

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 30°-31°C for bacteria and 72h at 27°-28°C 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 30°-31°C for 48h, while YMPG plates were incubated at 27°-28°C 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 4°C.

IPS medium contained (% w/v): glucose 2.0, yeast extract 0.2, KH_2PO_4 0.2, citric acid 0.1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 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 specificity 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 (tributylin, 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 30°-31°C (48h) for bacterial growth and at 27°-28°C (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	Tributyrim 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 ₂ O 0.05	K ₂ HPO ₄ 0.01	FeSO ₄ *7H ₂ O 0.001	-

Table 2. Environmental sampling sites and their natural source in Romania

Sample type	Sample location	Coordinates
Water samples		
<i>Salted lake</i>	Lacul Negru, Ocna Sibiului	45°52'29"N 24°4'0"E
<i>Salted lake</i>	Lacul Randunica, Ocna Sibiului	45°52'29"N 24°4'0"E
<i>Natural dam lake</i>	Lacul Rosu, Harghita	46°47'34"N 25°47'35"E
<i>Cold spring water</i>	Piatra Neamt, Neamt	46°55'39"N 26°22'15"E
Soil, mud and sand samples		
<i>Mud from salted lake</i>	Lacul cu namol, Ocna Sibiului	45°52'29"N 24°4'0"E
<i>Soil from an oil field</i>	Videle, Teleorman	44°16'N 25°32'E
<i>Soil from a limestone and marlstone extraction region</i>	Tasca, Neamt	46°52'49"N 25°58'52"E
<i>Soil</i>	Constanta	44°10'24"N 28°38'18"E
<i>Soil</i>	Fetesti, Ialomita	44°23'10"N 27°50'38"E
<i>Soil from cultivated field</i>	Mircesti, Olt	44°40'53"N 24°36'48"E
<i>Soil from cultivated field</i>	Petresti, Dambovita	44°38'59"N 25°20'27"E
<i>Garden soil</i>	Nicolae Balcescu, Tulcea	44°59'45"N 28°35'11"E
<i>Beach sand</i>	Constanta	44°10'24"N 28°38'18"E
Plant materials		
<i>Green moss from a rock</i>	Bicaz-Chei, Neamt	46° 91'08"N 26° 09' 11"E
<i>Spruce cones</i>	Piatra Neamt, Neamt	46°55'39"N 26°22' 15"E
<i>Beechnuts</i>	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).

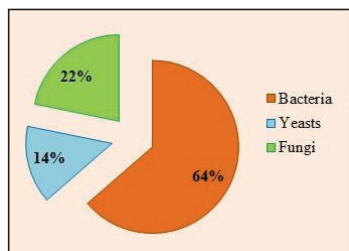


Figure 1. Microbial diversity of the newly isolated strains

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).

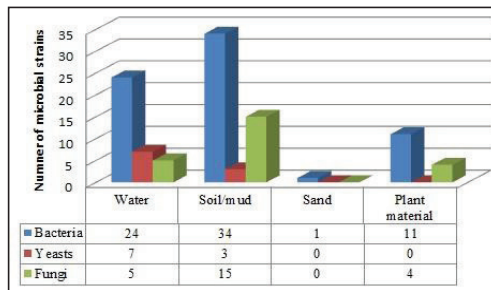


Figure 2. Ecology and microbial biodiversity of the newly isolated strains

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).

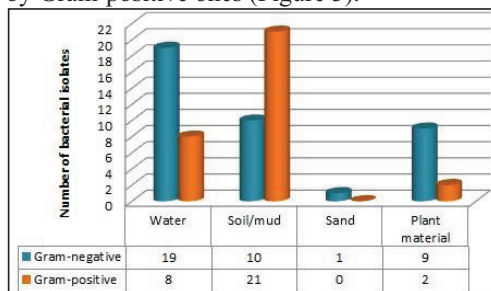


Figure 3. Gram-negative and Gram-positive isolates distribution in environmental collected samples

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).

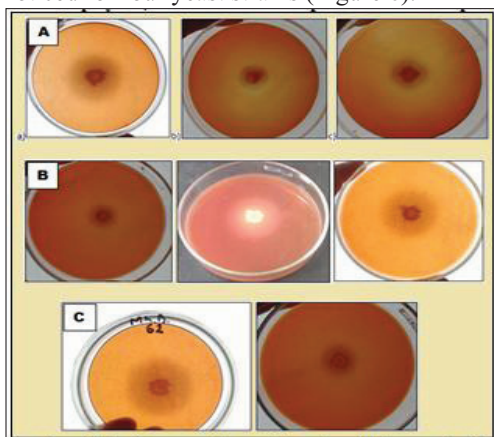


Figure 4. Different bacteria isolates on M4.B medium; opaque halos around colonies and colour variation formed due to the lipolytic activity

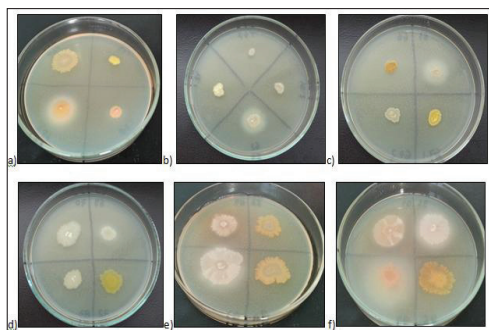


Figure 5. Different bacterial isolates on T80 medium; opaque halos around the colonies formed due to the lipolytic activity

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.

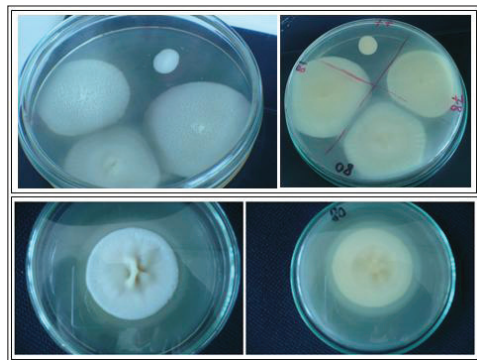


Figure 6. Isolated yeast strains on YS medium; opaque halos around the colonies formed due to the lipolytic activity.

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.

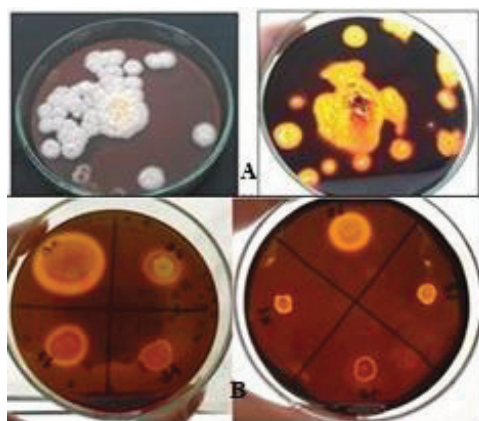


Figure 7. A) Fungal strain (isolation code 117) on CDA medium. B) Bacteria on GA medium. Plates flooded with Lugol's solution; clearly halos around colonies due to the amylolytic activity

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).

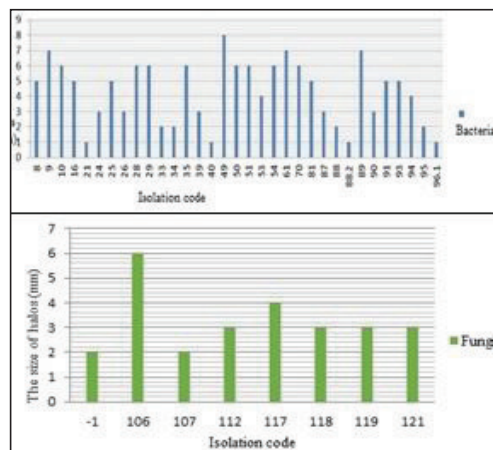


Figure 8. Bacteria and fungi – the size of halos (mm) corresponding to starch hydrolysis by amylase production

In figure 9 is presented the MALDI-TOF MS-dendrogram for some of the identified microbial strains; also it can be noticed that *Bacillus sp.* is the predominant genus isolated from nature.

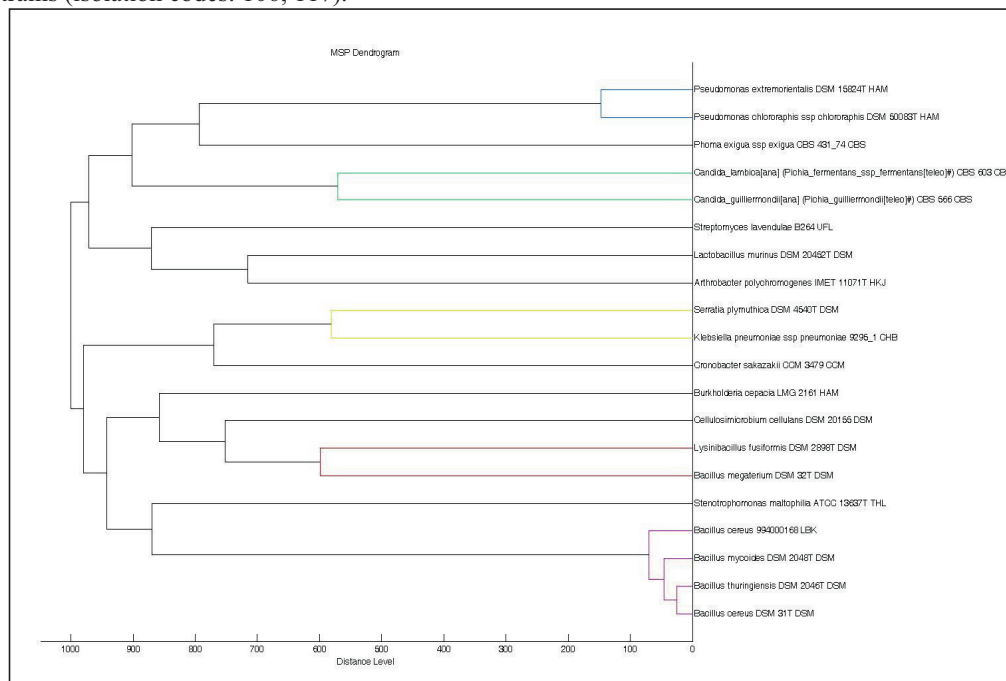


Figure 9. Identification of strains: the corresponding MALDI-TOF MS dendrogram

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).

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FOOD SAFETY

