Scientific Bulletin. Series F. Biotechnologies, Vol. XX, 2016
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INDOOR CULTIVATION OF SELECTED OIL – CONTAINING CONSORTIA OF PHOTOSYNTHETIC MICROORGANISMS FOR FURTHER BIODIESEL PRODUCTION; PRELIMINARY FINANCIAL EVALUATION

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Abstract

This paper presents results concerning the indoor cultivation of selected mixed populations of photosynthetic microorganisms using BG_{11}medium as well as a cheaper medium based on chemical fertilizers and residual glycerol. Preliminary financial evaluations are also presented in order to focus on the economical constraints. The growth of selected oil- containing consortia of photosynthetic microorganisms in 90L of BG_{11}(prepared in spring water) produced in 14 days 285 grams of dry weight biomass containing 28% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass means 83.3 lei; whereas the same consortium grown in 30L of alternative medium, prepared with agricultural fertilizers, residual glycerol and spring water produced in 14 days 150g grams of dry weight biomass containing 20% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass is 3.2 lei.

Key words*: photosynthetic microorganisms, biomass, lipids, agricultural fertilizers.*

INTRODUCTION

Biodiesel is produced by the transesterification of different origins triglyceride with monohydric alcohols such as methanol. One main advantage of biodiesel it that it can be blended in any proportion with fossil based diesel which is not possible with bioethanol. Classically, rapeseed, canola, sunflower, soybean oils, beef tallow and many other oils have been used for the production of bio-diesel esters. The ability of photosynthetic microorganisms, both prokaryotes and eukaryotes, to accumulate lipids inside the cell is very well known from more than a century, but a national program, in USA, was funded for the production of biodiesel from high lipid-content algae (Sheehan et al., 1998) only in 1978. Over the almost two decades of this program (1978-1996), tremendous advances were made in the science of manipulating the metabolism of algae and the engineering of microalgae algae production systems (Sheehan et al., 1998). Mainly there is a huge increase practically all over the globe for the use of photosynthetic microorganisms in the last two decades, both prokaryotes and eukaryotes, as sources of lipids for biodiesel (Thomas et., 1983; Chisti, 2007; Li et al., 2008; Brune et al., 2009; Griffiths and Harrison., 2009; Vijayaraghavan and Hemanathan K., 2009; Liang et al., 2009; Thurmond, 2009; Demirbas, 2010; Huang, 2010; Mata et al., 2010; Brennan and Owende, 2010; Amaro et al.,; 2011; Santibáñez et al., 2011; Kaiwan-arporn 2012; Schuhmann et al., 2012; Borowitzka, 2013; Rawat et al., 2013; Velea et al., 2014) as well as for other bio(nano)-tehnogical application (Borowitzka, 2013; Ardelean and Zarnea, 1998; Ardelean, 2006; Ardelean 2015a and b, and references herein).

This interest is based mainly on the followings (for more details see Demirbas, 2010; Amaro et al.; 2011; Rawat et al., 2013, and references herein):

Microalgae have rapid growth rates, thus they can to double their weight with respect to biomass within 24 h, some of them being able to grown strictly photoautotrophic but also mixotrophic or even heterotrophic (into darkness);

Photosynthetic microorganism which converts sunlight, water and CO_2 to sugars, from which macromolecules such as lipids and

triacylglycerides (TAGs) can be obtained which are promising and sustainable feedstock for biodiesel production;
Many micro algae have the ability to produce substantial amounts (20-50%) of TAGs as a storage lipid under photooxidative stress or other adverse environmental conditions;
Microalgae require less freshwater for cultivation than terrestrial plants (but water is still a problem for true economically applications!)
Lipids produced are generally neutral lipids that have a high level of saturation making it a suitable feedstock for biodiesel production; the chemical and physical properties of biodiesel produced from microalgae reach the international standard for cars (EN14214).
During their growth microalgae can use N and P in the form of phosphates and nitrates from wastewater and CO_2, even residual one, which is a major component of flue gases.
Apart of lipids, some microalgae produce other valuable substances such as proteins, pigments, biopolymers and carotenoids including antioxidant substances for commercial or pharmaceutical purpose.
However, so far there are some drawbacks (for more details see Demirbas, 2010; Amaro et al.; 2011; Rawat et al., 2013, and references herein): Recovery of microalgae from their growth media is seen as one of the major challenges of using microalgae for the production of biodiesel owing to their small size and planktonic distribution;
Cell wall breaking is difficult and leads to unspecific mixtures thus large scale extraction techniques for microalgal lipids are complex and expensive;
Most metabolic pathways in microalgae are not fully known, thus, biochemical optimization is difficult;
The knowledge of regulatory basis of metabolic networks in microalgae is incipient, so rational control of endocellular reactions is hard to accomplish;
Microalgae grown in open pond systems are prone to contamination, bacteria being strong competitors for nutrients, including organic ones (in the case of mixotrophic or heterotrophic growth of photosynthetic microorganisms);
Open systems are also susceptible to grazers in the form of protozoa and zooplankton which can devastate algal concentration in 2–3 days;
There is a huge need of nitrogen to sustain the intensive growth of photosynthetic microorganisms, many specialist thinking that the only true answer for that is the use of residual waters rich in inorganic nitrogen;
The conversion of lipids to biodiesel produce wastes, including residual glycerol whose destruction is an expensive step; alternatively, it could be used as row carbon source for growing other useful microorganisms, including lipid accumulation one.
The aim of this paper is to present original results concerning the in- door cultivation of selected mixed populations of photosynthetic microorganisms using BG_{11}medium as well as a cheaper medium based on chemical fertilizers and residual glycerol, in connection with the amount of biomass and oil produced. Preliminary financial evaluations are also presented in order to focus on the economical constraints.

MATERIALS AND METHODS

The consortia's selection of photosynthetic microorganisms relatively rich in lipids was done taking into account the fluorescence signal in the presence of the fluorochrome Nile red (Ardelean et al., manuscript in preparation)
Microscopic investigation of lipid content. The photosynthetic microorganisms consortia were treated with Nile red(9-(Diethylamino)-5H benzo [∞] phenoxazin- 5), one of the selective fluorescence markers for lipids (Greenspan et al., 1985; Chen et al., 2009). The microbial populations were incubated for 30-120 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 an epifluorescence microscop, with respect to fluorescence signal in the red region as well as in the green region of the spectrum.
The growth of photosynthetic populations. In agreement with the literature, BG_{11} was used to cultivate the mixtures of photosynthetic microorganisms in 20L PET bottles at 28-32^{o} C with natural 12 hours sunlight illumination (7am-7pm) and 12 hours (7pm-7am) artificial lighting (2400 luxes ?), air bubbling (240 L/hour), the pH varying between 8.0 and 9.0. The growth of photosynthetic populations was carried out also in alternative medium containing residual glycerol and chemicals

found in commercially available fertilizers for agriculture, thus decreasing the cost of the growing medium, in agreement with the proposals in the literature (Santibáñez et al., 2011). The growth conditions were as those for the growth in BG_{11}. Cell harvesting can be done very well at laboratory level by centrifugation; however, for larger volumes centrifugation is very costly; thus, in the experiments reported in this paper the harvesting was done by flocculation using $Al_2(SO_4)_3x18H_2O$ (SR EN 878/2004 A) (Teodosiu, 2001; Mâşu and Zamfiroiu, 2007) the stock solution (20g/L) was diluted 10 times with the microbial culture and in short time (5-10 minutes) the cells are gravitationally separated from the clear liquid supernatant (Figure 1).

Figure 1. The separation of microbial cell by flocculation.

Lipid extraction was done by Bligh and Dyer method (Bligh and Dyer 1959) and ultrasonication. For that microalgal biomass were collected by centrifuging the cells at 4000 *g* for 10 minutes. The cells were washed with distilled water, dried (48 hours at 85°C) and weighted. The known amount of biomass (100 mg) was then homogenized with chloroform: methanol 1:2 at 35°C and sonicated for 60 min. The extract was centrifuged for 7 minutes at 10,000 g and supernatant was collected in a separating funnel. The residue was further homogenized with chloroform and again centrifuged (10,000 g) to collect the supernatant. Now 0.9% NaCl solution was added to the filtrate and washed, lower layer of chloroform was separated and treated with anhydrous Na_2SO4 to remove the traces of water. The lipid content was determined gravimetrically and expressed as dry weight % after evaporating the chloroform calculated using the following equation: Y(%) = WL/WDA, where WL and WDA were the weights of the extracted lipid and the dry algae biomass, respectively.

RESULTS AND DISCUSSIONS

In Figure 2 one can see the PET bottles after 14 days of indoor cultivation.

Figure 2. PET bottles with selected consortia of photosynthetic microorganisms, both prokaryotes and eukaryotes, after 14 days of autotrophic cultivation (see Materials and methods).

In Figure 3 there are presented microscopic images both in bright field and in epifluorescence microscopy (Nile red labeling of lipid droplets- when present) of different microscopic fields; each microscopic field has three images; one in bright field and two fluorescent images: red and green portion of the fluorescence emission spectrum of the Nile red.

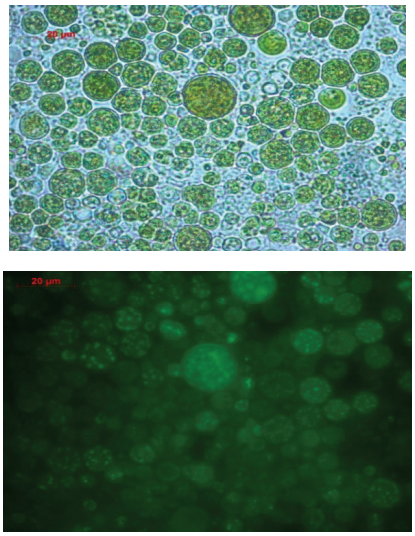

Figure 3. Bright filed and pifluorescence images (red and green filters, respectively) of different populations of photosynthetic microorganisms presented in the A9 mixture of populations

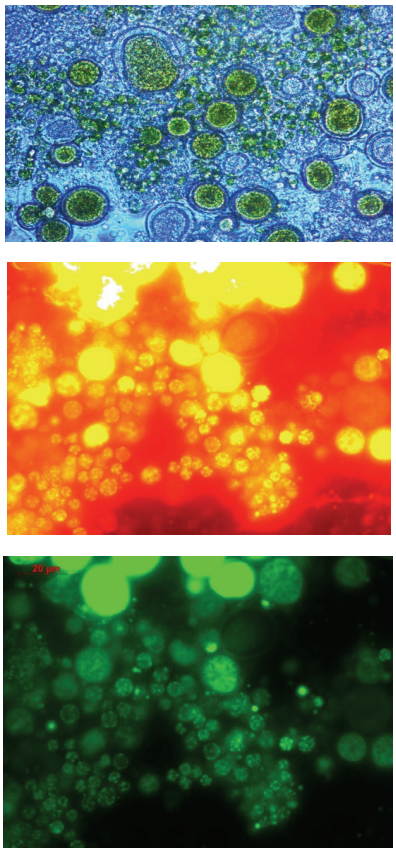

Figure 4. Bright filed and epifluorescence images (red and green filters, respectively) of different populations of photosynthetic microorganisms presented in the A3 mixture of populations

The lipid intracellular inclusions (lipids droplets) can be seen in some cells of photosynthetic microorganisms as red or green surfaces; the size and the intensity of the fluorescence varies from cell to cell. The size and the intensity emission are higher for cells containing larger quantities of lipid inclusions. These images show the diversity of the mixture of populations of photosynthetic populations with respect to lipid intracellular inclusions, strongly arguing for the necessity to further isolate in pure cultures the strains with high lipid content.

In Table 1 there are presented the synthetic results concerning the dry biomass of photosynthetic microorganisms obtained after 14 days of indoor cultivation either in BG_{11} prepared with tap water or in alternative medium, containing chemicals found in commercially available fertilizers for agriculture, and residual glycerol.

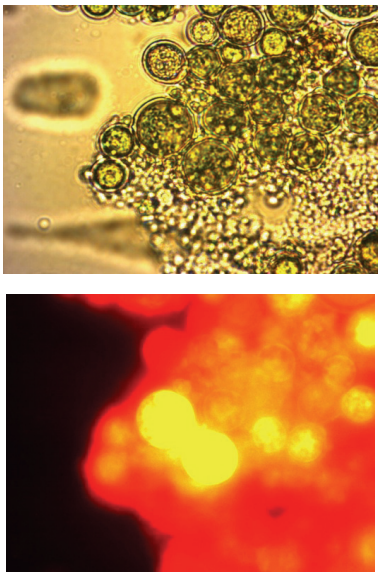

Figure 5. Bright filed and epifluorescence (red and green filters, respectively) images of different populations of photosynthetic microorganisms presented in the A7 mixture of populations

Table 1. The results concerning dry biomass and lipid content of selected consortia of photosynthetic microorganisms grown in $BG_{11}$and in alternative medium (with spring water and residual glycerol)

Growing medium	Volume of culture	Dry biomass	Lipid content %
BG_{11} (with spring water)	95 L	285 g	28%
Alternative medium	30 L	150 g	20 %

As one can see from Table 1, the results obtained in indoor cultivation are rather modest with respect to lipid content, probably because of the use of mixture of populations, containing strains with rather high lipid content (as can be seen during microscopic inspection- see Figure 3) and strain with practically no lipid deposits (as can be seen during microscopic inspection- see Figure 3). The use of alternative medium allows a higher dry biomass per liter, as compared with standard medium prepared in tap water, which seems to be promising for the future research. The cost of BG_{11} prepared in distilled water is 1.25 lei /L and prepared with

spring water 0.25 lei/L whereas the cost of alternative medium, prepared with agricultural fertilizers, residual glycerol and spring water is 0.0163 lei /L. Taking into account the biomass obtained by growing these populations of photosynthetic microorganisms in BG_{11} prepared in spring water the cost of 1 Kg of dry biomass is 83.3 lei whereas the use of alternative medium reduce the cost significantly down to 3.2 lei. We have to remember that the calculation concerns only cost of water and chemical ingredients, all other costs (illumination, continuous air-bubbling, flocculation, drying, chemical extraction – including chemicals and ultrasonication, labor etc.) were not take into account for the time being. It has to be said that the price of 83.3 lei/kg dry biomass of photosynthetic microorganisms cannot commercially compete with the price of oleaginous plants, which is 100 times lower!

CONCLUSIONS

The indoor cultivation of selected oil- containing consortia of photosynthetic microorganisms in 90L of BG_{11} (prepared in spring water) produced in 14 days 285 grams of dry weight biomas containing 28% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass means 83.3 lei;
The indoor cultivation of selected oil-containing consortia of photosynthetic microorganisms in 30L of alternative medium, prepared with agricultural fertilizers, residual glycerol and spring water produced in 14 days 150g grams of dry weight biomas containing 20% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass is 3.2 lei.
The future prospects for this research are the followings:
Selection and isolation of strains able to store lipids in high proportions and to rapidly grow in standardized media (BG_{11}, for example);
Further selection of the above (1) isolates but able to grow in cheaper media containing agricultural fertilizers or, better, in different types of waste waters;
The growth of the selected strain(s) in large volumes (1,000-10,000 L) only under solar irradiation, extraction of oil and its conversion to biodiesel
Cost calculations for the scaled up processes, and future improvements.

ACKNOWLEDGEMENTS

I.I.A. work was supported by Romanian Academy (Grant RO1567-IBB05/2015).This paper is dedicated to the 150th Anniversary of The Romanian Academy.

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