geveke D. J., 2014. Nonthermal Processing By Radio Frequency Electric Fields. Emerging Technologies for Food Processing (Second Edition), 259–269.

Uemura K., Takahashi C., Kobayashi I., 2010. Inactivation of Bacillus subtilis spores in soybean milk by radio-frequency flash heating. Journal of Food Engineering, 100, 622–626.

Valero A., Cejudo M., García-Gimeno R.M., 2014. Inactivation kinetics for Salmonella Enteritidis in potato omelet using microwave heating treatments. Food Control, 43, 175- 182.

Wang J., Wang K., Wang Y., Lin S., Zhao P., Jones G., 2014. A novel application of pulsed electric field (PEF) processing for improving glutathione (GSH) antioxidant activity. Food Chemistry, 161, 361–366.

Wang Y., Wig T.D., Tang J., Hallberg L.M., 2003. Sterilization of Foodstuffs Using Radio Frequency Heating. Journal Of Food Science 68, 2, 539- 544.

Yildirim S., 2011. Active packaging for food biopreservation. Woodhead Publishing Limited, 460-489.

Yildiz-Turp G., Sengun I.Y., Kendirci P., Icier F., 2013. Effect of ohmic treatment on quality characteristic of meat: A review. Meat Science 93, 441–448.

Zhao W. Z., Yu Z. P., Liu J. B., Yu Y. D., Yin Y. G., Lin S. Y., et al., 2011. Optimized extraction of polysaccharides from corn silk by pulsed electric field and response surface quadratic design. Journal of the Science of Food and Agriculture, 91, 2201–2209.

Zhao W., Yang R., Lub R., Wang M., Qianb P., Yang W., 2008. Effect of PEF on microbial inactivation and physical–chemical properties of green tea extracts. LWT, 41, 425–431.

Zhongli, P., Shih, C., McHugh, T.H., Hirschberg, E., 2008. Study of banana dehydration using sequential infrared radiation heating and freeze drying. LWT Food Science Technology, 41, 1944-1951.

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Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

LACTIC ACID BACTERIA INHIBITORY ACTIVITY ON THE PATHOGENS *SALMONELLA* AND *LISTERIA MONOCYTOGENES*

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Abstract

Throughout the technological process of obtaining dairy products occur unintentionally or not, microbial contamination, both internal and external with various pathogens that negative change their quality and threaten the health and safety of consumers. Consumption of dairy products contaminated with pathogens like Salmonella or Listeria monocytogenes causes unwanted effects: food poisoning, meningitis, cardiovascular disease, listeriosis, encephalitis, miscarriage, gastroenteritis, in some cases resulting in death etc.
Using LAB (lactic acid bacteria) in the dairy industry adds a plus protection and safety to consumer by preventing illness; also LAB have a beneficial role on longevity and helps to prolong preservation of dairy products.
Experimental research objectives are intended to level of inhibition by LAB (Delbrueckii subsp. Bulgaricus, Streptococcus thermophilus and Lactococcus subsp. Lactis) on some pathogenic Salmonella and Listeria monocytones twins.

Key words*: LAB, pathogens, degree of inhibition.*

INTRODUCTION

Throughout the technological process of obtaining dairy products microbial contamination could occur unintentionally or not, both internal and external with various pathogens that negatively change their quality and endanger the safety of consumers. Consumption of dairy products contaminated with pathogens like *Salmonella* or *Listeria monocytogenes* causes food illnesses such as: meningitis, cardiovascular disease, listeriosis, encephalitis, miscarriage, gastroenteritis, in some cases resulting in death .
Defined as "microorgansim whose main transmission pathways to humans is through contaminated food during production and processing" (World Health Organization Working Group, 1988), *Listeria monocytogenes* is thought to be the infectious agent that induces the highest mortalityrate. Also, an increasing segment of the population susceptible to infection (due to immune deficiency, malnutrition or aging) makes human listeriosis a very dangerous disease.

Listeria monocytogenes is a psychrotrophic bacteria that grow in food preserved by freezing, prepared and reheated foods - in which is producing listeriolysin; *Listeria monocytogenes* is the causative agent of human listeriosis, a potentially fatal food-borne infection. Clinical manifestations range from fever, gastroenteritis to severe invasive forms including meningitis, encephalitis, abortions and perinatal infections (Dussurget 2008).
Many cases of listeriosis have been associated with the consumption of milk and dairy products in 1980, thus causing a concern for the dairy industry due to increased overall mortality rate by 30%. Following the outbreak alert by *L. monocytogenes* contamination, hygiene measures were strictly enforced, which led to a satisfactory control of the pathogen.
Unhygienic practices cause indirect contamination of milk with *L. monocytogenes* present in the technological flow or feces. *L. monocytogenes* survives during the manufacture and ripening of several types of cheese and is probably to grow if the pH reaches higher values (Gaya et all. 1998).

L. monocytogenes can live as a saprophyte on different natural environments (soil, water, plants, manure, fodder, etc.) or as epiphyte in the body of different animal species. Unlike other non spore forming bacteria it is highly resistant to environmental factors. It resist 12 minutes at 60ºC and 10 minutes at 63ºC. It can be destroyed by the thermal treatments, applied in food technologies. (Bărzoi, 1985).

Lactic acid bacteria (LAB) population able to produce lactose fermentation ensure an acid protective environment by lowering the pH that reaches unfavorable levels for *Listeria* and other pathogens. Organic acids such as lactic acid and acetic acid produced by LAB were found to be considerably more effective as inhibitors of *Listeria* than anorganic acids such as hydrochloric acid (Farber et all., 1989).

Salmonella includes species that are important agents of food poisoning: *Salmonella enteridis, S. dublin, S. Virchow, S. typhymurium* etc. The toxins are intracellular, so its are formed and remain in the cell of the bacterium. After consumption of the product takes place, under the action of HCl in the stomach, bacterial cell are destroyed and the toxin from cells are eliminated. These bacteria can multiply in food but no sensory changes occur. Frequently, contaminated foods are dairy products, chicken, eggs. In gastroenteritis, bacteria multiply in the intestinal lumen and the syndrome occurs after 12 to 24 hours after consumption.

Using LAB in the dairy industry adds a plus of protection and consumer safety by preventing illness, has a beneficial role to the longevity and contributes at extension dairy preservation.

The objectives of the experimental research aimed to determine the degree of inhibition produced by LAB (*L. delbrueckii subsp. bulgaricus*, *Streptococcus thermophilus* and *Lactococcus subsp. lactis*) on *Salmonella* and *Listeria monocytones* pathogens.

MATERIALS AND METHODS

Experiments were performed in SC ICA Research & Development SRL Bucharest - Microbiology Laboratory.

Before starting experiments samples were tested to establish the initial microbial load by lactic acid bacteria. The determination has not confirmed the presence of lactic acid bacteria (*L. delbruecki subsp. bulgaricus*; *S. thermophilus*; *Lactococccus lactis subsp. lactis*) in any samples.

Materials: 36 samples of fresh milk, 42 samples of yogurt, 16 samples of fresh cheese. Samples for analysis were purchased from various local farms.

Equipments: Laboratory instruments, culture media and reagents and reference strains (*Salmonella typhimurium* ATCC 14028, *Listeria monocytogenes* ATCC 13932, Cultures of lactic acid bacteria – *L. delbruecki subsp. bulgaricus*; *S. thermophilus*; *Lactococccus lactis subsp. lactis*)

Methods: SR EN ISO 11290-1/2000/A1/2005, SR EN ISO 6579:2003/AC:2006, ISO 15214:1998, SR EN ISO 6887-1:2002, SR EN ISO 7218:2007, SR CEN ISO/TS 11133-1: 2009, ISO/TS 11133-2:2003, SR CEN ISO/TS 11133-1: 2009

Experiments on the initial microbiological load determination for:

- 36 samples of fresh milk
- 42 samples of yogurt
- 16 samples of fresh cheese

1. Determination of the pH value at the time t_0 (immediately after opening the package).
2. Determination of microbial load on *Salmonella* contamination at the time t_0.
3. Determination of microbial load on *Listeria monocytogenes* contamination at the time t_0.

RESULTS AND DISCUSSIONS

A. Inhibition of pathogen *Salmonella* by lactic acid bacteria

Steps:

o Establishing the nutritional value of the culture medium used in determining *Salmonella* (SVR MKTTn, XLD, BGA)

o Inoculation P1, P2 and P3 with *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus* and *Lactococccus lactis subsp. lactis.* The samples were inoculated with lactic acid bacterial cultures at 10^8 and *Salmonella typhimurium* ATCC 14028 at 10^6.

- Inoculation P1, P2 and P3 with reference strain of *Salmonella typhimurium* ATCC 14028 (a reference strain inoculation 10^2) (Figure 1)

- Incubation for 24 hours at 37 ^{0}C
- *Salmonella* microbial load determination after incubation at 37^{0}C for 24h
- A replay of determining the microbial load after 48h and 72h.

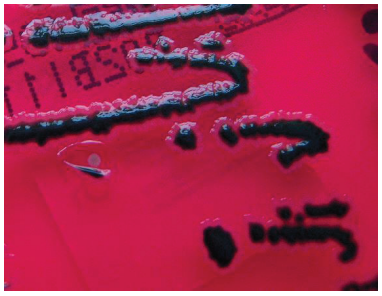


Figure 1. *Salmonella typhimurium* ATCC 14028

Inhibition levels of *Salmonella* by LAB obtained in the experiments carried out on 36 samples of fresh milk (Tabel 1, Figure 2).

Table 1. *L. delbrueckii* subsp. *bulgaricus*

Time of incubation at 37°C (after inoculation *with L. delbrueckii. subsp. bulgaricus*)	Number of samples analyzed	Results
24 h	25	Total inhibition
	11	Partial inhibition
48 h	27	Total inhibition
	9	Partial inhibition
72 h	32	Total inhibition
	4	Partial inhibition

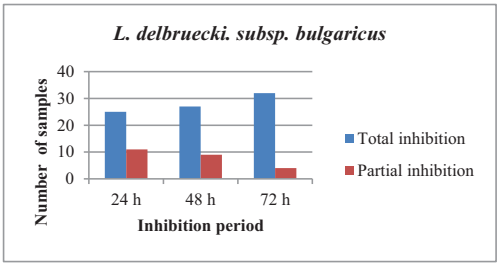


Figure 2. Inhibitory action caused by *L. delbrueckii subsp. Bulgaricus*

Level inhibition on the development of *Salmonella* by LAB obtained in the experiments carried out on 42 samples of yogurt (Tabel 2, Figure 3).

Table 2. *S. termophilus*

Time of incubation at 37°C (after inoculation *with S. thermophilus*)	Number of samples analyzed	Results
24 h	26	Total inhibition
	16	Partial inhibition
48 h	29	Total inhibition
	13	Partial inhibition
72 h	38	Total inhibition
	4	Partial inhibition

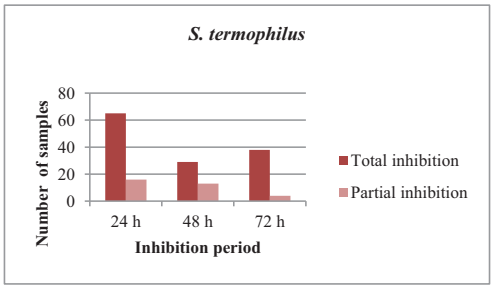


Figure 3. Inhibitory action caused by *S. thermophilus*

Level inhibition on the development of *Salmonella* by LAB obtained in the experiments carried out on 16 samples of cheese (Tabel 3, Figure 4).

Table 3. *Lactococccus lactis subsp. lactis*

Time of incubation at 37°C (after inoculation *with Lactococcus lactis subsp. lactis*)	Number of samples analyzed	Results
24 h	6	Total inhibition
	10	Partial inhibition
48 h	9	Total inhibition
	7	Partial inhibition
72 h	11	Total inhibition
	5	Partial inhibition

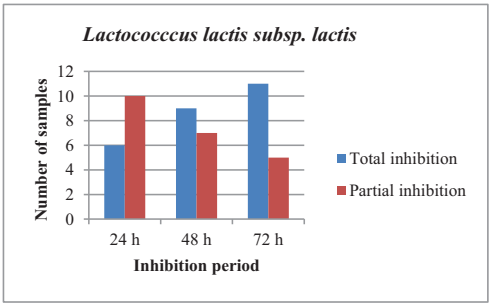


Figure 4. Inhibitory action caused by *Lactococccus lactis subsp. lactis*

B. Inhibition of pathogen *Listeria monocytogenes* by lactic acid bacteria

Steps:

- Establishing the nutritional value of the culture medium used in determining *Listeria monocytogenes* (half Fraser, Fraser, ALOA, Pavement)
- Check *Listeria monocytogenes* ATCC reference strain 13932
- Inoculation P1, P2 and P3 with *L. delbruecki* subsp. *bulgaricus*, *S. thermophilus* and *Lactococccus lactis subsp. lactis* cultures
- Inoculation P1, P2 and P3 with reference strain of *Listeria monocytogenes* ATCC 13932 (a reference strain inoculate 10^2) (Figure 5)
- Incubation for 24 hours at 37 ^{0}C
- Determination of microbial load of *Listeria monocytogenes* after thermostat (24h)
- Repeating the determination of microbes after 48 hours and 72 hours.

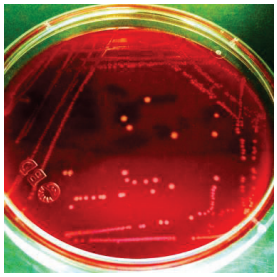


Figure 5. *Listeria monocytogenes* ATCC 13932

Level inhibition on the development of *Listeria monocytogenes* by LAB obtained in the experiments carried out on 36 samples of fresh milk (Tabel 4, Figure 6).

Table 4. *L. delbrueckii subsp. bulgaricus*

Time of incubation at 37°C (after inoculation *with L. delbruecki. subsp. bulgaricus*)	Number of samples analyzed	Results
24 h	22	Total inhibition
	14	Partial inhibition
48 h	28	Total inhibition
	8	Partial inhibition
72 h	31	Total inhibition
	5	Partial inhibition

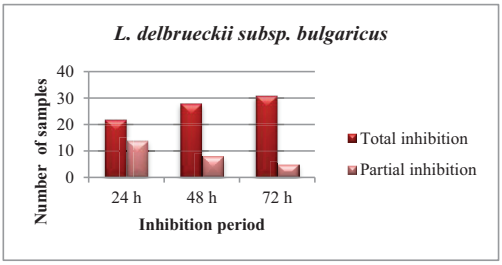


Figure 6. Inhibitory action caused by *L. delbruecki. subsp. bulgaricus*

Level inhibition on the development of *Listeria monocytogenes* by LAB obtained in the experiments carried out on 42 samples of fresh yoghurt (Table 5, Figure 7).

Table 5. *Streptococcus termophilus*

Time of incubation at 37°C (after inoculation *with S. thermophilus culture*)	Number of samples analyzed	Results
24 h	23	Total inhibition
	19	Partial inhibition
48 h	26	Total inhibition
	16	Partial inhibition
72 h	34	Total inhibition
	8	Partial inhibition

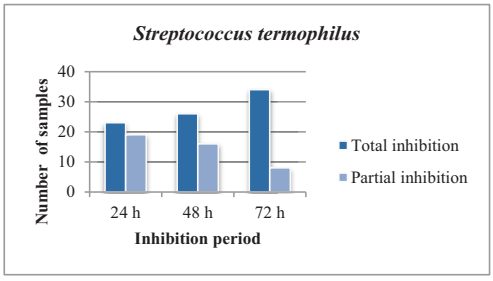


Figure 7. Inhibitory action caused by *S. thermophilus*

Level inhibition on the development of *Listeria monocytogenes* by LAB obtained in the experiments carried out on 16 samples of fresh cheese (Tabel 6, Figure 8).

Tabel 6. *Lactococccus lactis subsp. lactis*

Time of incubation at 37°C (after inoculation *with Lactococccus lactis subsp. Lactis*)	Number of samples analyzed	Results
24 h	9	Total inhibition
	7	Partial inhibition
48 h	11	Total inhibition
	5	Partial inhibition
72 h	14	Total inhibition
	2	Partial inhibition

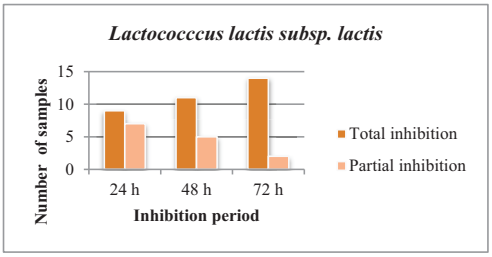


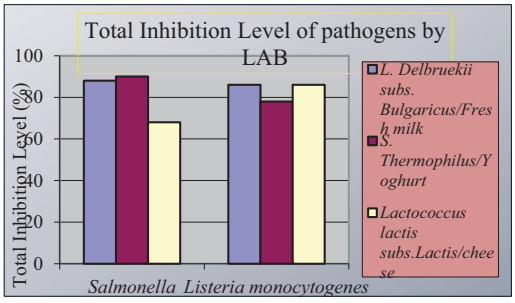
Figure 8. Inhibitory action caused by *Lactococccus lactis subsp. lactis*

CONCLUSIONS

From experiments resulted the following conclusions:

– all types of lactic acid bacteria used in experiments led to the inhibition of the analyzed pathogens - *Listeria monocytogenes* and *Salmonella* - in more than 70% of the samples, after 72h;

– maximum efficiency of inhibition was observed at *L. delbrueckii. subsp. bulgaricus and S. thermophilus* in the case of *Salmonella* and at *L. delbrueckii. subsp. bulgaricus, Lactococccus lactis* in the case of *Listeria monocytogenes*



ACKNOWLEDGEMENTS

This research work was carried out with the support of University of Agronomic Sciences and Veterinary Medicine of Bucharest, in Doctoral School of Engineering and Resource Management Plants and Animals.

REFERENCES

Beresford, T. P., N. A. Fitzsimons, N. L. Brennan and T. M. Cogan, 2001. *Recent advances in cheese microbiology*. *Int. Dairy J.*, 11: 259-274.

Carr, F.J., Chill, D., Maida, N. 2002. *The lactic acid bacteria: a literature survey. Crit. Rev. Microbiol.* 28(4):281–370.

Dussurget O. (2008*): New insights into determinants of Listeria monocytogenes virulence. International Review of Cell and Molecular Biology*, 270: 1–38.

El Soda, M., A. S. Medkor and P. S. Tong, 2000. *Marschall Rhodia International Dairy Science Award Lecture. Adjuncnt Cultures: Recent Developments and Potential Significance to the Cheese industry. J. Dairy Science,* 83: 609-619.

Farber, J. M., G. W., Sanders, S. Dunfield, R. Prescott. 1989. *The effect ofmvarious acidulants on the growth of Listeria monocytogenes.* Lett. Appl. Microbiol.9:181.

Gaya P., Sanchez J., Medina M., Nunez M. (1998): *Incidence of Listeria monocytogenes and other Listeria species in raw milk produced in Spain. Food Microbiology*, 15: 551–555.

Guzun V. - *Industrializarea laptelui*, Chişinău: Editura: Tehnica-Info, 2001, p. 488

ISO 15214:1998 - Metoda orizontală de enumerare a bacteriilor lactice mezofile

ISO/TS 11133-2:2003 - Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media. Part 2: Practical guidelines on performance testing of culture media. Partea 1: Reguli generale pentru pregatirea suspensiei initiale si a dilutiilor decimale.

McCormick, J. K., A. Poon, M. Sailer, Y. Gao, K. L. Roy, L. M. McMullen, J. C. Peterson, S. D., R. T. Marshall and H. Heyman, 1990. *Peptidase profiling of lactobacilli associated with Cheddar cheese and its application to identification and selection of strains of cheeseripening studies. J. of D. Science,* 73: 1454-1464.

S. Ivana, G. Câmpeanu, A.T. Bogdan, I. Ţogoe, T. Enache, S. Bărăitarenu, I. Iudith, A. Popescu – *Microbiologia alimentelor*, pp. 211, Ed. Asclepius, Bucureş ti 2011

Somers, E. B., M. E. Johnson and A. C. L. Wong, 2001. *Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in dairy environment. J. Dairy Science*, 84: 1926-1936.

SR CEN ISO/TS 11133-1: 2009 - Microbiologia alimentelor si furajelor. Ghid de preparare si obtinere a mediilor de cultura. Partea 1: Ghid general de asigurarea calitatii pentru pregatirea mediilor de cultura in laborator.

SR CEN ISO/TS 11133-1: 2009 - Microbiologia alimentelor si furajelor. GHID DE PREPARARE SI OBTINERE A MEDIILOR DE CULTURA. Partea 1: Ghidul general pentru asigurarea calitatii pentru pregatirea mediilor de cultura in laborator.

SR EN ISO 11290-1/2000/A1/2005 - Microbiologia alimentelor si furajelor. Metoda orizontala pentru detectarea si numararea *Listeria monocytogenes*. Partea 1. Metoda de detectie. Amendament 1. Modificarea mediului de izolare si a testului de hemoliza si includerea datelor de precizie.

SR EN ISO 6579:2003/AC:2006 - Microbiologia produselor alimentare şi furajere. Metodă orizontală pentru detectarea bacteriilor din genul *Salmonella.*

SR EN ISO 6887-1:2002 - Microbiologia alimentelor si furajelor. Pregatirea probei pentru analiza, a suspensiei initiale si a dilutiilor decimale pentru examenul microbiologic.

SR EN ISO 7218:2007 - Microbiologia alimentelor si furajelor. Cerinte generale si ghid pentru examenele microbiologice

Stiles, M. E. and W. H. Holzapfel, 1997. *Lactic acid bacteria of foods and their current taxonomy*. *Int. J. Food Microbiology*, 36: 1-29.

Vederas, M. E. Stiles, M. J. VanBelkum. 1998. *Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by Brochotrix campestris ATCC 43754.* Appl. Environ. Microbiol. 64:4757-4766.

MISCELLANEOUS

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MAINTAINING AND IMPROVING THE TRACE ELEMENTS PARAMETERS RATIO OF THE MICROBIAL POPULATION IN A BIOGAS PLANT

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Abstract

The purpose of this research is to identify the influence of trace elements concentrations in fermentation tanks on the biogas yield and plant efficiency. In order to make Anaerobic Digestion more efficient and optimized, measures of the process are often made. In this way the biogas plant managers are trying to produce as much biogas from the given substrate as possible, while at the same time maintaining a reasonable level of process stability. Trace elements (ex. Cobalt, Iron, Selenium, Zinc, etc.) are needed for the growth of the microorganisms involved in biogas biosynthesis. This is mainly related to the fact that most of them are situated in active parts of enzymes, thus having a strong influence on their activity. Maintaining a normal concentration of those elements inside the Fermentation medium is a vital part of the Anaerobic Digestion process and adding supliments of trace elements in the biogas fermenter has often proved beneficial by increasing the biogas production, resulting in a more stable and efficient methane production. The importance of the biogas production and its components are well known because they are strictly related to the operation of the Cogeneration Module which uses it as a fuel. Also very important is to increase the CH4 percentage contained in one cubic meter of biogas in order to have a superior calorific power and a good cogeneration efficiency.

Key words*: Anaerobic Digestion, Biogas Production, Cogeneration.*

INTRODUCTION

The anaerobic metabolism takes place in four big steps with specific enzymes and bacteria: hydrolysis, acidification, acetonogenesis and methanogenesis. The supply with nutrient powder is crucial for the biogas production in Anaerobic Fermentation because they contain elements like cobalt, nickel, iron, zinc, molybdenum which are important to be apart of the biogas plant substrate in order to have an efficient and continuous biogas production[4].
The limitation or undersupplied of these elements influences the conversion considerably, resulting in a disturbed process.
This limitation leads to reduced methan yields and considerable problems due to increasing process instability [1].
The bioavailability of trace elements for metabolic pathways of the anaerobic bacteria it is strictly related to the feeding plan of the biogas plant. Also, many parameters such as variations of the pH-value or the working temperature inside the Fermentation tanks may lead to a diminish of trace elements.
The biogas plant which is operating only with energy crops (in our research, maize silage) as single substrates in the feeding plan, after a short period since its startup, show lack of trace elements and an inefficient biogas production. In this work, we studied the influence of the macro/micro elements inside a biogas plant substrate which uses only maize silage and the results after correcting the values with supliments.

MATERIALS AND METHODS

Many biogas plants in Europe are operating with maize silage as solo substrate. Often these plants are suffering from a dramatic drop of biogas production after a certain time of operation. This phenomenon is usually related due to the lack of trace elements such as nickel and cobalt which are essential factors of the enzymes involved in the anaerobic digestion of the biomass inside the fermentation tanks [1].
We have studied this issue in one Romanian biogas plant ($1MW_{el}$ plant), where after one year of operating with maize silage (32-35 %

dry matter and 95% organic dry matter) the effiency of the biogas production dropped considerably and the operator was forced to increase the feeding amount in order to maintain a 100% biogas production for the needs of the CHP (Combined Heat and Power) with 10 % from 53 tons to 59 tons per day of maize, resulting in an inefficient input-output economic calculation and also increasing the FOS/TAC ratio.

The CH_4 percentage was analysed and it's value was 52 % in accordance with Pronova SM6000 gas analyser.

It was necessary to make some analysis of the substrate inside the fermenters in order to see what is missing from the Fermentation medium and to apply as soon as possible the needed trace elements supliments. We made those analysis with 2 different laboratories from Germany: MT-Energie GmbH and bonalytic GmbH. They determined our trace elements results in accordance with test methods ICP-OES (inductively coupled plasma atomic emission spectrometry): EN ISO 11885:1997; DM (Dry Matter): EN 122880:2000 accredited test methods.

RESULTS AND DISCUSSIONS

The samples were analysed in two separated months, January and March in order to see the concentration of the elements. The results are listed below and in Table 1:

DM content: 9,15 k/kg
pH: 7,50
FOS: 4.336 mg/l
TAC: 10.891 mg/l

Table 1.Analysis results regarding the concentration of trace elements in medium digester

Element	Unit	Content of DM	
		bonalytic (January)	MT-En. (March)
Cobalt (Co)	mg/kg	1.11	0,88
Copper (Cu)	mg/kg	32.1	31
Iron (Fe)	mg/kg	2530	1730
Manganese (Mg)	mg/kg	491	229
Molybdenum (Mo)	mg/kg	1.95	2
Nickel (Ni)	mg/kg	8.84	8.4
Sulphur (S)	mg/kg	4870	4860
Selenium (Se)	mg/kg	<15	0.25
Zinc (Zn)	mg/kg	196	181

According to both of the laboratories knowledge, there was a partial lack of trace elements inside the microbial population. The analysis shown low contents of the elements iron, molybdenum, selenium and especially cobalt. The high acid concentrations in the digester illustrates that the microorganisms do not have an optimal nutrient supply. To improve the nutrient concentrations the laboratories recommended to add a special trace elements powder for biogas plant customized to our needs and based on the analysis.

The issue we were dealing with was normal because we feed only maize silage and , in accordance with the analysis of maize silage [3] and shown in Table 2, the values of the elements containted in it will be everytime in an 'undersupplied' range.

Table 2. Maize silage nutrients content of Fresh Mass

Element	Nutrient content [mg/kg DM]
Cobalt	0.84
Copper	4
Iron	111
Manganese	32
Molybdenum	0.78
Nickel	<1
Zinc	38

These values show lack of the nutrients needed for a normal anaerobic microbial digestion[2], that is why we complied with their recommendations and started to use the supliments which contained concentrations as Table 3:

Table 3. Elements and concentrations for the trace elements powder supplied

Element	**Amount**	**Unit**
Iron	21.7	%
Cobalt	200	ppm
Copper	490	ppm
Manganese	0.91	%
Molybdenum	326	ppm
Sodium	0.98	%
Nickel	1030	ppm
Selenium	23	ppm
Tungsten	60	ppm
Zinc	3010	ppm

pH-value (in aqueous solution): 6-8;
Density: 800-1000 g/l.

We introduced a daily dosage of this product of 2kg/100kW_{el}, meaning 20kg for 1 MW_{el} for 3 months in order to see the results for a long

period and, of course, we monitored the results monthly like Table 4:

Table 4. Evolution of the elements during the period April-June 2014

		Content of DM		
Element	Unit	April	May	June
Cobalt (Co)	mg/kg	0.94	1	1.3
Copper (Cu)	mg/kg	32	36	38
Iron (Fe)	mg/kg	1590	1700	2100
Manganese (Mg)	mg/kg	266	253	284
Molybdenum (Mo)	mg/kg	2.6	3.2	2.4
Nickel (Ni)	mg/kg	9.9	8.1	11.4
Sulphur (S)	mg/kg	5290	5040	4260
Selenium (Se)	mg/kg	0.24	0.31	0.36
Zinc (Zn)	mg/kg	231	171	166

CONCLUSIONS

After 3 months of dosing the trace elements powder, there was still a partial lack of cobalt but the other ones were in a medium range of the recommended values (in accordance with the laboratories recommended values). The limitation of cobalt inside the digester decreases the conversion of acetic and propionic acids into biogas. The recommended value of Cobalt was obtained after five months of feeding the powder [4].

The improving of the trace elements concentration was also seen comparing the old feeding plan versus the new feeding plan: now the biogas production process is more stable and the daily amount feeded inside the digester is 52 tons per day. Also, we improved the methane concentration with 1%, from 52 % to 53 % according to the gas analyses from Table 5 [5]:

Table 5. Variation of the methane content between January and June

Month	Monthly average values of CH4 [%]
January	51.58
February	51.97
March	52.23
April	53.23
May	52.85
June	53.02

In this moment, the biogas plant runs with a daily dosage of powder of 10kg in order to supply continuously the lack of elements contained in the maize silage and to have an efficient biogas production all over the year.

Feeding micronutrients and active ingredients blends may provide an optimum output of the methane production. They balance deficiencies as well as unfavorable ratios and availabilities of micro nutrients in the digesters of biogas plants. Thereby they support an ecological recycling management.

Like all biological processes, biogas plants are relating to Liebig's law, which means that due to the limitation of only one essential nutrient no full scale of yields can be obtained. An individually tailored trace element dosage thus provides the stabilization and optimization of the methane production by supporting the growth of the microorganisms being active in the degradation process as well as enables the production of necessary enzymes and coenzymes. A synchronization of single steps in the biogas production and a long-term increase of the digester performance are obtained by the application of trace elements.

ACKNOWLEDGEMENTS

This research was carried out with the support of Genesisbiopartner.

REFERENCES

[1] Hinken, L., Haun, E., Urban, I., Weichgrebe, D. & Rosenwinkel, K.H. 2008 The valuation of malnutrition in the monodigestion of maize silage by anaerobic batch test. Water Science and Technology

[2] ISF GmbH & University of Hohenheim 2008 Method for Producing Biogas in Controlled Concentrations of Trace Elements. Patent, published by WIPO/PCT

[3] Keymer, U., 2004. Biogasausbeuten verschiedener Substrate, Institut fur Landliche Strukturentwicklung, Betriebswirtschaft und Agrainformatik, Munchen

[4] Pobeheim, H., Munk, B. Lindorfer, H. & Guebitz, G.M. 2010 Impact of nickel and cobalt on biogas production process stability during semi-continuous anaerobic fermentation of a model substrate for maize silage. Water Research 45

[5] Pobeheim, H., Munk, B., Johansson, J., Guebitz, G.M., 2010. Influence of trace elements on methane formation from a synthetic model substrate for maize silage. Biores. Technol

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

A CASE STUDY ON MITIGATION STRATEGIES OF ACRYLAMIDE IN BAKERY PRODUCTS

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Abstract

Acrylamide is a chemical compound first detected in food in 2002 by the Swedish National Food Administration (NFA) and the University of Stockholm. Acrylamide was added on the list of substances of very high concern and was also classified as a probable carcinogenic substance to humans.
Acrylamide is mainly formed in the Maillard reaction when asparagine reacts with the reducing sugars, especially glucose and fructose that are present in food. This usually takes place during high temperature processing of food: frying, baking, roasting and grilling.
This work presents a review of scientific literature published between 2003 and 2014 presenting different methodologies and experimental approaches to reduce acrylamide levels in bakery products. The main pathways for acrylamide mitigation in bakery products take into account three important issues: raw materials, recipes and food processing conditions.

Key words*: acrylamide, asparagine, bakery products, mitigation strategies.*

INTRODUCTION

Acrylamide (acrylic acid amide, 2-propenamide) is a colorless to white and odorless chemical compound having molecular weight 71.08 g/mol (Lewis, 2000; WHO, 2003; Lewis, 2007; HSDB, 2009; Zhang et al., 2009). Its physical state is crystalline solid (WHO, 2003; Lewis, 2003). After publishing the information announcing the presence of acrylamide in some food products processed at high temperatures in 2002 (Sweedish National Food Administration, 2002), and being already known the classifycation of acrylamide as "probably carcinogenic to humans" made by the International Agency for Research on Cancer (IARC) (IARC, 1994), the interest of scientific community all over the world increase significantly (Ahn et al., 2002; Becalski et al., 2003; Hartig et al., 2002; Hofler et al., 2002; Nemoto et al., 2002; Tareke et al., 2002; Biedermann et al., 2002; Tateo et al., 2003; Clarke et al., 2002).
During the last years researches were conducted in the field of finding new pathways for the reducing of acrylamide content and developing mitigation strategies (Zhang et al., 2009; Amrein et al., 2007; Sadd et al., 2008; Claus et al., 2008).

MATERIALS AND METHODS

Data from specialized literature regarding acrylamide formation and available mitigation strategies for reducing acrylamide levels was used in this work.

RESULTS AND DISCUSSIONS

Acrylamide formation

Asparagine is the only amino acid capable of directly generating acrylamide; consequently it is considered the main source of acrylamide in food. The studies related to the detailed mechanism of this transformation have indicated that sugars and other carbonyl compounds play a specific role in the decarboxylation process of asparagine - a necessary step in the generation of acrylamide.
Mass spectral studies showed that the three C atoms and the N atom of acrylamide were all

derived from asparagine (Zyzak, D.V, 2002). In several studies (Stadler et al., 2002, Zyzak, 2002, Mottram et al., 2002, Becalski et al., 2003) it was also found that reducing sugars containing an aldehyde group such as glucose react with asparagine above 100 °C to form an N-glycoside, which is then cleaved at the C-N bond to an intermediate that can be transformed to acrylamide (Figure 1)

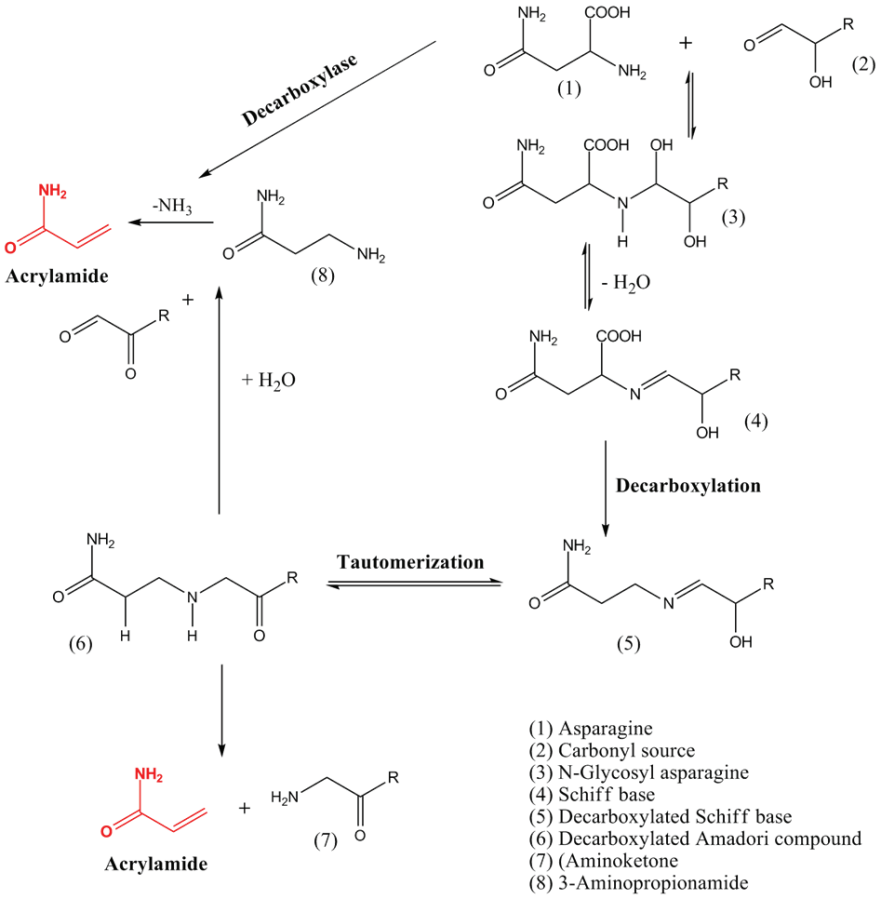

Figure 1. Acrylamide formation via asparagine route

It is well known that recent studies had clarified the formation mechanism of acrylamide via asparagine and carbohydrate route, but is also necessary to refer strictly to a certain food matrix to investigate corresponding factors influencing acrylamide generation.

In bakery products, the most important source of high amounts of acrylamide are bread, biscuits and cookies (Brathen et al, 2005; Konings et al., 2003; Amrein et al., 2004; Fink et al., 2006; Gokmen et al., 2007; Gokmen et al., 2008).

In Table 1 are presented some of acrylamide formation conditions in cereals and bakery products, including acrylamide analysis determination method and acrylamide content.

Table 1. Acrylamide formation conditions

Product	Frying/baking conditions	Pretreatment	Acrylamide determination method	Acrylamide content	Reference
Cereals	Baking at 190 °C for 9 min.	--	-	Inorganic salts declined acrylamide content	Kukurova et al., 2009
Flours	Toasting at 180 °C for 22 min.	---	LC-MS/MS	260 – 300 µg/kg	Capuano et al., 2009
Wheat	Drying at 105 °C for 24 hours	---	GC-MS	-	Weber et al., 2008
Bread	Baking at 180-280 °C for 15-45 min. in a fan oven	One half-baked immediately and other freeze-dried prior to baking	HPLC	-	Brathen et al., 2005

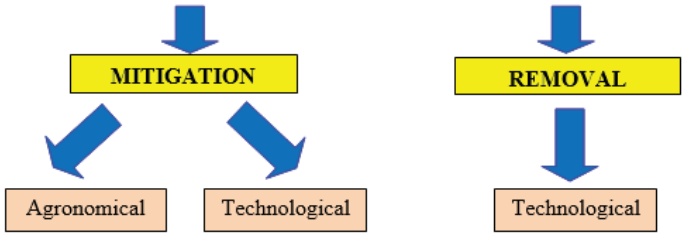

Figure 2. Strategies to reduce acrylamide levels

Mitigation

The possibilities of reducing acrylamide levels in food have to be regarded by two conceptually different approaches, as presented in Figure 2 (Anese et al., 2009): Mitigation strategies – used to keep as low as possible acrylamide formation

Removal strategies – used to move away the already formed acrylamide Based on Trade Guidelines on Reducing Acrylamide in Food, published by Centre for Food Safety – Food and Environment Hygiene Department in 2011 and revised in 2013, there are three main strategies for reducing acrylamide formation to be covered, which are applied to raw materials, recipes and food processing conditions.

The concentration of the precursors in the raw materials is the essential factor in acrylamide formation. Free asparagine and reducing sugars, the main precursors of acrylamide formation, are essential compounds in several biochemical pathways (Matthaus and Haase, 2014). In cereals, the reducing sugars concentration is much higher than asparagine (Muttucumaru et al., 2006; Granvogl et al., 2007; Curtis et al., 2009) meaning that asparagine content is the limiting factor to form acrylamide. Taking this into account, in order to reduce acrylamide level in bakery products, you have to consider replacing proportion of high asparagine level cereals with those with low asparagine. Asparagine level in commonly cereals used in bakery is presented in Table 2.

Table 2. Asparagine level in cereals

Cereal	Asparagine level, mg/kg
Wheat	75 – 2200
Oat	50 – 1400
Maize	70 – 3000
Rye	319 – 880
Rice	15 – 25

Another promising strategy for acrylamide mitigation is asparaginase pre-treatment. The main role of asparaginase is to convert asparagine in aspartic acid with maintaining intact the sensorial attributes of the final product (Pedreschi et al., 2014). Asparaginase can achieve a 60 to 90% reduction level (Kukurova et al., 2009).

The asparagine level can be decreased by using fermentation. Longer fermentation time may reduce acrylamide formation as shown by Haase et al., 2012. Yeast fermentation may assimilate up to 80% of asparagine in dough, while sourdough fermentation can only assimilate only up to 17% of asparagine (Pedreschi et al., 2014).

The type of flour to be used is also important because high extraction flours contain less asparagine than whole meal flour. However, reducing whole meal content will decrease the nutritional benefits of the final product.

Reducing the use of ammonium bicarbonate as raising agent also reduce acrylamide level. Other alternatives are: sodium bicarbonate + acidulants, disodium diphosphate + sodium bicarbonate + organic acids, potassium bicarbonate + potassium bitartrate or sodium bicarbonate + sodium acid pyrophosphate (Biederman and Grob, 2003; Weisshaar, 2004; Amrein et al., 2004, 2005; Sadd et al., 2008).

Fermentation time and addition of cysteine decrease acrylamide level in bread. Claus et al., 2008, conducted an experiment in which breads were brushed with an aqueous solution of cysteine prior to and after baking. Obtained results were in agreement with those obtained by Clayes et al., 2005, who also reported a significantly decrease in acrylamide level when cysteine was used.

pH modification is also a way to reduce acrylamide formation in food (Zhang et al., 2009). Several studies were conducted in order to explain how a lower pH can mitigate acrylamide level. It was found that citric acid can be used to modify pH (Low et al., 2007).

Addition of NaCl as well as $CaCl_2$ seems to have also mitigation effect in acrylamide generation. Ou et al., 2008, tested the effect of $CaCl_2$ on mitigating acrylamide and found that Ca^{2+} prevent acrylamide formation completely.

In Table 3 are presented recent studies regarding acrylamide mitigation in several food matrixes (bread, cereals, and bakery products). Information in Table 3 is adapted from Zhang et al., 2009, and completed with other recent studies.

Table 3. Studies on mitigation of acrylamide in different food matrix

Food matrix	Mitigation recipe and acrylamide reduction percentage	Reference
Bread	Long time fermentation - 87% and 77% reduction in whole grain and rye bran bread	Friedriksson et al., 2004
Baked, fried and roasted products	Addition of asparaginase - Significant reduction of acrylamide	Hendriksen et al., 2005
Wheat	Agronomic and genetic approaches - Reducing acrylamide precursors	Muttucumaru et al., 2008
Bakery products	Removing ammonium-based raising agents, long yeast fermentation, fortification of flour with $CaCO_3$ and lowering the dough pH - Summarizing the effectiveness of mitigation methods	Sadd et al., 2008
Wafers	When increasing extraction rate from type 550 to 1050, acrylamide level is almost doubled	Haase et al., 2003
Wheat crackers	Replacing invert sugar syrup by sucrose - 60% reduction	Vaas et al., 2004
Gingerbread	Replacing NH_4HCO_3 with $NaHCO_3$ - Acrylamide is significantly reduced when using $NaHCO_3$	Amrein et al., 2004
Wheat bread	Different baking temperatures - Linear increasing of acrylamide with time and temperature	Surdyk et al., 2004
Wheat crackers	Changing linear temperature profile (220 °C) to a gradient temperature profile (230 – 190 °C) – up to 60% reduction	Vaas et al., 2004
Breakfast cereals	Direct expansion extrusion cooking process gives raise to acrylamide content compared to pellet-to-flaking extrusion cooking	Rufian-Henares et al., 2006
Crisp bread	Lactic acid fermentation – 70% reduction	Baardseth et al., 2004
Wheat bread	Addition of glycine (high doses) – more than 80% reduction	Brathen at al., 2005
Cereals	Addition of legume proteins reduces acrylamide level	Vattem et al., 2005

CONCLUSIONS

The aim of this review is to summarize the studies regarding mitigation of acrylamide in bread and bakery products published in the last 12 years. Three main strategies for acrylamide mitigation are covered: raw materials, recipes and processing conditions.

The main precursors in acrylamide formation are asparagine and reducing sugars. Thus, strategies regarding possibility of reducing the content of these acrylamide precursors are taking into account.

By reducing time and temperature during baking the acrylamide content can be significantly reduced.

Among other mitigation strategies, asparaginase was extremely effective at different formulation and temperatures.

Fermentation time and addition of cysteine significantly lowered acrylamide in bakery products.

Replacing ammonium bicarbonate as raising agent, modifying pH, adding NaCl or $CaCl_2$, or adding legume proteins are other mitigation strategies for reducing acrylamide in bakery products.

A lot of works has been done in the last 12 years to mitigate acrylamide formation in bakery products. Nevertheless, this problem is not solved, yet.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

REFERENCES

Ahn J.S., Castle L., Clarke D.B., Lloyd A.S., Philo M.R., Speck D.R., 2002.Verification of the findings of acrylamide in heated foods. Food Addit. Contam., 19:1116–1124.

Amrein T.M., Andres L., Escher F., Amado R., 2007. Occurence of acrylamide in selected foods and mitigation options, Food Addit. Contam., Supplement I, 24(S1): 13-25.

Amrein T. M., Andres L., Schonbachler B., Conde-Petit B., Escher F., Amado R., 2005.Acrylamide in almond products, European Food Research and Technology, 21:14-18.

Amrein T. M., Andres L., Schonbachler B., Escher F., Amado R., 2004.Acrylamide in gingerbread: Critical factors for formation and possible ways for reduction, Journal of Agricultural and Food Chemistry, 52: 4282-4288.

Anese M., Suman M., Nicoli M. C., 2009.Technological strategies to reduce acrylamide levels in heated foods, Food Eng Rev, 1:169-179.

Baardseth P., Blom H., Enersen G., Skrede G., Slinde E., Sundt T., Thomassen T., 2004. Reduction of acrylamide formation in cereal-based food processing, Patent WO2004028276.

Becalski A, Lau B.P.Y., Lewis D., Seaman S., 2003. Acrylamide in foods: Occurrence, sources and modelling. J. Agric. Food Chem., 51:802–808.

Biedermann M., Biedermann-Brem S., Noti A., Grob K., Egli P., Mandli H., 2002. Two GC- MS Methods for the Analysis of Acrylamide in Foods, Mitt. Lebensm. Hyg., 93:638–652.

Brathen E., Knutsen S. H., 2005. Effect of temperature and time on the formation of acrylamide in starch-based and cereal model systems, flat breads and bread. Food Chem., 92, 693–700.

Clarke D.B., Kelly J., Wilson L.A., 2002. Assessment of performance of laboratories in determining acrylamide in crisp bread. J. AOAC Int., 85:1370–1373.

Claus A., Mongili M., Weisz G., Schieber A., Carle R., 2008.Impact of formulation and technological factors on the acrylamide content of wheat bread and bread rolls, Journal of Cereal Science 47, 546-554.

Curtis T.Y., Muttucumaru N., Shewry P.R., Parry M.A. et al., 2009. Effects of genotype and environment on free amino acid levels in wheat grain: Implications for acrylamide formation during processing. J. Agric. Food Chem., 57, 1013–1021.

Fink M., Andersson R., Rosen J., Åman P., 2006. Effect of Added Asparagine and Glycine on Acrylamide Content in Yeast-Leavened Bread, Cereal Chem., 83, 218-222.

Friedriksson H., Tallving J., Rosen J., Aman P., 2004.Fermentation Reduces Free Asparagine in Dough and Acrylamide Content in Bread, Cereal Chem., 81, 650-653

Gokmen V., Acar O.C., Arribas-Lorenzo G., Morales F.J., 2008. Investigating the correlation between acrylamide content and browning ratio of model cookies, J. Food Eng., 87, 380-385.

Gokmen V., Acar O.C., Koksel H., Acar J., 2007.Effects of dough formula and baking conditions on acrylamide and hydroxymethylfurfural formation in cookies, Food Chem., 104, 1136-1142.

Granvogl M., Wieser H., Koehler P., Von Tucher S., Schieberle P., 2007. Influence of sulfur fertilization on the amounts of free amino acids in wheat. Correlation with baking properties as well as with 3-aminopropionamide and acrylamide generation during baking. J. Agric. Food Chem., 55, 4271–4277.

Haase N.U., Matthaus B., Vosmann K., 2003.Acrylamid in BackwareneinSachstandbericht, Getreide, Mehl und Brot 57, 180-184.

Haase N.U., Grothe K.H., Matthaus B., Vosmann K., Lindhauer M.G., 2012. Acrylamide formation and antioxidant level in biscuits related to recipe and baking. Food AdditContam A., 29:1230-1238.

Hartig L. Hummert C., Buhlert J., von Czapiewski K., 2002. Detection of acrylamide in starch enriched foods with HPLC-MS/MS. 17th Symposium on LC-MS, Montreux, Switzerland.

Hendriksen H.V., Kornbrust B., Ernst S., Stringer M., Heldt-Hansen H.P., Østegaard P., 2005.J. Biotechnol., 118 (S1), S135

Hofler F., Maurer R., Cavalli S., 2002. SchnelleAnalyse von Acrylamid in Lebensmittelnmit ASE und LC/MS. GIT Labor-Fachzeitschrift, 48:986–970.

Konings E.J.M., Baars A.J., van Klaveren J.D., Spanjer M.C., Rensen P.M., Hiemstra M., van Kooij J.A., Peters P.W., 2003.Acrylamide exposure from foods of the Dutch population and an assessment of the consequent risks, J. Food Chem. Toxicol., 41, 1569-1579.

Kukurova K., Morales F., Bednarikova A., Ciesarova Z., 2009.Effect of L-asparaginase on acrylamide mitigation in a fried-dough pastry model, MolNutr Food Res., 53: 1532-1539.

Lewis R.J., 2000. Acrylamide. In: Sax's dangerous properties of industrial materials. 10th Ed. New York, NY: John Wiley & Sons, Inc., 66-67.

Lewis R.J., 2007. Acrylamide. In: Hawley's condensed chemical dictionary. 15th ed. Hoboken, NJ: John Wiley & Sons, Inc., 19-20.

Low M.Y., Koutsidis G., Parker J.K., Elmore J.S., Dodson A.T., Mottram D.S., 2006.Effect of citric acid and glycine addition on acrylamide and flavor in a potato model system, J. Agric. Food Chem., 54: 5976-5983.

Matthaus B., Haase N.U., 2014.Acrylamide – Still a matter of concern for fried potato food?, Eur. J. Lipid. Sci. Technol., 116, 675-687.

Muttucumaru N., Elmore J.S., Curtis T., Mottram D.S., Parry M.A.J., Halford N.G., 2008. Reducing Acrylamide Precursors in Raw Materials Derived from Wheat and Potato, J. Agric. Food Chem., 56, 6167-6172.

Muttucumaru N., Halford N.G., Elmore J.S., Dodson A. T. et al., 2006.The formation of high levels of acrylamide during the processing of flour derived from sulfate-deprived wheat. J. Agric. Food Chem.,54, 8951–8955.

Nemoto S., Takatsuki S., Sasaki K., Maitani T., 2002, Determination of acrylamide in food by GC/MS using 13C-labelled acrylamide as internal standard. J. Food Hyg. Soc. Japan, 43:371–376.

Ou S.Y., Lin Q.L., Zhang Y.P., Huang C.H., Sun X., Fu L., 2008. Reduction of acrylamide formation by selected agents in fried potato crisps on industrial scale, Innov. Food Sci. Emerg. Technol., 9:116-121.

Pedreschi F., Mariotti M.S., Granby K., 2014.Current issues in dietary acrylamide: formation, mitigation and risk assessment, J Sci Food Agric., 94:9-20.

Rufian-Henares J.A., Delgado-Andrade C., Morales F.J., 2006.Relationship between acrylamide and thermal-processing indexes in commercial breakfast cereals: a survey of Spanish breakfast cereals. Molecular Nutrition and Food Research, 50: 756-762.

Sadd P.A., Hamlet C.G., Liang L., 2008.Effectiveness of methods for reducing acrylamide in bakery products, J. Agric. Food Chem., 56, 6154-6161.

Surdyk N., Rosen J., Andersson R., Aman P., 2004.Effects of asparagine, fructose and baking conditions on acrylamide content in yeast-leavened wheat bread, Journal of Agricultural and Food Chemistry, 52: 2047-2051.

Tareke E., Rydberg P., Karlsson S., Eriksson M., Tornqvist M., 2002.Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J. Agric. Food Chem., 50:4998–5006.

Tateo F.,Bononi M., 2003. A GC/MS method for the routine determination of acrylamide in food. Italian J. Food Sci., 15:149–151.

Vaas M., Amrein T.M., Schonbachler B., Escher F., Amado R., 2004.Ways to reduce acrylamide formation in cracker products, Czech Journal of Food Science, 22:19-21.

Vattem D.A., Shetty K., 2005. Composition of legume proteins and methods of use thereof for reducing acrylamide in cooked foods. Patent US20050048172.

Zhang Yu, Ren Y., Zhang Yi., 2009.New research developments on acrylamide: analytical chemistry, formation mechanism and mitigation recipes, Chem. Rev., 109, 4375-4397.

*** HSDB., 2009. Acrylamide. Hazardous Substances Data Bank. National Library of Medicine. http://toxnet.nlm.nih.gov

*** International Agency for Research on Cancer (IARC)., 1994.Some industrial chemicals, IARC Monographs on the evaluation for carcinogenic risk of chemicals to humans. vol. 60.

*** Swedish National Food Administration., 2002. Information about acrylamide in food.
www.slv.se

*** WHO, 2003. Acrylamide in drinking-water. World Health Organization. WHO/SDE/ WSH/03.04/71. www.who.int/water_sanitation_health/dwq/chemicals/acrylamide.pdf

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PROXIMATE ANALYSIS OF SEEDS FROM SOME FIELD BORDER FLOWERING STRIPS

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Abstract

Field border flowering strips are commonly grown throughout the world mainly to enhance biodiversity. However besides their basic function they can also yield numerous compounds which could be interesting for wide range of industries such as food, pharmaceutical, etc. With the aim of valorization, proximate compositional analysis of seeds from some commonly grown flowering strips: Galium verum, Hypericum perforatum, Leontodon hispidus, Lotus corniculatus, Lythrum salicaria, Origanum vulgare and Trifolium pratense was realized. The protein content of residue left after the lipid extraction was also determined for exploring possibilities of its utilization as a protein source. Results suggest that seeds from some of these plants can be a potential source to render food compounds.

Key words: *wildflower strips, proximate composition, valorization.*

INTRODUCTION

Field border flowering strips are grown throughout the world and serve for a wide range of functions in agronomy, environment management, recreation/rural development, nature conservation, and adorn aesthetic value of landscape (Marshall & Moonen, 2002); most important being enhancement of biodiversity (Haaland et al., 2011). In recent years these factors have encouraged the emergence of a new industry engaged in commercial production of seeds from field border flowering strip. This development of agricultural practices to produce seeds in such a pure form will certainly promote other possibilities to utilize these seeds such as their ability to render food or pharmaceutical compounds.

Seeds aid in plant reproduction and for this purpose they carry reserves such as proteins, lipids and carbohydrates which are also an

important part of human diet (Bewley & Black, 1994). Seeds of some plants are directly used in human diets as cereals and legumes, while others such as oilseeds are source of lipids. These lipids are used as food and they also serve for a wide range of industrial purposes.

Earlier reports present reasonable data that seeds from some plants which are commonly grown in the strips can yield a broad spectrum of compounds which could be interesting for food and pharmaceutical industries (Azcan et al., 2004; Kocak et al., 2011). However analysis of seeds for compounds, from a large majority of plants in these strips still needs to be realized. Owing to the fact that seeds from some plants in the strips have already been explored to contain useful compound and the emergence of new sector which has the ability to commercially produce seeds, it could be really interesting to analyze the chemical composition of seeds from other plants in the field border flowering strips.

In the present research the seeds of some commonly grown plants in field flowering strips i.e. *Galium verum* L., *Hypericum perforatum* L., *Leontodon hispidus* L., *Lotus corniculatus* L., *Lythrum salicaria* L., *Origanum vulgare* L. and *Trifolium pratense* L. were investigated for moisture, ash, protein and lipid content to explore possibilities of utilizing seeds from field border flowering plants to render food compounds.

MATERIALS AND METHODS

Seeds of *G. verum*, *H. perforatum*, *L. hispidus*, *L. corniculatus*, *L. salicaria*, *O. vulgare* and *T. pratense* used for the investigation were procured from ECOSEM, Belgium.

Weight of the seeds, moisture content, protein content, ash content and lipid content was analyzed in the present investigation. Moreover the residue left after lipid extraction was also analyzed for protein content to explore possibilities of its valorization as a protein source.

Weight per 20 seeds was determined using KERN ALT 100-5AM electronic balance (KERN & Sohn GmbH, Germany). Seeds were grinded for 30 s in IKA A-10-B mill (IKA-Werke GmbH & Company, Germany) to obtain a fine powder which was used for further analysis.

Moisture and ash contents were estimated by official methods (AOCS, 2012). Protein content was calculated by multiplying the nitrogen content estimated using the rapid N cube (Elementar Analysensysteme GmbH, Germany) by 6.25.

For lipid extraction a slightly modified previously reported protocol utilizing 2:1 chloroform/methanol (Folch et al., 1957) was adopted. Supernatant was collected three times after repeated cycle of washing 5 g ground seeds with 25 ml of solvent subjected to 10 min of handshaking and then to centrifugation for 10 min at 1800 g in Avanti J-E (Beckman Coulter Inc., Belgium). This supernatant was filtered (5 µm Whatman filter paper, Sigma-Aldrich Company, Belgium) and collected in a 250 ml separation funnel. The washing of the filter paper was done using the same solvent until the solution in separation funnel was 160 ml. Following this 40 ml of 0.58 % NaCl solution was added in the funnel. The mixture was thoroughly mixed and allowed to stand overnight (Figure 1). The lower phase was then collected and 40 ml chloroform was added to the funnel. After standing for 5 hours, again the lower non-aqueous phase was removed. The solvent from this non-aqueous phase was removed by RE-121 Rotavapor (BUCHI Labortechnik GmbH, Netherlands) and nitrogen flushing. The seed residue left after lipids extraction was store at -20 °C and later analyzed for protein content using same method mentioned above.

Figure 1. Lipid Extraction Process

RESULTS AND DISCUSSIONS

The weight per 20 seeds is mentioned in the table 1. Some of the seeds were really small in size specially those of *O. vulgare*, *L. salicaria* and *H. perforatum*, this is also visible from the results. In some cases the standard deviation was high; this indicates that the weight of seeds were quite variable.

Table 1. Weight per 20 seeds. Results are expressed as mean ± standard deviation, n=3

S.NO.	Species	Weight per 20 seed (mg)
1	*G. verum*	8.4 ± 1.2
2	*H. perforatum*	1.7 ± 0.4
3	*L. hispidus*	33.9 ± 3.3
4	*L. corniculatus*	27.9 ± 2.0
5	*L. salicaria*	1.2 ± 0.4
6	*O. vulgare*	0.6 ± 0.1
7	*T. pratense*	37.5 ± 2.8

This seed weight data is in accordance to data previously published in literature i.e. mean seed weight of 0.4 mg for *G. verum*, 0.1 mg for *H. perforatum*, 0.8 mg for *L. hispidus*, 1.2 mg for *L. corniculatus*, 0.1 mg for *L. salicaria*, 0.1 mg for *O. vulgare* and 1.5 mg for *T. pratense* (Kuhn et al., 2004). Moisture content of the seeds is mentioned in Table 2.

Table 2. Moisture content (%) of seeds. Results are expressed as mean ± standard deviation, n=2

S.NO.	Species	Moisture content (%)
1	*G. verum*	9.21 ± 0.03
2	*H. perforatum*	6.51 ± 0.26
3	*L. hispidus*	7.85 ± 0.21
4	*L. corniculatus*	8.04 ± 0.04
5	*L. salicaria*	7.37 ± 0.26
6	*O. vulgare*	7.94 ± 0.72
7	*T. pratense*	8.70 ± 0.60

Table 2 indicates that moisture content of all the seven variety of seeds analyzed was roughly between 6-9 %. This moisture content should not be confused with the moisture in fresh seeds. This is the moisture content which is present in seeds after being subjected to different treatments by the supplier followed by packaging and storage in optimum conditions (one year in a cool and dry place in this case). Moisture content of oilseeds is an important parameter; it plays an important role not only in storage but also in industrial processes such as drying, milling and oil extraction (Lubatti & Bunday, 1960). It is really important to maintain optimum moisture content in seeds during storage which will cause minimum damage to the seed components and maintain seed viability. For oilseeds like rape the recommended moisture content during storage is between 7 to 10 % (Gawrysiak-Witulska et al., 2012) which is quite comparable to the moisture content in seed investigated. Ash content of the seeds on dry basis is mentioned in table 3.

Table 3. Ash content (%) of seeds on dry basis. Results are expressed as mean ± standard deviation, n=2

S.NO.	Species	Ash content (%)
1	*G. verum*	6.65 ± 0.05
2	*H. perforatum*	4.99 ± 0.18
3	*L. hispidus*	4.56 ± 0.06
4	*L. corniculatus*	4.67 ± 0.13
5	*L. salicaria*	5.46 ± 0.03
6	*O. vulgare*	3.03 ± 0.15
7	*T. pratense*	3.88 ± 0.08

Ash content of a sample is the measure of minerals present in the sample. It is well known that some minerals have special significance in proper body functioning. In the present investigation ash content of seeds from all the species were between 3-7 %. Further it could be interesting to analysis individual minerals present in the seeds.

Protein content of the seeds on dry basis is mentioned in figure 2, which clearly indicates that seeds from some plants in field border flowering strips offer an interesting source of proteins. The protein content of *T. pratense* and *L. corniculatus* were quite comparable to the proteins content of other high protein commonly consumed legume such as soybean i.e. 35.8 % (Christensen, 2009). Both of these plants belong to Leguminosae (Fabaceae) family which is known to have nitrogen fixing bacteria in their roots. These bacteria uptake atmospheric nitrogen and convert it into more usable form for the plants (Mafongoya et al., 2004), which maybe one of the reason behind the high protein content of the seeds from these plants. Seeds of *T. pratense* were found to have highest amount of proteins amongst all investigated. Previous

research reported maximum protein content of 17.3 % on dry basis in the seeds of *T. pratense* (Kratovalieva et al., 2012) which is much lower than the findings of current research. However analysis on other species in *Trifolium* family have supported the fact that protein content in plants of this family could even exceed 36 % (Kokten et al., 2011).

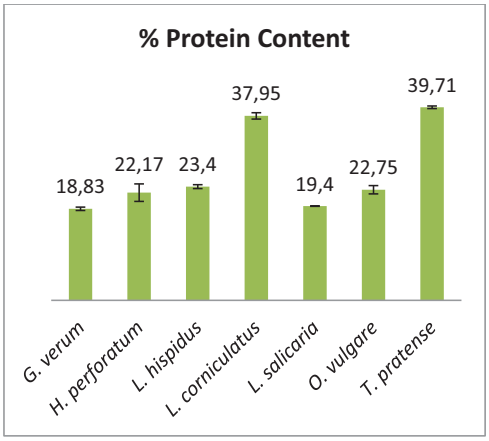

Figure 2. Protein content (%) of seeds on dry basis. Results are expressed as mean ± standard deviation, n=2

The chemical composition of seeds is related to the genetics, environment, climate and minerals in the soil (Mayer & Poljakoff-Mayber, 1982). This could also be one of the reasons behind variation of present results from the earlier published literature. The protein content of *L. corniculatus* seeds was in close proximity to the earlier investigation published (Kocak et al., 2011). However in order to introduce them as novel protein sources it is important to investigate their amino acid profile, protein digestibility and even the presence of antnutritional factors.

Lipid content of the seeds on dry basis is mentioned in figure 3. Some seeds have been extensively investigated and utilized by humans for edible and industrial oils. The seeds from some plants commonly grown in field border flowering strips are really interesting sources of lipids as indicated in figure 3. Particularly *H. perforatum*, *O. vulgare* and *L. salicaria* have considerably high amount of lipids.

Seeds from all three species contain more lipids then proteinaceous seeds such as soybean i.e. 18.4 % (Christensen, 2009) but less then oleaginous seeds such as rapeseed i.e. 41% (Wang, 2010). The lipids from *H. perforatum* were similar to the results documented earlier i.e. 27.2-30.6 % (dry basis) using diethyl ether and methyl-tert-butyl ether as solvent (Fontanel, 2013). For *O. vulgare* the lipid content was higher than the earlier reports (20.09 % on dry basis) where hexane was used for extraction (Azcan et al., 2004).

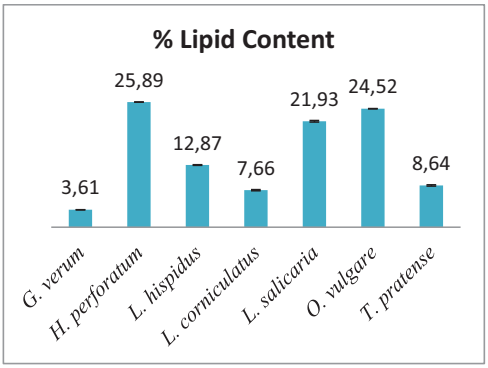

Figure 3. Lipid content (%) of seeds on dry basis. Results are expressed as mean ± standard deviation, n=2

In the above statement, the value was converted from wet basis (as reported in literature) to dry basis considering the experimental results of moisture content in *O. vulgare* seeds of 7.94 %. Further it could be interesting to investigate the lipid content of some other plants in the flowering strips and also the composition (fatty acid composition, triacylglycerol profile and other minor compounds) of these lipids to explore their food or industrial usage. Protein content of seed residue left after lipid extraction on wet basis is mentioned in the Table 4.

Table 4. Protein content (%) of seed residue left after lipid extraction on wet basis. Results are expressed as mean ± standard deviation, n=2

	Species	Protein content (%) in seed residue left after lipid extraction
1	*G. verum*	17.49 ± 0.20
2	*H. perforatum*	19.08 ± 0.32
3	*L. hispidus*	14.63 ± 1.24
4	*L. corniculatus*	32.63 ± 0.19
5	*L. salicaria*	18.69 ± 0.28
6	*O. vulgare*	23.63 ± 1.60
7	*T. pratense*	37.07 ± 0.51

Defatted seeds cakes are by-products of oil extraction industry. They have been utilized in a wide range of application such as food, feed, fuel and raw material of biotech products (Ramachandran et al., 2007). The seed residue left after lipid extraction from *T. pratense* and *L. corniculatus* has protein in considerably high quantities.

These results are in accordance to the protein content in non-defatted seed powder reported earlier in this paper. Protein content of defatted seed residue from *T. pratense* and *L. corniculatus* are quite comparable to the protein content of mustard and sunflower seeds press-cakes i.e. 38.5 % and 34.1 %. (Ramachandran et al., 2007). It is important to note that during the process of lipid extraction some non-lipid compounds (soluble in chloroform and methanol) are washed away. The residue left after lipid extraction from some of the high lipid containing seeds could be used to prepare protein concentrates, animal feeds, etc.

CONCLUSIONS

Field border flowering strips play important roles in agronomic practices, environmental management and other functions. Seeds from some of these field border flowering strips could be an interesting source of food compounds. Investigation in the present research revealed that seeds from *H. perforatum*, *O. vulgare* and *L. salicaria* contains considerable high amount of lipids, while the seeds of *T. pratense* and *L. corniculatus* are rich in proteins.

Results indicate that seeds from some of these plants could be potential alternate to commercially used oil source such as soybean. However besides these quantitative parameters, it is also important to study the quality of proteins (amino acid profile, digestibility and antnutritional factors) and lipids (fatty acid profile, triacylglycerol profile and minor components) in these seeds. Further, more research is required on compounds from seeds of these plants and some other plants which are grown in wildflower strips to explore their food and industrial potential.

ACKNOWLEDGMENTS

This research was made possible from the financial support of the CARE Agriculture Is Life, Gembloux Agro-Bio Tech, University of Liege, Belgium.

REFERENCES

AOAC, 2012. Official Methods of Analysis of AOAC International 19th ed., AOAC International, Maryland.

Azcan N., Kara M., Demirci B., Baser K., 2004. Fatty Acids of the Seeds of *Origanum onites L.* and *O. vulgare L.* Lipids, 39, 487-489.

Bewley J.D., Black M., 1994. Seeds. In: Seeds: Physiology of Development and Germination. Springer USA, New York, 1-33.

Christensen T., 2009. Nutritional Information, The Danish Food Composition Databank. Available at: http://www.foodcomp.dk/v7/fcdb_details.asp?FoodId=0032 [Accessed February 7, 2015].

Folch, J., Lees M., Stanley G.H.S., 1957. A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues.

Fontanel D., 2013. Exceptionally High Content of Unsaponificable Matter in Plant Species with High Fat Content. In: Unsaponificable Matter in Plant Seed Oils. Springer, Heidelberg, 317-318.

Gawrysiak-Witulska M., Rudzinska M., Wawrzyniak J., Siger A., 2012. The Effect of Temperature and Moisture Content of Stored Rapeseed on the Phytosterol Degradation Rate. Journal of the American Oil Chemists' Society, 89, 1673-1679.

Haaland C., Naisbit R.E., Bersier L.-F., 2011. Sown Wildflower Strips for Insect Conservation: A Review. Insect Conservation and Diversity, 4, 60-80.

Kocak A., Kokten K., Bagci E., Akcura M., Hayta S., Bakoglu A., Kilic O., 2011. Chemical Analysis of Seeds of Some Forage Legumes from Turkey. A Chemotaxonomic Approach. Grasas y Aceites, 62, 383-388.

Kokten K., Kocak A., Kalpan M., Akcura M., Bakoglu A., Bagci E., 2011. Tannin, Protein Content and Fatty Acid Composition of the Seeds of Some *Trifolium L*. Species from Turkey. Asian Journal of Animal and Verterinary Advances, 6, 88-95.

Kratovalieva S., Popsimonova G., Ivanovska S., Jankuloski L., Meglic V., 2012. Macedonian Genebank: Seed Protein Content of Wild Red Clover (*Trifolium pratense L.*) Accessions. Agriculturae Conspectus Scientificus, 77, 199-202.

Kuhn I., Durka W., Koltz S., 2004. BiolFlor – A New Plant-trait Database as a Tool for Plant Invasion Ecology. Diversity and Distribution, 10, 363-365.

Lubatti O.F., Bunday G., 1960. The Water Content of Seeds. I.- The Moisture Relations of Seeds Peas etc. Journal of the Science of Food and Agriculture, 11, 685-690.

Mafongoya P.L, Giller K.E., Odee D., Gathumbi S., Ndufa S.K., Sitompul S.M., 2004. Benefiting from N_2-Fixation and Managing Rhizobia. In: van Noordwijk M., Cadisch G., Ong C. (Eds.). Below-Ground Interactions in Tropical Agroecosystems 1st ed. CABI, Oxfordshire, 227-242.

Marshall E.J.P., Moonen A.C., 2002. Field Margins in Northern Europe: Their Functions and Interaction with Agriculture. Agriculture, Ecosystems and Environment, 89, 5-21.

Mayer A.M., Poljakoff-Mayber a., 1982. Chemical Composition of Seeds. In: The Germination of Seeds 3rd ed. Pergamon Press, Oxford, 10-21.

Ramchandran S., Singh S.K., Larroche C., Soccol C.R., Pandey A., 2007. Oil Cakes and their Potential Biotechnological applications- A Review. Bioresource Technology, 98, 2000-2009.

Wang H., 2010. Review and Future Development of Rapeseed Industry in China. Chinese Journal of Oil Crop Sciences, 2, 300-302.

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

LIFE CYCLE ASSESSMENT AND POTENTIAL OF CAMELINA MEAL FOR DAIRY CATTLE NUTRITION IN ROMANIAN COMMERCIAL FARMS

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Abstract

In Romania, up to 90% of the farms have linked the agricultural and animal husbandry business in order to reduce the inputs and to increase profitability and business sustainability. Camelina sativa is also an rotational oil seed crop, harvested and used after oil extraction as a protein meal in romanian dairy commercial farms
This study shows the nutritional potential of Camelina sativa meal, by-product from Camelina seeds after oil extraction, oil used as renewable source for several types biofuels.
Feeding is the biggest input in dairy cattle farms, due to higher worldwide demand, protein is one of biggest financial inputs in dairy cattle nutrition of commercial farms. Moreover, traditional protein meals are not available for whole seasons and solutions are sought permanently.
In order to reduce the inclusion rate of traditional meals such as soybean meal, rapeseed meal and sunflower meal, any source of protein is welcomed, especially when is a by-product from renewable biofuels production that enters in the food production chain.
We performed a study for assessing the inclusion rate of traditional meals in several commercial dairy farms with more than 300 milking cows, were the consumption of protein meals are important due to high milk production.
Camelina meal has big potential to replace up to 15% of the protein concentrate which will provide a better utilisation of biofuels technology by-products, from financial point of view and more important, there might be an environmental protection due to reduction of Greenhouse gases resulted from biofuels whole chain production, determined with Life Cycle Assessment method.

Key words: *camelina meal, dairy cows, life cycle assessment.*

INTRODUCTION

Nowadays, globally the trend in commercial dairy farms is to produce more milk in order to cover demand for milk and dairy products consumption generated by developing countries (OECD-FAO, 2010). This trend also applies to Romania, due to increased milk production of commercial dairy herds, farmers will need to expand the surfaces cultivated for forages, energy and protein feeds, for correlation of the feed and forages consumption with increased milk production. This will generate an land use change in favour of green forages surfaces but the demand for cereals will remain the same. In this situation any by-product from industries with nutritional value for ruminants can be a good solution for keeping the balance of land use change.

Any increasing of feed and forages consumption needs to be close followed by environmental considerations. Dairy production systems are main sources of greenhouse gas (GHG) emissions from animal husbandry business, most important in this days is methane (CH4).

According to a global Life Cycle Assessment (LCA) of the cattle dairy sector performed in 2007, the sector emitted around 2000 million tonnes CO2-eq, of which more than 70% were attributed to milk production. The contribution of the global milk production, processing and transportation to total anthropogenic emissions was estimated around 3 percent (FAO, 2010) of the total anthropogenic GHG emissions reported (IPCC 2007).

Methane emissions are by far the largest contributor, accounting for more than 50 percent of the total emissions of this sector, followed with a lower contribution by nitrous

oxide and then carbon dioxide (S. Gerosa and J. Skoet 2012).

Globally, emissions per unit of milk product are estimated at 2.4 kg CO2-equivalent per kg of fat and protein-corrected milk (FPCM) at the farm gate (FAO, 2010). However, values can have big differences among different regions of the world, due to different climate and continuing with different feeding and management systems.

Transportation of protein sources from all over the world to farms also contributes to GHG emissions. If the direct emissions from milk productions cannot be avoided, all other factors such as transportation and preparation of feeds can be reduced if more of the feeds are produced locally or at least regionally.

Main reason than stimulates this development is ability to increase profitability by increasing feed efficiency and productivity of the herds, adopting the many technological innovations which often require high capital and therefore bigger herds to be profitable. At the same time, genetic haves several improvements and also feeding has seen a tendency to shift the ratio between roughages and feed concentrates in order to reach higher yields. However, when it comes to industrialization, milk production is lagging production systems for poultry and pigs, partly because dairy cows normally are fed more bulky and fresh fodder and because of the relative labor intensity of dairy production, which makes economies of scale more difficult to achieve when higher yields and better feed efficiency is a moving target.

Today, there are still major differences between developed and developing countries, where most milk in developing countries still are produced in traditional small scale system minimum mechanization and no feeding and management innovations, although large-scale units can be found also in developing countries (S. Gerosa and J. Skoet 2012).

Most developed countries have seen increasing herd size and higher annual milk production per cow by increasing feed efficiency of energy and protein sources.

This reflects different production systems, correlated with the nutrition of the cows and also to different genetic potential of the animals. The feeding strategy has a major impact on the production obtained. The system in Romania is based on in barn feeding with medium energy-protein rich total mixed rations.

A good opportunity for Romanian farmers who are combining agricultural with animal husbandry businesses can be cultivation of *Camelina sativa* meal as oilseed rotational crop and moreover, the use of Camelina meal after oil extraction as a feed for ruminants.

Camelina is primarily an energy crop, with high oil content for industrial use, as a feedstock to produce renewable fuels, mainly for kerosene used in aviation.

The *by-product* obtained from the oil extraction (camelina cake/meal) has the opportunity to be succesfully used as high protein animal source feed.

Camelina is suitable to be used as an oilseed *rotational crop* with wheat and more important, it has a good potential for cultivation on marginal, uncultivated and contaminated land.

Camelina, though cultivated for over 2,000 years in the area for its seeds containing 30-45% oil, it was used as adjuvant food, in the dye industry, in the soap industry, and more recently, for bio fuel. However, it is little known at present in Romania, though soil and climate conditions are favourable for this crop. Camelina yield potential and optimum cultivation technology was already studied (Moraru A. et al., 2013).

The production was influenced by the sowing date and the climatic conditions of the soils. Nutritional value of Camelina meal (Hurtaud and Peyraud, 2007).

Table 1. Camelina meal nutritional value for ruminants

Nutrional properties	Value
DM %	91,3
CP, g/Kg DM	411
NDF, g/Kg DM	269
ADF, g/Kg DM	144
Fat, g/Kg DM	139
PDIN, g/Kg DM*	257
PDIE, g/Kg DM**	125
NEL, Mcal/kg of DM	2,02

*PDIN = protein digested in the small intestine supplied by rumen-undegraded dietary protein and by microbial protein from rumen-degraded organic matter (INRA, 1989).
**PDIE = protein digested in the small intestine supplied by rumen-undegraded dietary protein and by microbial protein from rumen-fermented organic matter (INRA, 1989).

The inclusion rate of the Camelina meal as a protein source in ratio can be up to 5% of the total ratio, on dry matter basis (Chedea et all., 2014). However, more trials with several cultivars are needed in order to evaluate the increasing of the inclusion rate at a maximum level, according with nutritional safety requirements.
One trial performed in Finland, using red clover silage-based diets and supplemented with Camelina meal at 20% of the concentrate, had no negative effect on silage feed intake, milk yield, or milk composition, but did had a small impact on changing the milk fatty acid composition of the milk (Halmemies-Beauchet-Filleau et all, 2011).

MATERIALS AND METHODS

In this study, we evaluated the usage and the types of protein sources used in one of the biggest commercial dairy farms from Romania and nutritionally potential of Camelina meal to be included in cows daily ratio.
The period of data gathering and farm visit is described in table below.

Table 2. Protein sources and daily feeding quantities

Date	Farm	County	Herd size[1]	Protein sources (meal/cake)	EDF[2]
Dec. 2014	Farm 1	IL	>500	Soybean, Rapeseed, Sunflower, Corn DDGS	1,0
Dec. 2014	Farm 2	GR	<500	Soybean, rapeseed	0,7
Dec. 2014	Farm 3	GR	<500	Soybean, rapeseed	0,6
Dec. 2014	Farm 4	DJ	<500	Rapeseed, sunflower	0,8
Dec. 2014	Farm 5	VN	<500	Rapeseed, sunflower	0,7
Dec. 2014	Farm 6	PH	<500	Rapeseed, sunflower	0,8
Dec. 2014	Farm 7	AB	<2000	Rapeseed, sunflower	1,0
Dec. 2014	Farm 6	IL	<500	Rapeseed, sunflower	0,7
Dec. 2014	Farm 9	PH	<1000	Rapeseed, sunflower	0,9
Feb. 2014	Farm 10	AR	>1000	Soia, DDGS	1,0
Feb. 2014	Farm 11	AR	<300	Rapeseed, sunflower	1,0
Feb. 2014	Farm 12	TM	<1000	Soybean	0,50
Feb. 2014	Farm 13	SV	<100	Soybean	1,0
Feb. 2014	Farm 14	BT	<100	Sunflower	0,5
Feb. 2014	Farm 15	IS	<500	Soybean	1,50
Feb. 2014	Farm 16	IS	<500	Soybean	1,0
Feb. 2014	Farm 17	VS	<500	Soybean	0,50

[1] Herd size is reffered to milking cows only.
[2] EDF=Estimated daily consumption expressed in Kg/cow/day, according with milk yield and currently protein sources.

RESULTS AND DISCUSSIONS

Camelina meal has an good opportunity to be used as a protein source for ruminants in Romania due to a particular dairy farms market, where commercial compound feed is not used by big dairy farms, in order to obtain a better price of making the energy-protein feed in directly in farm.
Nutritional profile is favourable for using it at an inclusion rate of at least 10% of the total dry matter intake of the cow.
Inclusion rate in daily ratio of dairy cows can safely replace up to 20% of protein sources in cow's ratio, as an alternative to seasonal prices of the protein sources.

CONCLUSIONS

The utilisation of this by-product as a protein source in dairy farms can only improve Life Cycle Assessment for *Camelina sativa*, by increasing the value of this crop, starting from agricultural phase, oil extraction, and protein meal utilisation in animal feed, all with less impact for the environment.
All farms will increase milk yield and generate bigger inputs for energy and protein sources. This demand will have an negative impact over the GHG emissions and we must limit this effect by using local and regional by-products in total mixed rations. By utilisation of the local and regional by-products after oil extraction, the economically and environmental impact of cultivation of Camelina will be maximized. All advantages from environmental point of view will be evaluated by applying Life Cycle Assessment method.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no.
POSDRU/159/1.5/S/132765.

REFERENCES

A. Halmemies-Beauchet-Filleau, T. Kokkonen, A.-M. Lampi, V. Toivonen, K.J. Shingfield, A. Vanhatalo, Effect of plant oils and camelina expeller on milk fatty acid composition in lactating cows fed diets based on red clover silage, Journal of Dairy Science, Volume 94, Issue 9, September 2011, Pages 4413–4430

Bulletin of the International Dairy Federation 445/2010. A common carbon footprint approach for dairy. The IDF guide to standard lifecycle assessment methodology for dairy sector.

Chedea V., Pelmus R., Toma S., Taranu I., Grosu H., & Dragomir C. "Evaluation of Camelina Meal as a Dietary Source of Polyphenol for Dairy Cows." Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies [Online], 71.2 (2014): 279-280. Web. 7 May. 2015

FAO, 2010. Greenhouse gas emissions from the dairy sector: A life cycle assessment. Rome 2009.

Hurtaud C. and J. L. Peyraud, Effects of Feeding Camelina (Seeds or Meal) on Milk Fatty Acid Composition and Butter Spreadability, Journal of Dairy Science, Volume 90, Issue 11, November 2007, Pages 5134–5145.

IPCC, 2007. Climate change 2007: IPCC Fourth Assessment Report. Cambridge University Press, UK.

Moraru A., Jurcoane S., Dimitriu D. 2013, Camelina cultivation for biofuels production. Scientific Bulletin. Series F. Biotechnologies, Vol. XVII, ISSN 2285-1364, pages 24-28.

OECD-FAO, 2010. OECD-FAO Agricultural Outlook: 2010-2019, http://www.agri-autlook.org/45599621.pdf

S. Gerosa and J. Skoet, Milk availability, Trends in production and demand and medium-term outlook, Agricultural Development Economics Division Food and Agriculture Organization of the United Nations, ESA Working paper No. 12-01 February 2012, www.fao.org/economic/esa

Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

GROWING SPECIES *PLEUROTUS OSTREATUS* M 2175 ON DIFFERENT SUBSTRATES UNDER HOUSEHOLD

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Abstract

Cultivation of winter oyster mushroom is realised in an increasing number and variety of ways, due to minimum environmental requirements, and its high productivity. The research was made on different substrates, in order to identify an efficient formula for the Pleurotus ostreatus M 2175 species cultivation under household. The sawdust, available in Nehoiu, Buzău was mainly used. Heat-sterilized substrate was placed in heat-resistant plastic bags. The inoculum was obtained on sterilized wheat seeds, at a ratio of 1 - 2%. The average temperature of colonization/fructification varied between 17 – 20^0 C. It was noted that all substrate formulas could support cultivation of the P. ostreatus M 2175 species, including formula partially containing softwood sawdust. The number of primordia was comparable to controls developed exclusively on beech sawdust and chopped straw. The study proved that this mushroom species has a significant adaptability to different types of substrate.

Key words*: mushroom, substrate, sawdust, productivity.*

INTRODUCTION

The benefic effects of mushrooms consumption are due to significant content in protein, dietary fibers, mineral salts and various bioactive compounds (phenolic compounds, flavonoids, vitamins, carotenoids, tocopherols, essential amino acids). They have a low fat content generally up to 2.5%. Among the mushroom species chemical composition differs significantly, wild edible mushrooms having a more complex composition in bioactive compounds. For cultivated species, the biological value of fruit bodies depends on the substrate formula (Tudor, 2007).

In Romania, Oyster mushroom has a consumption similar to *Agaricus bisporus* species because it is considered to have a more pleasant aroma and the biological value is higher (Vamanu et al., 2012). *P. ostreatus* cultivation is facilitated by the isolation of species adapted to atmospheric conditions, thereby growing during the year without requiring a particular technological effort. Normal substrate (straw) is a much cheaper and readily available, fact that eliminates imports. It can be obtained without investment in equipment or special processing conditions in any individual household. Spawn necessary for substrate inoculation is also cheap (Kwon and Kim, 2004).

Since sawdust is the most valuable substrate for the cultivation of the species, the aim of the paper was the identification of original formulas to valorize woody biomass in Nehoiu area, Buzau county, Romania to support the obtaining of high productivity of Winter Oyster Mushroom (*P. ostreatus* M 2175), under household. Another objective was to obtain a fresh production for personal use.

MATERIALS AND METHODS

Culture preparation. The species *P. ostreatus* M 2175 was obtained from Mycelia BVBA, Belgium.

The mycelium was stored on wheat grains, in glycerol, at -20^0 C. Revitalizing was realised by the cultivation on PDA medium (http://en.wikipedia.org). Other Petri dishes with PDA medium for spawn preparation (sterilized wheat grains) were inoculated with this culture. The wheat grains were sterilized at 121^0 C, for two hours, in a glass jar with air filter. In both cases, the propagation of mycelium was realised for 7-10 days, at 23^0 C,

in the dark in a Labtech laboratory incubator (Moonmoon et al., 2010; Pathmashini et al., 2008).

Table 1. Substrate formulas

No. Crt.	Substrate formula
M1	Straw 1000 mL, 25 mL gypsum.
M2	Cobs 1000 mL, 25 mL gypsum.
S1	Beech 1000 mL, cobs 1000 mL, 50 mL gypsum
S2	Poplar 1000 mL, cobs 1000 mL, 50 mL gypsum
S3	Poplar 1000 mL, cobs 1000 mL, husked rice 500 mL, 50 mL gypsum
S4	Poplar and resinous mixture (1:1), cobs 1000 mL, 50 mL gypsum
S5	Poplar and resinous mixture (1:1), cobs 1000 mL, husked rice 500 mL, 50 mL gypsum
S6	Acacia 1000 ml, cobs 1000 mL, 50 mL gypsum
S7	Spruce 1000 mL, cobs 1000 mL, 50 mL gypsum

Substrate preparation. Vegetal biomass (sawdust) came from individuals who process timber in Nehoiu, Buzau county, Romania. To obtain the substrates there was used sawdust of the following species: beech, aspen, mixed conifer, poplar, acacia, spruce (**Table 1).** As control, straw and chopped stalks (with a shredders OK, bauMax Romania) were used.
Incubation during the colonization of the substrate was at 20 – 24^0 C, in the dark, while the fruiting period was maintained constant at 17^0 C, humidity of 50 - 60%, light 800 lumen. The moisture was maintained by means of a sprayer, up to 2-3 watering / day. Natural ventilation was used. The flushes successive were at an interval of 7-10 days, depending on the substrate. The required environmental conditions were made in a sterile enclosure inside a house in Nehoiu, Buzau County, Romania **(Figure 1)**.
Determining productivity. The productivity of the species during the 3 or 4 flushes was calculated using the following formula: Productivity (%) = (amount of fresh mushrooms / weight of fresh substrate) × 100. Biological efficiency was also, determined by the following formula: BE (%) = (amount of fresh mushrooms / weight of dry substrate) × 100 (de Andrade et al., 2007).

Figure 1. The fructification enclosure under domestic condition

Determination of total protein content of mushrooms. The Lowry method was used, and the results were expressed as mg protein / g fresh mushroom (Vamanu et al., 2010).
Statistical analysis. The results are the mean of at least three separate experiments.

RESULTS AND DISCUSSIONS

The most rapid colonization was observed for the control, exclusively containing straw, while the two substrates from coniferous species showed a higher period for colonization of approximately 40%. For the straw mycelium run was of 1.5 - 2 cm /day, respectively maximum 0.5 cm /day for substrate formulas containing coniferous species.
In the first flash, the productivity of the two controls had significant differences, over 50% (**Table 2**). Chopped stalks led to a reduction in productivity, and in biological efficiency on average of about two thirds. Using beech and poplar sawdust determined an average increase of about 50% of productivity compared to M2 and 25% lower than that achieved in the case of wheat straw (M1 – **Figure 2**). The biological effectiveness was approximately 10% lower than that obtained in the case of M1. The biological effectiveness was proved superior in the case of using poplar sawdust to the beech. Substrate supplementation with rice caused an increase of biological efficiency and productivity on average 10%. Thus, the average

value of the substrate S3 productivity was of 30.00 ± 1.00% and 52.00 ± 2.00% of biological efficiency (**Figure 3** and **Figure 4**).

Figure 2. Fruiting substrate M1

Figure 3. Fruiting substrate S2

Figure 4. Fruiting substrate S3

Figure 5. Fruiting substrate S6

Figure 6. Fruiting substrate S7

It was noticed that the exclusive use of poplar sawdust lead to four flushes, which was interpreted as the maximum utilization of the substrate, even if the average value of productivity was lower than in the presence of husked rice (substrate S3). The last flush had a low average productivity of 5.00 ± 1.00%. In general, it was noted that the presence of a single type of sawdust, without supplements, causes a slower and more efficient use, even if such behavior decreases productivity in favor of biological efficiency. The study is supported by previous research that interprets the behavior of *P. ostreatus* mycelium as one response to the content of lignin present in the substrate. Although the enzymatic mechanism is not clear yet, it seems to be dependent on the metabolic activity of the species used (Ruiz-Rodríguez et al., 2011).

A reduced time of colonization and fructification (first flush) was registered for the use as a substrate of straw and stalks, with an average of 10 days. For use in substrate of deciduous species the first flush was determined after 15 days. For husked rice supplementation an average decrease of 2 to 3

day of theperiod of the first fructification was observed. There was recorded instead, that use of spruce and acacia determined first fruiting after about 20 days, the period being divided equally between colonization and the emergence of primordial stage. If mixed with aspen, spruce sawdust use had not negatively affected the range of colonization and fructification. For the rest of the flushes (max three) the interval between harvest and appearance of the first primordia averaged 7 days. The fourth flush occurred after an interval of 10 to 15 days and was not obtained when using resin substrate formula. This last flush, at the use of poplar sawdust, was directly influenced by temperature because the increase of 2 to 3 degrees had a direct influence through the absence of the fourth fructification. The results proved that sawdust supports a final competitive production cost (**Figure 5** and **Figure 6**). Although wheat straw is the main component in Romania, the mixture of different species of hardwood and softwood causes high productivity. This substrate as compared to the straw is used directly, whereas the straw requires a further stage consisting in grinding and special equipment. Using sawdust competes with another use that got more ground in recent years, with the advent of more sophisticated heating systems, pellet manufacture. They are considered, a renewable green fuel.

Table 2. Effect of substrate formula on the productivity and the average value of the biological efficiency of *P. ostreatus* M 2175 species

Substrate formula	Productivity (%)	Biological efficiency (%)
M1	32.00 ± 3.70 %	50.35 ± 2.80 %
M2	11.50 ± 2.00 %	17.00 ± 1.00 %
S1	24.00 ± 0.80 %	44.70 ± 1.00 %
S2	26.50 ± 1.00 %	46.75 ± 6.00 %
S3	30.00 ± 1.00 %	52.00 ± 2.00 %
S4	23.00 ± 2.00 %	39.00 ± 3.00 %
S5	20.66 ± 1.90 %	42.96 ± 1.60 %
S6	12.50 ± 5.00 %	30.50 ± 8.50%
S7	07.00 ± 1.00%	18.00 ± 1.50%

Another observation was that the maximum protein content was obtained when substrate formulas were supplemented with husked rice. Instead, the resinous substrate causes a reduction in the protein content of mushrooms, about 25.00 ± 0.30% compared to the control (wheat straw) - M1 substrate. The values do not exceed 5.00 ± 0.60 mg /g mushroom.

CONCLUSIONS

In conclusion, the study proved the efficiency of softwood sawdust in cultivation of *P. ostreatus* M 2175 species, especially if supplemented with poplar sawdust. Husked rice increases with minimum 10% substrate utilization, expressed by the value of biological efficiency. Poplar sawdust was found to be more effective than the beech one, which is commonly used for the production of mushrooms. This sawdust is at least as effective in obtaining Winter Oyster mushrooms in household arrangements. The biological value of this sawdust is demonstrated by the fact that the combination with resin can sustain high productivity. These findings result in a higher valorification of woody biomass species of conifers.

ACKNOWLEDGEMENTS

This research was financed through the project PNCDI II CNCSIS – Human Resources, Theme 9/2010 (https://sites.google.com/site/proiectte9/).

REFERENCES

de Andrade M.C.N., Filho J.K., de Almeida T.M.M., Coutinho L.N., Figueiredo M.B., 2007. Productivity, biological efficiency, and number of *Agaricus blazei* mushrooms grown in compost in the presence of *Trichoderma sp*. and *Chaetomium olivacearum* contaminants. Brazilian Journal of Microbiology, 38:243-247.

Kwon H., Kim B.S., 2004. Mushroom Growers' Handbook . MushWorld Publishing House.

Moonmoon M., Uddin Md. N., Ahmed S., Shelly N. J., Khan Md. A., 2010. Cultivation of different strains of king oyster mushroom (*Pleurotus eryngii*) on saw dust and rice straw in Bangladesh. Saudi Journal of Biological Sciences, 17:341–345.

Pathmashini L., Arulnandhy V., Wilson Wijeratnam R.S., 2008. Cultivation of oyster mushroom (*Pleurotus ostreatus*) on sawdust. Ceylon Journal of Science, 37:177-182.

Ruiz-Rodríguez A., Polonia I., Soler-Rivas C., Wichers H.J., 2011. Ligninolytic enzymes activities of Oyster mushrooms cultivated on OMW (olive mill waste) supplemented media, spawn and substrates. International Biodeterioration & Biodegradation, 65:285-293.

Tudor I., 2007. Edible and medicinal mushrooms. Lucman Publishing House, Bucharest.

Vamanu E., Nita S., Vamanu A., 2010. Practical - Plant and animal biotechnology. Ars Docendi Publishing House, Bucharest.

Vamanu E., 2012. Determination of antioxidant and antimicrobial properties of *Agaricus bisporus* from Romanian markets. Ovidius University Annals of Chemistry, 23, 47-52.