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SELECTION OF MICROALGAL STRAINS WITH LOW STARCH CONTENT AS POTENTIAL HIGH LIPID - CONTAINING ISOLATES

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Abstract

In the last forty years there is an increased scientific interest in deeper understanding lipid metabolism in photosynthetic microorganisms, aiming at using the most lipid-rich strains as source for biodiesel production. One of the many constraints is the selection of strains with high lipid content. This paper presents the isolation, purification and selection of three strains of photosynthetic microorganisms by using an already known method, iodine vapour method, (Work et al., 2010) which allows to rapidly and easily select those colonies which low starch content as potential high lipid- containing isolates. These results show that in the three selected strains, the fluorescence emission after Nile red addition is increased with different values: 7.597; 10.832 and 11.428 for strains 9.3.1, 9.8.2 and 12.9.0, respectivley.

Key words: *photosynthetic microrganisms, iodine method, Nile red, fluorescence.*

INTRODUCTION

The ability of photosynthetic micro-organisms to accumulate lipids inside the cell received in the last four decades attention with respect to the use of photosynthetic microorganisms as sources of lipids for biodiesel (Sheehan et al., 1998; Chisti, 2007; Li et al., 2008; Liang et al., 2009; Demirbas, 2010; Huang, 2010; Mata et al., 2010; Amaro et al., 2011; Schuhmann et al., 2012; Borowitzka, 2013; Rawat et al., 2013; Velea et al., 2014; Ardelean and Manea, 2016).

This interest is based mainly on the their metabolic advantages over higher plants mainly with respect to shorter generation time (Demirbas, 2010; Amaro et al., 2011; Rawat et al., 2013; Ardelean and Manea, 2016). However, so far, there are some drawbacks (Demirbas, 2010; Amaro et al., 2011; Rawat et al., 2013; Ardelean and Manea, 2016).

In the last two decades there is an increased interest in understanding the competition between starch and lipids for intermediary metabolites (Libessart et al., 1995; Ball, 1998; Hu et al, 2008; Blaby, 2013; Davey et al, 2014; Tamayo-Ordóñez et al., 2017), including the selection of clones with a low starch content.

There are many reports arguing that the clones with low starch content have a higher lipid content (Ramazanov and Ramazanov, 2006; Wang et al., 2009; Li et al 2010 a and b; Siaut et al., 2011; Work et al, 2010; de Jaeger et al., 2014; Sirikhachornkit et al., 2016).

However, there are also results showing that mutants with low starch content have the same lipid content as the wild type cells (Vonlanthen et al., 2015).

The aim of this paper is to present original results concerning the screening of naturally occurring photosynthetic microorganisms with low starch content, as possible potential high lipid containing strains, using the iodine vapour method (Work et al., 2010).

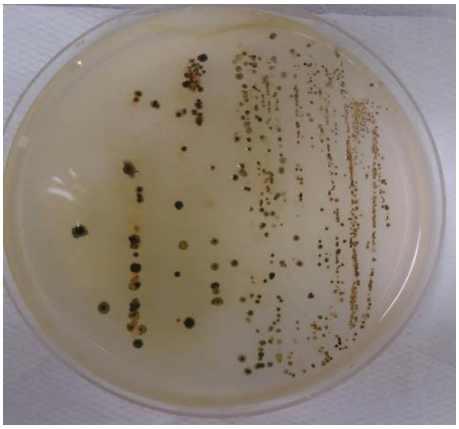
MATERIALS AND METHODS

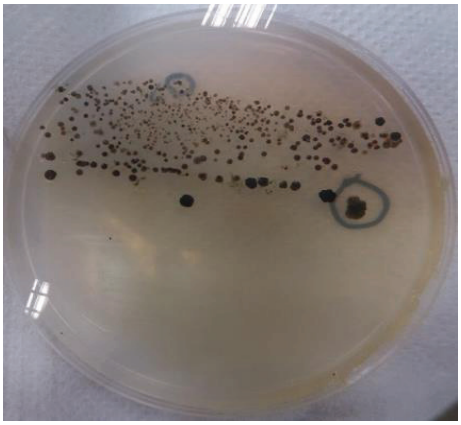
Populations of photosynthetic micro-organisms relatively rich in lipids previously selected (Ardelean, 2015; Ardelean and Manea, 2016) were used as biological material in these experiments. Isolation and purification of

photosynthetic microorganisms from the consortia of populations was achieved by dilution method on classically solidified BG_{11} (Ardelean, 2015).
Selection of colonies with low starch content was done using the qualitative iodine vapour method by placing solid I_2 pellets on the surfaces of agar plates to initiate sublimation (Work et al., 2010).
Estimation of lipid content was done both by microscopic method and by fluorescence quantification. The colonies of isolated and purified photosynthetic microorganisms were treated with Nile red (9-(Diethylamino)-5H benzo [∞] phenoxazin- 5) (Sigma Aldrich), one of the selective fluorescence markers for lipids (Greenspan et al., 1985; Chen et al., 2009).
The cells were incubated for 30 minutes in the presence of Nile red in order to allow as much as possible the penetration of cell wall and cell membrane; then, the microbiological samples were inspected using a fluorescence microscope, with respect to fluorescence signal in the red region as well as in the green region of the spectrum.
As with the microscopic method, for fluorescence quantification, the cells suspensions (OD_{730nm} 1,0 units) were incubated with Nile red for 30 minutes and then washed with fresh BG_{11} medium.
The fluorescence emission of these cell suspensions was analyzed before Nile red addition (fluorescence emission of only photosynthetic pigments) and after Nile red addition (fluorescence emission of photosynthetic pigments and Nile red in the presence of cellular lipids).
The fluorescence spectra were recorded with spectrofluorometer FP-8300, excitation at 530nm and emission 600-750nm, PMT voltage 500 V, data interval 0.5nm and screen speed 100nm/min; the surface of the spectrum was calculated with Spectra analysis software associated to this instrument.

RESULTS AND DISCUTIONS

In the figure 1 there are presented the macroscopic images of Petri dishes of three different populations, mixture of species, (9.3; 9.8 and 12.9 respectively) containing colonies (species) with different affinities for iodine vapour.
One can see that the majority of colonies are dark brown whereas few colonies are less coloured, as expression of lower starch content.
It has to be said that there are wild type strains, naturally occurring in the mixed populations when grown in the so- called normal conditions, without the occurrence of any thermal, nutritional or osmotic stress which could increase the lipid content of the cells (Chisti, 2007; Li et al., 2008; Amaro et al., 2011).
The colonies with lower affinity for iodine vapours were further cultivated in liquid medium to increase their biomass, to microscopically check morphologic uniformity and to label the lipids with the lipid-specific marker Nile red.
In the figures 2, 3 and 4 there are presented the results concerning the microscopic images (both in bright field and in fluorescence microscopy) of the purified (but non axenic) strains (so called unialgal strains) 9.3.1., 9.8.2 and 12.9.0, respectively.
Each microscopic field has three images: one in bright field and two images of the fluorescent emission: red and green portion of the fluorescence emission spectrum of the Nile red.
One can see that the red fluorescence signal is distributed distinctly within the cells of strain 9.8.2 and, especially, 12.9.0, suggesting the occurrence in these two strains of lipid droplets detectable by the use of classical optical microscopes.
Interestingly, these two strains gave higher enhancement of the fluorescence signal, after Nile red labelling (see table 1).





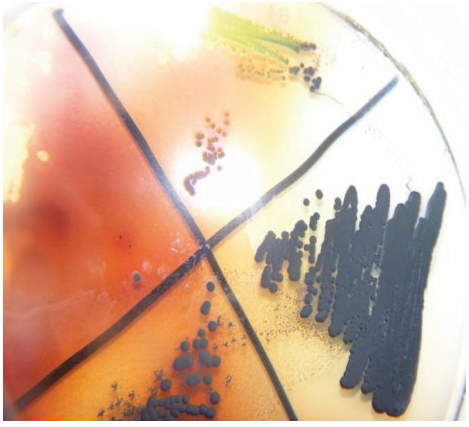
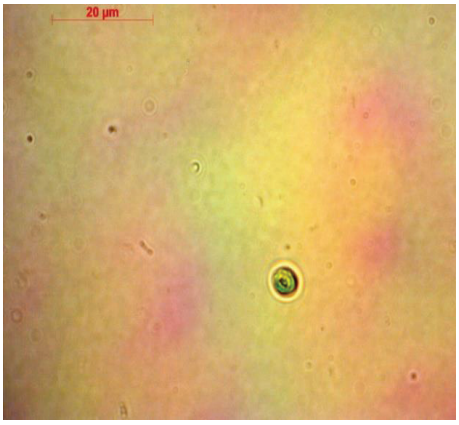
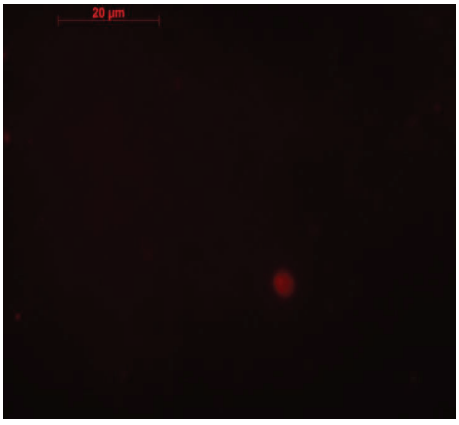


Figure 1. Colonies with different affinities for iodine vapour, after 14 days o autotrophic cultivation in normal conditions; from up to the bottom : mixed population 9.3; mixed population 9.8. and mixed population 12.9





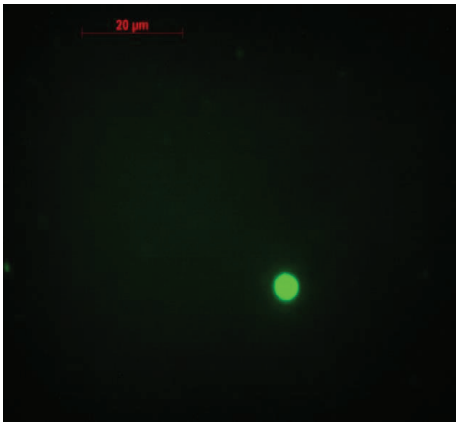
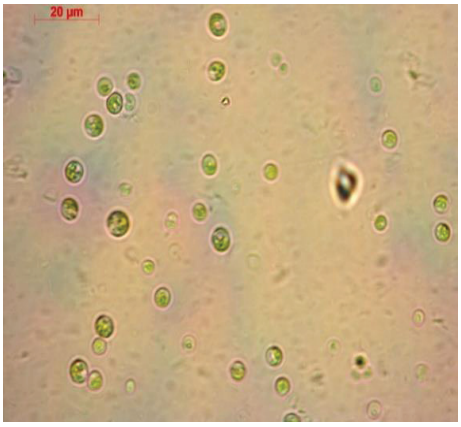
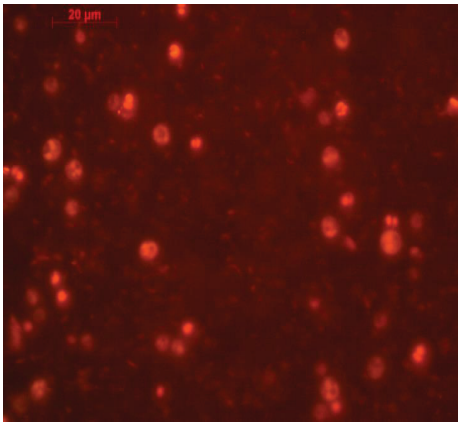


Figure 2. Bright filed and epifluorescence images (green filter and red filter) of cells belonging to the purified strain 9.3.1.





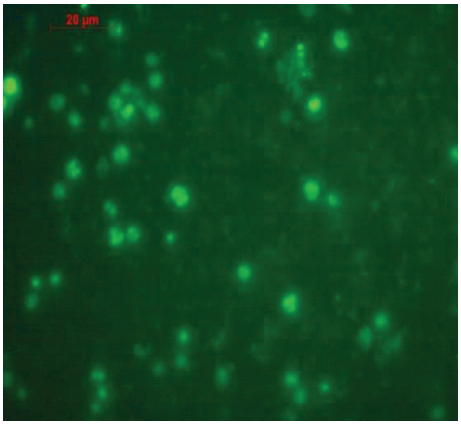
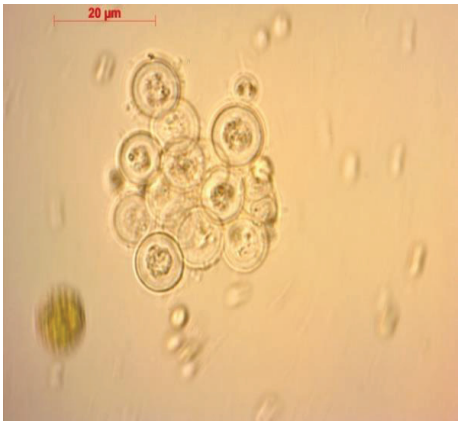
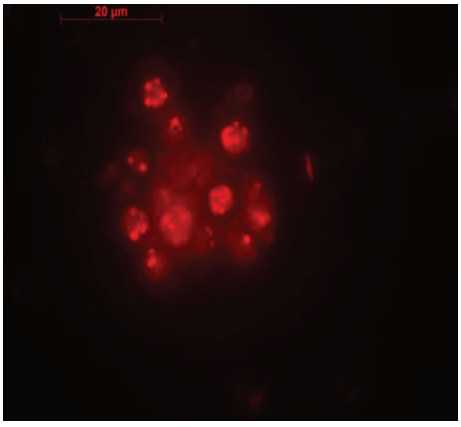


Figure 3. Bright filed and epifluorescence images (green filter and red filter) of cells belonging to the purified strain 9.8.2





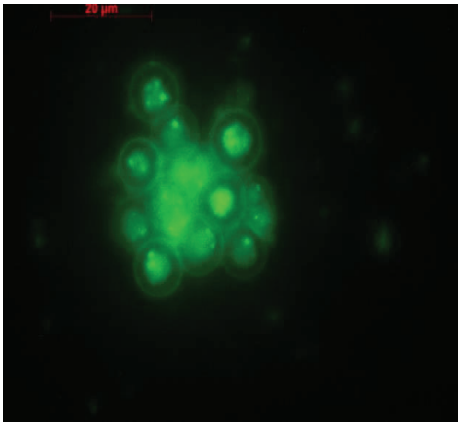


Figure 4. Bright filed and epifluorescence images (green filter and red filter) of cells belonging to the purified strain 12.9.0

Further estimation of lipid content was done by measuring the emission fluorescence spectrum of isolates before and after Nile red labelling. In table 1 there are presented the results concerning the fluorescence emission intensity of the three isolates before- and after Nile red labelling.

Table 1. Fluorescence emission (arbitrary units) of purified strains (9.3.1; 9.8.2 and 12.9.0) in the absence and in the in the presence of Nile red (NR)

Strains	Fluorescence emission, without NR	Fluorescence emission, with NR
9.3.1	5.813	13.410
9.8.2	7.176	18.028
12.9.0	4.586	15.914

These results show that in the three selected strains, the fluorescence emission after Nile red addition is increased with different values: 7.597; 10.832 and 11.428 for strains 9.3.1, 9.8.2 and 12.9.0, respectivley.
These differences represents the differnce between fluorescene emission with Nile red and fluorescence emission without Nile red and are, probably, a expression of different lipid content of each strain.

CONCLUSION

Isolation, purification and selection of three clones of photosynthetic micro-organisms with estimated low starch content and, probably, different lipid content.

PERSPECTIVES

These isolates, as well as those to be obtained using the same method, are the biological material for further experiments concerning quantitative determination of biological parameters important for true candidates for lipid production.
These parameters concern lipid content and growth rate, both in the so-called normal conditions and under different type of stress (nitrogen or other nutrient limitation etc.,) which, generally, increases the lipid content.
Furthermore, the selection method could be improved by the use of different strategies to decrease chlorophyll fluorescence signal which overlaps with red fluorescence of Nile red.

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REFERENCES

Amaro H.M., Guedes A.C., Malcata F.X., 2011. Advances and perspectives in using microalgae to produce biodiesel. Appl Energy, 88: 3402–3410.

Ardelean I.I., Manea M., 2016. Indoor Cultivation Of Selected Oil – Containing Consortia Of Photosynthetic Microorganisms For Further Biodiesel Production; Preliminary Financial Evaluation Scientific Bulletin. Series F. Biotechnologies, Vol. XX,1372, ISSN-L 2285-1364, 237-242.

Ardelean I.I., 2015. Selection of oil-containing populations of phototrophic microorganisms for further biodiesel production. International Conference of the RSBMB , Bucharest, 17-18 September 2015, AGIR Publishing House, ISBN 978-973-720-605-3, p.53.

Ball S., 1998. Regulation of starch biosynthesis, in: J.-D. Rochaix, M. Clermont- Goldschmidt, A. Merchant (Eds.), Mol. Biol. Chloroplasts Mitochondria Chlamydomonas, Kluwer Academic Publishers 549–567.

Blaby I.K., Glaesener A.G., Mettler T., Fitz-Gibbon S.T., Gallaher S.D., Liu B., et al., 2013. Systems-level analysis of nitrogen starvation-induced modifications of carbon metabolism in a *Chlamydomonas reinhardtii* starchless mutant, Plant Cell 25: 4305–4323.

Borowitzka M.A., 2013. High-value products from microalgae-their development and commerce-alisation. J. Appl.Phycol. 25: 743–756.

Chen W., Zhang C., Song L., Sommerfeld M., Hu Q., 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods, 77: 41–47.

Chisti Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25: 294–306.

Davey M.P., Horst I., Duong G.-H., Tomsett E.V., Litvinenko A.C.P., Howe C.J., et al., 2014. Triacylglyceride production and autophagous responses in *Chlamydomonas reinhardtii* depend on resource allocation and carbon source, Eukaryot. Cell 13: 392–400.

de Jaeger L., Verbeek R.E., Draaisma R.B., Martens D.E., Springer J., Eggink G., et al., 2014. Superior

triacylglycerol (TAG) accumulation in starchless mutants of *Scenedesmus obliquus*: (I) mutant generation and characterization, Biotechnol. Biofuels 7:69.

Demirbas M.F., 2010. Microalgae as a feedstock for biodiesel [J]. Energy Education Science and Technology Part A, 25(1−2): 31−43.

Greenspan P., Mayer E.P., Fowler S.D., 1985. Nile red—A selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100: 965–973.

Hu Q., Sommerfeld M., Jarvis E., Ghirardi M., Posewitz M., Seibert M., et al., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances, Plant J. 54: 621–639.

Huang G., Chen F., Wei D., Zhang X., Chen G., 2010. Biodiesel production by microalgal biotechnology. Appl Energy. Elsevier Ltd, 87(1): 38–46.

Li Y., Han D., Hu G., Dauvillee D., Sommerfeld M., Ball S., et al., 2010. Chlamydomonas starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol, Metab. Eng. 12: 387–391.

Li Y., Han D., Hu G., Sommerfeld M., Hu Q., 2010. Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*, Biotechnol. Bioeng. 107: 258–268.

Li Y., Horsman M., Wang B., Wu N., Lan C.Q., 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans.* Appl Microbiol Biotechnol.; 81(4): 629–636.

Liang Y.N., Sarkany N., Cui Y., 2009. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett, 31: 1043–1049.

Libessart N., Maddelein M.L., Koornhuyse N., Decq A., Delrue B., Mouille G., et al., 1995. Storage, photosynthesis, and growth: the conditional nature of mutations affecting starch synthesis and structure in *Chlamydomonas*, Plant Cell 7: 1117–1127.

Mata T.M., Martins A.A., Caetano N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy Rev. 14: 217–232.

Ramazanov A., Ramazanov Z., 2006. Isolation and characterization of a starchless mutant of *Chlorella pyrenoidosa* STL-PI with a high growth rate, and high protein and polyunsaturated fatty acid content, Phycol. Res. 54: 255–259.

Rawat I.R., Ranjith Kumar T., Mutanda F., 2013. Biodiesel from microalgae: A critical evaluation from laboratory to large scale production Applied Energy, 103: 444–446.

Schuhmann H., Lim D.K.Y., Schenk P.M., 2012. Perspectives on metabolic engineering for increased lipid contents in microalgae. Biofuels 3: 71–86.

Sheehan J., Dunahay T., Benemann J., Roessler P., 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae; National Renewable Energy.

Siaut M., Cuine S., Cagnon C., Fessler B., Nguyen M., Carrier P., et al., 2011. Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves, BMC Biotechnol. 11: 7.

Sirikhachornkit A., Vuttipongchaikij S., Suttangkakul A., Yokthongwattana K., Juntawong P., Pokethitiyook P., Kangvansaichol K., Meetam M., 2016. Increasing the triacylglycerol content in *Dunaliella tertiolecta* through isolation of starch-deficientmutants. *J.Microbiol. Biotechnol. 28*, 854–866.

Velea S., Ilie L., Stepan E., Chiurtu R., 2014. New photobioreactor design for enhancing the photosynthetic Productivity of *Chlorella homosphaera* culture. Revista de Chimie (Bucharest) 65: 56-60

Vonlanthen S., Dauvillée D., Purtona S., 2015. Evaluation of novel starch-deficient mutants of *Chlorella sorokiniana* for hyper-accumulation of lipids. Algal Research 12: 109–118.

Wang Z.T., Ullrich N., Joo S., Waffenschmidt S., Goodenough U., 2009. Algal lipid bodies: stress induction, purification and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*, Eukaryot. Cell 8: 1856–1868.

Work V.H., Radakovits R., Jinkerson R.E., Meuser J.E., Elliott L.G., Vinyard D.J., et al., 2010. Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7–10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains, Eukaryot. Cell 9: 1251–1261.

Tamayo-Ordóñez Y.J., Ayil-Gutiérrez B.A., Sánchez-Teyer F.L., De la Cruz-Arguijo E.A., Tamayo-Ordóñez F.A., Córdova-Quiroz V. and Tamayo-Ordóñez M.C., 2017. Advances in culture and genetic modification approaches to lipid biosynthesis for biofuel production and in silico analysis of enzymatic dominions in proteins related to lipid biosynthesis in algae, Phycological Research, 65: 14–28.