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Influence of Culture Medium on *Desmodesmus* sp. Growth and Lipid Accumulation for Biodiesel Production

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Biodiesel production is currently a highly researched topic due to environmental issues as well as a possible biofuel to contribute with the energetic matrix regarding transportation. Among potencial alternative feedstock for biodiesel production microalgae appear as a possibility in view of its advantages in comparison to conventional crops, since they present high photosynthetic activity, exponential growth rates and high biomass productivities. In this investigation, the influence of two culture media (BG-11 and Guillard f/2) on the growth and lipid accumulation of the *Desmodesmus* sp. microalgae was assessed. Microalgal growth in BG-11 medium reached higher cell count in the considered time period and the microalgae presented higher lipid fraction in relation to the Guillard f/2 medium, thus being the most suitable medium for *Desmodesmus* sp. cultivation.

1. Introduction

Biodiesel is a biodegradable fuel produced from different renewable feedstock, such as animal fats, vegetable oils and biomass. Biodiesel use reduces emissions of CO, CO_2 and sulfur compounds, gases harmful to the environment (Pragya et al., 2013). Different vegetable oils and its derivatives are used as feedstock for the obtention of biofuels with great potential to substitute conventional fuels (Issariyakul and Dalai, 2014). Vegetable oils usually employed in biodiesel production are soybean, palm, coconut, corn and canola oils (Bergmann et al., 2014), among others. Current large-scale biodiesel production is carried out from the cited oils, although the global output of this fuel is not able to supply its worldwide demand despite improvements in the production process. Besides there is a concern about food security when crops used for food are employed for biofuel production. Therefore, there is an incentive to new oil sources that need to be sought including those that increase the global process yields. Third generation biodiesel is produced from the biomass of microbes and microalgae, which are unicellular microorganisms able to grow in fresh and salt water environments. The production of metabolites by microalgae has been extensively studied and is of high interest to several industrial areas. Such compounds include carbohydrates, proteins, sterols, amino acids, fatty acids and carotenoids (Kirrolia et al., 2013). Several types of biofuels can be produced from different fractions of the microalgal biomass, such as bioethanol, syngas, hydrogen and biodiesel. Concerning the production of biodiesel from microalgae, the metabolite of interest are the lipids contained within the cell. Microalgae have the advantage of presenting high rates of cell growth, high biomass production and the ability of accumulating high quantities of lipids when compared to conventional feedstock for biodiesel production - namely soybean and palm oils. For the production of biodiesel from microalgal biomass, several consecutive steps are needed (Sing, S. et al., 2013): microalgae growth, biomass separation unit, biomass drying, lipid extraction and transesterification reaction for the conversion of triglycerides into esters. Regarding microalgae

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growth, microalgae are able to thrive in a broad range of culture medium conditions and temperatures, including nutrient scarcity (Rios et al., 2014) and other adverse conditions, such as contamination. In large scale, microalgae cultivation can be carried out with seawater, with water from estuaries and with wastewater from agricultural and industrial operations and with domestic wastewater. The induction of metabolite accumulation in microalgae, such as of carbohydrates and lipids, is performed through the manipulation of cultivation conditions, such as temperature, light, pH and nutrients (Mata et al., 2013). Most microalgae species present a complete life cycle in few hours, a factor which favors the selection of strains and genetic improvement. The unicellular nature of microalgae ensure an homogeneous biochemical composition unlike terrestrial plants, in which compounds of interest are often located in specific parts of leafs, seeds, roots and fruits (Cohen, 1986). In general, microalgae cultivation can be carried out in open or closed systems of various designs and operation modes. However, this route still requires high capital investment (Ningam and Singh, 2011), thus needing improvements in order for the scale up of the process to be of economic interest (Rawat et al., 2013). Bearing all this in mind this work focused in the improvement of biomass production (first step of the process) through investigation the use of two culture media with the goal of achieving higher microalgae growth rates and lipid accumulation in *Desmodesmus* sp. in closed systems,

2. Materials and methods

2.1 Culture medium

The identified *Desmodesmus* sp. strain is a green microalga donated by the Laboratory of Research on Aquatic Organisms (LAPOA) of the Integrated Group on Aquiculture and Environmental Studies (GIA) of the Federal University of Paraná (UFPR). Two culture media were employed: BG-11 and Guillard f/2. BG-11 medium presents the following composition (Rippka et al., 1979): $NaNO_3$ (1500 mg L^{-1}), K_2HPO_4 (40 mg L^{-1}), $CaCl_2.2H_2O$ (30 mg L^{-1}), Na_2CO_3 (19 mg L^{-1}), $MgSO_4.7H_2O$ (8 mg L^{-1}), $C_6H_8O_7.H_2O$ (7 mg L^{-1}), ammonium ferric citrate (6 mg L^{-1}), H_3BO_3 (3 mg L^{-1}), $MnCl_2.4H_2O$ (2 mg L^{-1}), $Na_2EDTA.2H_2O$ (0.7 mg L^{-1}), $Na_2MoO_4.2H_2O$ (0.4 mg L^{-1}), $ZnSO_4.7H_2O$ (0.2 mg L^{-1}), $CuSO_4.5H_2O$ (0.1 mg L^{-1}) and $Co(NO_3)2.6H_2O$ (0.05 mg L^{-1}). The Guillard f/2 medium contains the following compounds (Guillard e Ryther, 1962): $NaNO_3$ (750 mg L^{-1}), $NaH_2PO_4.H_2O$ (5 mg L^{-1}), $Na_2SiO_3.9H_2O$ (300 mg L^{-1}), $FeCl_3.6H_2O$ (3.15 mg L^{-1}), $Na_2EDTA.2H_2O$ (4.36 mg L^{-1}), $CuSO_4.5H_2O$ (9.8 mg L^{-1}), $Na_2MoO_4.2H_2O$ (6.3 mg L^{-1}), $ZnSO_4.7H_2O$ (22 mg L^{-1}), $CoCl_2$ $6H_2O$ (10 mg L^{-1}), $MnCl_2.4H_2O$ (180 mg L^{-1}) and vitamins thiamine hydrochloride (vitamin B1, 200 mg L^{-1}), biotin (vitamin H, 0.1 mg L^{-1}) and cyanocobalamin (vitamin B12, 1 mg L^{-1}). Culture medium pH was adjusted to 7.5 (BG-11) and 8.0 (Guillard f/2) and the mixtures were autoclaved at 121 ºC for 15 min.

2.2 Experimental procedure

Microalgae growth was carried out in 500 mL Erlenmeyer flasks. To each flask, 300 mL of culture medium and a 10% microalgae inoculum were added. The assays were carried out in a light bank with light flux of 62 $\mu Em^{-2} s^{-1}$, photoperiod of 12h, temperature of 26 ± 4 °C and forced aeration. Figure 1 shows the assembly of the cultivations, which were carried out for a period of seven days. The experiments were performed in duplicate. After growth, biomass was separated from the spent medium through centrifugation (Eppendorf, model 5810R) at 1843 × *g* for 15 min. The recovered biomass was washed with distilled water several times to ensure nutrient removal prior to lipid extraction.

Figure 1: Microalgae cultivation in both culture media

2.3 Analytical methods

2.3.1 Cell dimensions

Cell dimensions were measured with an optical microscope (Leica DMLM) coupled with a digital camera and a computer, which allowed image acquisition, cell count and average size distribution determination with a dedicated software (Image Tool). Microalgae average width and length were determined in this way.

2.3.2 Microalgae biomass

Microalgae growth was determined through direct cell count with a microscope (Olympus CX21) using a Neubauer chamber. Cell quantity was analyzed twice a day throughout a cultivation period of seven days. Results were obtained in number of cells per mL.

2.3.3 Lipid extraction and quantification

Lipid extraction was carried out with the Bligh and Dyer method (Bligh and Dyer, 1959). Microalgae biomass was separated from the culture medium after seven days of cultivation through centrifugation (Eppendorf, model 5810R) at 3500 rpm for 15 min and dried in an oven at 105 ºC for 24h. A 50 mg sample of dry biomass was added to distilled water and a 2:1 methanol/chloroform mixture and vigorously agitated for 25 min. Afterwards, the resulting suspension was centrifuged at 4500 × *g* for 10 min. Three phases are obtained: a top phase, containing water and methanol; a middle phase, consisting in a protein disk; and a lower phase, with the extracted lipids and chloroform. Lipid quantification was carried out through drying in an oven at 105 ºC until constant weight was reached. Lipid mass fraction was calculated as in Eq(1):

$$\%Lipids = \frac{lipid\ mass\ after\ extraction}{initial\ sample\ mass} \quad (1)$$

2.3.4 Growth rate calculation

For growth rate determination, the growth curve is linearized by plotting it in a semi-log graph. Growth rate was calculated by Eq(2):

$$\frac{dX}{dt} = \mu \quad (2)$$

where X is the cell concentration, t is the time and μ is the specific growth rate. The definition of μ is shown in Eq(3):

$$\mu = \frac{ln\frac{X_t}{X_{t0}}}{t - t_0} \quad (3)$$

From the semi-log graph, the growth rate is the inclination of the curve in the exponential growth phase, as shown in Eq(4):

$$X = X_0 + \mu t \quad (4)$$

2.3.5 Doubling time calculation

The doubling time (t_d) is the amount of time a microalgal cell takes to divide and the total biomass in the cultivation doubles. Its calculation is given by Eq(5):

$$t_d = \frac{ln\,2}{\mu} \quad (5)$$

2.3.6 Fatty acid profile

Fatty acid identification was carried out through their esterification and quantitative determination with capillary gas chromatography, according to AOCS (1997) and Hartmann and Lago (1973).

3. Results

Figure 2 shows the *Desmodesmus* sp. cells with an amplification of 500x. Cells are oval, with an average length of 6.25 µm and an average width of 6.22 µm.

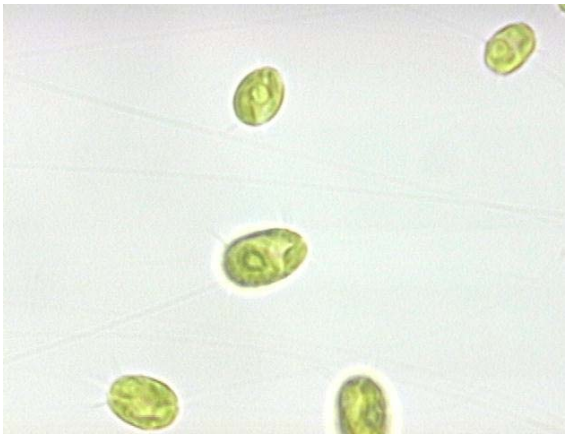

Figure 2: Cells of Desmodesmus sp.

Desmodesmus sp. growth in both culture media was compared throughout seven days of cultivation. Initial cell concentration in the cultivations differed by around 1 MCells/mL, which do not influence growth profiles. Figure 3 presents microalgae growth behavior for both culture media. Both curves depict the shape of a characteristic S-curve, which is composed by four defined phases: an initial lag phase, during which cells adapt to the culture medium; a following exponential growth phase (log phase), where cells multiply at their maximum rate; an ensuing stationary phase, in which cell growth rate equals cell death rate; and a death phase (not show in Figure 3), in which cell concentration declines. It can be observed that microalgae growth in BG-11 medium is faster than in Guillard f/2 throughout the whole period of cultivation. In day two, cell number in both experiments is nearly the same, although cultivation in BG-11 starts its exponential growth phase from day three. With the Guillard f/2 medium, exponential microalgal growth is shorter and starts before. As a result, microalgae cultivation in BG-11 medium achieves cell concentration values 2.44 times higher than in the Guillard f/2 counterpart.

Maximum cell concentration in BG-11 medium was of 23.5 MCells/mL. It can be observed that the exponential growth phase starts from day three, with a maximum growth rate (μ_{max}) of 0.10 day^{-1}. For the Guillard f/2 medium, cell concentration in day seven of the cultivation is of 9.63 MCells/mL. The exponential growth phase starts in day two with a μ_{max} of 0.05 day^{-1}. Thus, for process scale-up, cultivation in BG-11 should be considered since biomass concentration in this medium was 13.9 MCells/mL higher than in the Guillard f/2 medium.

Lipid extraction with the Bligh and Dyer method yielded similar results. Lipid fraction in the microalgae biomass was of 19% and 21% for Guillard f/2 and BG-11 media, respectively. This shows that both media were efficient in keeping lipid amounts nearly constant. The lipid values agree with recent studies (Komolafe et al., 2014) , though, according Xie et al. (2013) the cellular composition of microalgae can change with the growth medium. Fatty acid profile (Table 1) shows a distribution which favors molecules with lower molar masses, such as C16:0 (palmitic acid) and C14:0 (myristic acid), with 45.27% and 30.27% respectively. It is also interesting to note that the microalgae also produce fatty acids with longer chains, such as C20:4 and C24:1.

Table 1: Fatty acid profile of the Desmodesmus sp. *microalga*

Fatty acid	C6:0	C8:0	C12:0	C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:2	C18:3	C20:4	C24:1
Mass fraction (%)	0.18	0.32	0.32	30.27	5.27	45.27	0.64	0.24	0.31	6.21	1.39	3.81	0.19

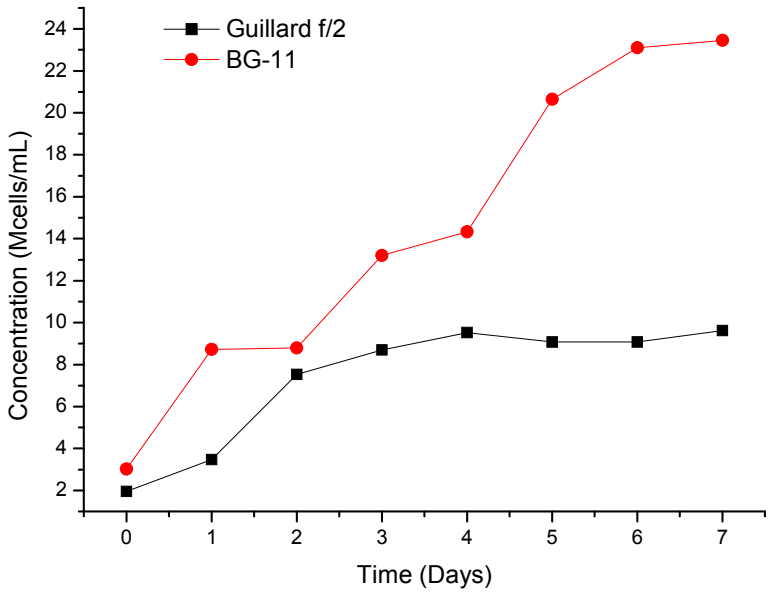

Figure 3. Comparison between cell growth in BG-11 and Guillard f/2 culture media

4. Conclusion

From this work, it can be seen that the BG-11 culture medium is interesting for *Desmodesmus* sp. cultivation, since high growth rates can be achieved. Concentration values of up to 2.44 times higher than in the comparison to the Guilard f /2 medium were obtained, with an average of 23.5 MCells/mL. Besides, reasonable lipid fractions were also leads obtained with this medium (around 21%), which is inside the values reported in the literature. It is also interesting to notice that the fatty acid profile produced by *Desmodesmus* sp. is highly diversified. This also leads to the potential of separating and commercializing selected fatty acids with high market value to improve the economic feasibility of an industrial unit producing both biofuels and bioproducts from this microalgae species.

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