Cultivation of microalgae *Phaeodactylum tricornutum in* mixotrophic medium with photoperiod and addition of glycerol for obtain biolipids

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Abstract. *The development of technologies for producing biodiesel from oilseed plants has been presented as a promising alternative for replacement parts for fossil fuels used currently. But this still is not presented as a viable competitor to diesel due to its low productivity and the competition that exists for the production of food for the population. Thus, one way of solving this problem is to use biolipídios from microalgae oil grown in photobioreactors, they allow the compaction of floor space, better control of the system and obtain high rates of biomass. To achieve higher biomass productivity as well as the amount of lipids per unit dry biomass, it is necessary to optimize the system of cultivation of microalgae in a photo bioreactor, which involves, besides the improvement of equipment and acquisition of microalgae to produce ideal biolipídios, develop the ideal culture medium for the microalgae to be grown. Therefore, this research took the cultivation of the microalga* Phaeodactylum tricornutum *in culture medium Guillard, in a scheme mixotrophy, photoperiod 12/12. The cultivation was carried mixotrophy with glycerol as carbon source at the beginning of cultivation, the following concentrations: 0M, 0.05 M, 0.1 M and 0.15 M, glycerol was chosen because it is a byproduct of biodiesel. The experiments were maintained in a room with temperature around 21 º C in order to provide suitable conditions for the development of microalgae. Farming with a concentration of 0.1 M had a higher rate than the other lipids, reaching an increase of approximately 20% of the total rate of lipids produced by the cells, when compared to growth without glycerol. Indicating that the addition of glycerol at high concentrations may be detrimental to the microalgae during storage of lipids by the cell.*

Keywords: *Biodiesel, glycerol, mixotrophy,* Phaeodactylum tricornutum *and photoperiod.*

1. INTRODUCTION

Biodiesel is a liquid biofuel that can be used in diesel engines without any modification of it, defined chemically as esters of long chain fatty acids derived from renewable biolipídios. It is produced by transesterification reaction of an oil with an alcohol in the presence of a catalyst, to obtain esters and glycerine (Demirbas, A., 2008). As feedstock for biodiesel production can be used multiple sources of fatty acids as animal fats, vegetable oils, processed or not, come from plants such as corn, soybean, canola, palm oil, among others. But the use of these involve the use of fertile land, leading to reduction of areas available for food crops for the population. This problem disappears when using fatty acids from microalgae.

Microalgae are microscopic unicellular organisms, mostly aquatic, with relatively simple nutritional requirements, since they have photosynthetic capacity (Andrade & Costa, 2008). They can be grown in areas unsuitable for agriculture in the traditional ponds and tanks used in aquaculture. But the floor area required to obtain significant production of lipids, can become quite low if the algae are grown in photobioreactors compact.

The cultivation of microalgae in photo bioreactor needs to be optimized to achieve greater productivity of biomass and the amount of lipids per unit dry biomass. This involves, in addition to obtaining ideal for production of microalgae biolipídios and develop in a photo bioreactor that best represents the natural habitat, develop the culture medium in a way that allows the algae to produce a higher rate of lipids.

The literature shows that the depletion of nutrients from the environment can affect the cellular multiplication of microalgae (Lourenço, 2006 and Penteado, 2010) thereby reducing the amount of biomass produced per liter of culture medium. Carbon is the macronutrient used in higher concentrations by microalgae, it is the most important element of all organic substances synthesized by cells, which limits cell multiplication (Lourenço, 2006).

Carbon dioxide – CO_2 is the preferred carbon source of microalgae, it diffuses passively from the medium to the intracellular medium, and used directly in the processes of carbon fixation (Derner, 2006). Therefore, the addition of CO_2 in the culture medium can increase up to seven times the cell multiplication while reducing the availability of carbon may limit microalgal growth (Ishida e*t al,* 2000). In cultivating microalgae *Phaeodactylum tricornutum* under mixotrophic with glycerol, and CO_2 injection, Cerón García *et al.* (2006), got a 100% increase in biomass crops compared with autotrophic

As injecting carbon dioxide into a system of large-scale cultivation increases the cost of the process, it may be replaced in part by other sources of carbon as carbonate salts, bicarbonate, among others.

Also the type of cultivation, autotrophic, heterotrophic, mixotrophic, may influence the accumulation of reserves by the cells. In a growing mixotrophic where the autotrophic and heterotrophic metabolism are operating simultaneously, the algae can absorb as much carbon as inorganic and organic, considerably increasing the production of biomass.

Thus, this study sought to evaluate the results achieved through the cultivation of the microalgae *Phaeodactylum tricornutum* amid Guillard mixotrophic, adding glycerol as additional carbon source at the beginning of cultivation.

2. METHODOLOGY

In order to evaluate the amount of glycerol that the microalga *Phaeodactylum tricornutum* can assimilate in such a way that contributes to the increase of lipid content in cells, the microalga was cultivated in mixotrophy in cultivation medium Guillard, with a salinity of 15 ‰ and glycerol. This was chosen as a source of additional carbon, because it is a byproduct of biodiesel production

The trial was performed to approximate the maximum of the experiment the actual conditions of cultivation and provide appropriate conditions for the development of microalgae. The experiments were maintained in a room with a temperature of approximately 21 °C with a photoperiod of 12/12 (12 hours light and 12 hours with no backlight).

The planning of the experiment, based on different concentrations of glycerol applied at the beginning of cultivation, with the treatments and their respective concentrations of glycerol is presented in Tab. 1. The experiments were performed in duplicate to reduce possible experimental errors.

Table 1. Planning of experiment for the treatments.

Treatments	Glycerol concentration	Type of cultivation
1	0 M	Autotrophic
2	0.05 M	Mixotrophic
3	0.10 M	Mixotrophic
4	0.15 M	Mixotrophic

Dry biomass, grams per liter of culture medium (g / L) was determined by vacuum filtration. And the determination of fat, milligrams per liter the cultivation medium (mg / L), was based on the Bligh & Dyer method involving the use of a monophasic mixture of chloroform, methanol and water. A mixture of chloroform / methanol ($CHCl_3$/CH_3OH) used

this methodology was used in the ratio 2:1 (v: v) because second Soares (2010) this proportion is most suitable for the microalga *Phaeodactylum tricornutum*.

The cell density, number of cells per liter of culture medium (N cel. / L), was obtained from the cell counts for each treatment in a Neubauer chamber, over the 20 days of experiment. Employed in the construction of growth curves (number of cells per volume of cultivation medium versus cultivation hours), required to determine the stationary growth phase, where there is storage of lipids in the cells.

The logistic and exponential equations (Eq. 2 and 4 respectively) were fitted to experimental data by nonlinear regression with the aid of the program TK Solver 5.0, in order to select the best equation to describe the profile of cell growth (Soares, 2010).

Logistic Equation in Differential Form

$$\frac{dN}{dt} = \mu.N.\left(1 - \frac{N_0}{N_{max}}\right) \tag{1}$$

where:
N- cell density (cel/mL);
N_0- initial cell density (cel/mL);
N_{max}- maximum cell density (cel/mL);
μ- specific growth speed (hours^{-});
t- cultivation time (hours);

Logistic Equation in Integrated Form

$$N = \frac{N_{max}}{\left[1 + \left(\frac{N_{max}}{N_0} - 1\right).e^{(-\mu.t)}\right]} \tag{2}$$

Exponential Equation in Differential Form

$$\frac{dN}{dt} = \mu.N \tag{3}$$

Exponential Equation in Integrated Form

$$N = N_0.e^{(\mu.t)} \tag{4}$$

3. RESULTS AND DISCUSSIONS

The kinetic analysis is important to identify the phases of growth, the cultivation takes time to achieve maximum cell density and the choice of the ideal equation for routine evaluation in the experiments. And in the case of experiments aimed at obtaining lipids, the identification of the period in which to store them.

Cell growth in each treatment was evaluated from the determination of cell density versus time. The Tab. 2 presents the parameters obtained by nonlinear regression from data of cell density of each treatment applied to the experiment.

Table 2. Parameters obtained by nonlinear regression.

Parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Logistic Adjust				
μ (hours^{-1})	0.03879	0.03489	0.03391	0.03624
N_0 (x10^4 cel.mL^{-1})	29.17	63.24	39.64	45.36
N_{max} (x10^4 cel.mL^{-1})	1715.39	2157.44	2009.69	1901.59
Time (hours)	0 – 432	0 – 432	0 – 432	0 – 432
Exponential Adjust				
μ (hours^{-1})	0.04045	0.03406	0.03896	0.03750
N_0	17.26	40.00	19.01	26.32
Time (hours)	0 – 96	0 – 96	0 – 96	0 – 96

The kinetics of growth of the microalga *Phaeodactylum tricornutum* for each treatment, adjusted from the logistic and exponential equations, as a function of time is given in Fig 1, 2, 3 and 4.

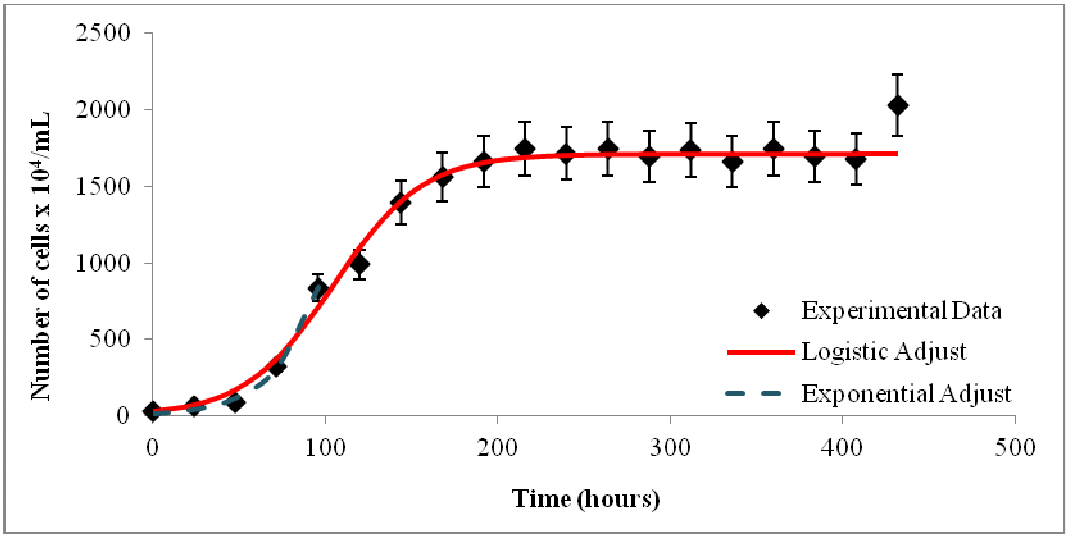

Figure 1. Growth curve of the treatment 1

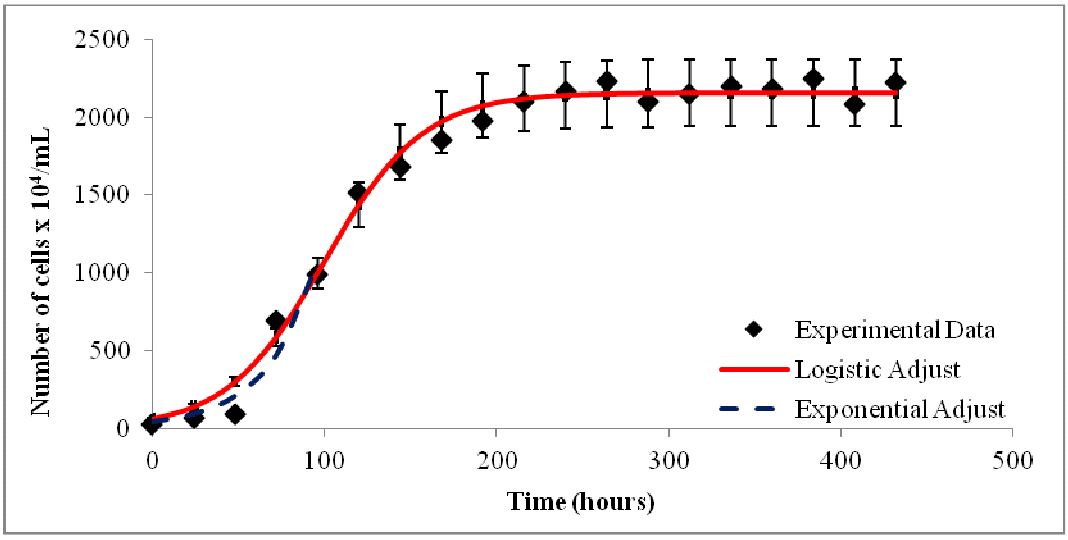

Figure 2. Growth of curve the treatment 2

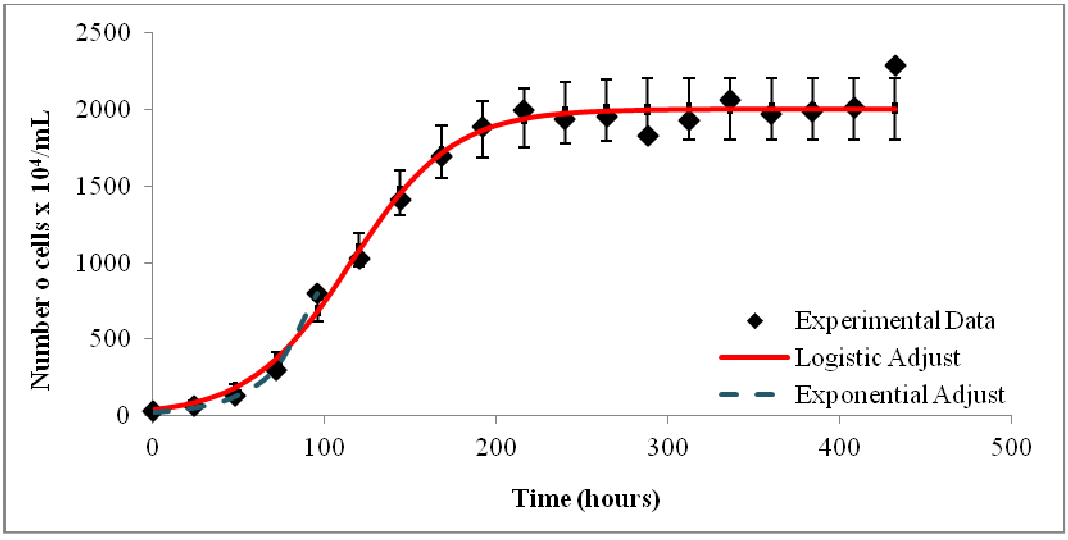

Figure 3. Growth curve of the treatment 3

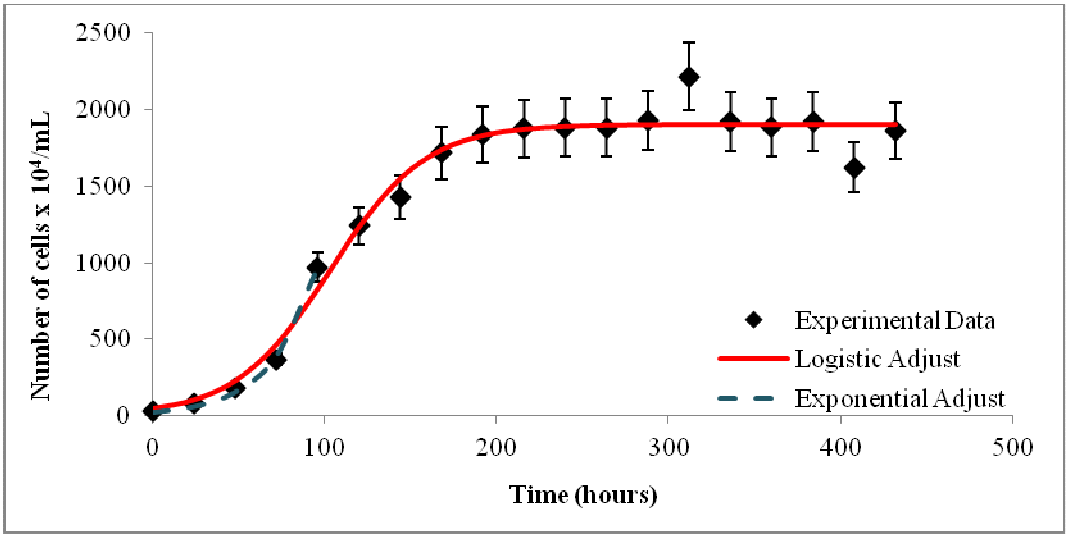

Figure 4. Growth curve of the treatment 4

The exponential equation described the growth only until the fourth day of cultivation in all treatments. However, the logistic equation fit the data well until the last day of culture evaluated. Demonstrating that cell replication begins to decrease from 96 hours with 216 hours and wax, probably due to the lack of some nutrient necessary for growth.

From the 216 hours the cultures enter the stationary phase at this stage the cell multiplication is practically nil and the cells begin to store reserves for periods of nutritional deprivation. This is where there is storage of lipids in the cells.

By analyzing at Tab. 3, which shows the values of biomass obtained in each treatment (shown highlighted the best result for dry biomass), and Tab. 4, which shows the average productivity, it is possible to see a considerable increase in the amount dry biomass between the 12nd and 20th days for all treatments.

Table 3. Dry biomass (g/L).

Cultivation time	Treatment 1	Treatment 2	Treatment 3	Treatment 4
12nd day	0.517 ±0.0127	0.626 ±0.0100	0.585 ±0.0300	0.574 ±0.0100
14th day	0.563 ±0.0100	0.767 ±0.0200	0.767 ±0.0200	0.730 ±0.0300
16th day	0.690 ±0.0300	0.815 ±0.0500	0.878 ±0.0200	0.817 ±0.0200
18th day	0.725 ±0.0300	0.919 ±0.0400	0.868 ±0.0200	0.846 ±0.0200
20th day	0.909 ±0.0300	1.013 ±0.0500	**1.030 ±0.0500**	1.020 ±0.0200

Table 4. Productivity of dry biomass (g/L).

Cultivation time	Treatment 1	Treatment 2	Treatment 3	Treatment 4
12nd day	0.043 ±0.0011	0.052 ±0.0010	0.049 ±0.0023	0.048 ±0.0012
14th day	0.040 ±0.0008	0.055 ±0.0014	0.055 ±0.0015	0.052 ±0.0019
16th day	0.043 ±0.0017	0.051 ±0.0029	0.055 ±0.0015	0.051 ±0.0012
18th day	0.040 ±0.0014	0.051 ±0.0022	0.048 ±0.0011	0.047 ±0.0010
20th day	0.045 ±0.0016	0.051 ±0.0023	0.051 ±0.0024	0.051 ±0.0010

As the chart shows in Fig. 5 for all treatments biomass almost doubled over the last eight days of culture where, according to the growth curve, all treatments were in stationary phase. Thus, as there is no cell division, it appears that assimilation is occurring nutrient which causes an accumulation of cellular reserves, including increased lipid content.

The graph in Fig. 6 allows us to observe the increase in the biomass crops mixotrophy compared to autotrophic. Even for treatment 4, which, for the three treatment suffered a decrease, the increase of dry biomass was considerable. The yield remained approximately constant over the period.

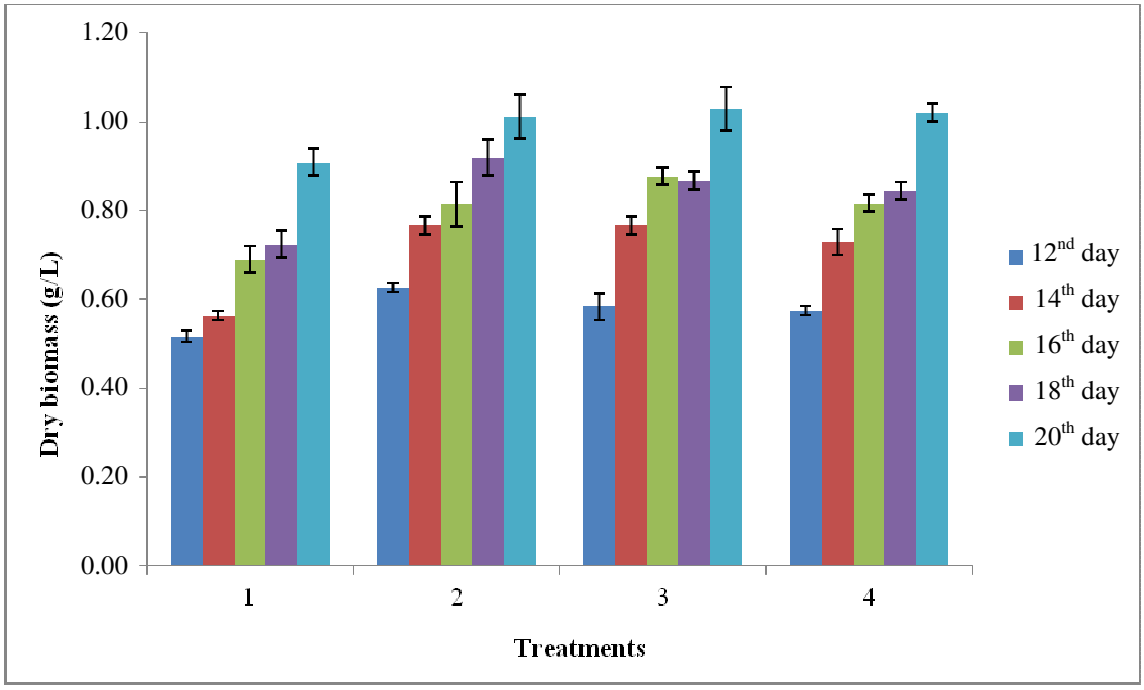

Figure 5. Dry biomass between 12nd and 20th days

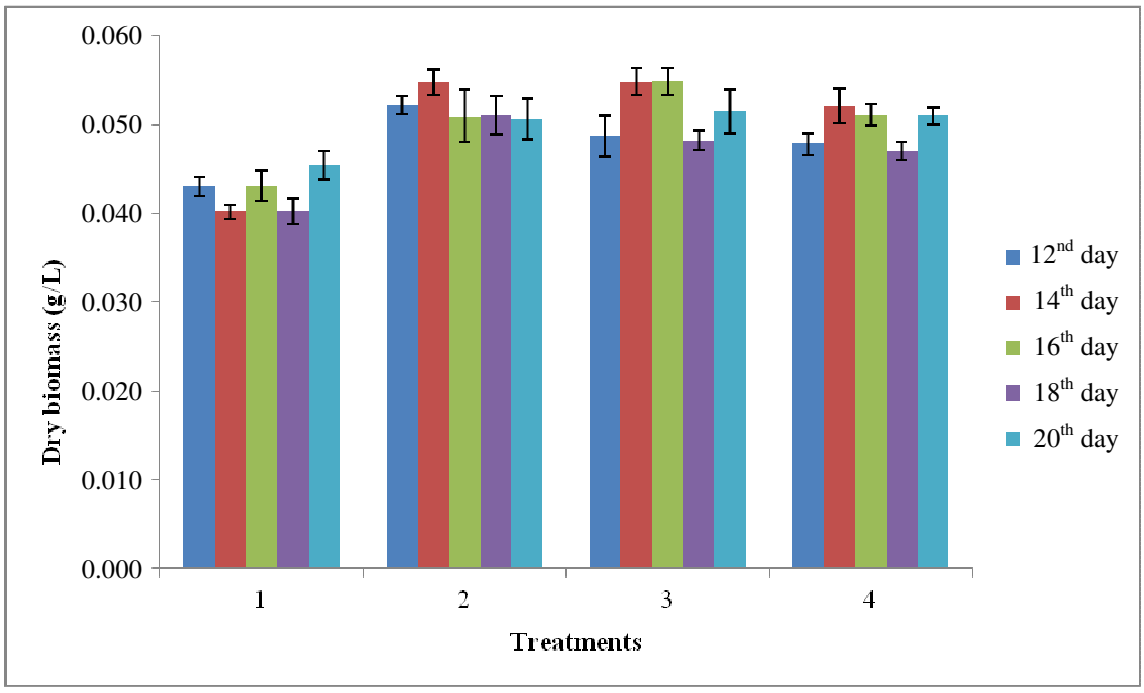

Figure 6. Productivity of dry biomass between 12nd and 20th days

On the 20th day of cultivation the greatest increase in biomass was observed in treatment 3, a concentration of 0.1 M glycerol, with 1.03 g / L. However, compared to control (treatment 1) the increase was only 12%, indicating that the photoperiod was unfavorable for the period of carbon assimilation.

You can see a smaller increase of biomass in experiments with photoperiod 12/12 compared with experiments without photoperiod (light exposure for 24 hours) and keeping the other variables of cultivation (Radmann, 2009). This difference was identified in biomass, between the two experiments, demonstrates the importance of light as a variable of cultivation, because exposure to light during a 12 hours more a gain of 27% in biomass

According to the results obtained by Cerón García *et al*. (2006), to cultivate microalgae *Phaeodactylum tricornutum* under mixotrophy, adding glycerol in concentrations of 0M; 0.005 M; 0.01 M; 0.05 M and 0.1 M, without injecting CO2 and photoperiod, the best results were also found to concentration of 0.1 M. The cultivation of biomass reached a rate equal to 2.99 g / L, 100% more than in the autotrophic cultivation, possibly due to excellent metabolic assimilation

of this species of microalgae in relation to exposure to light and CO_2 injection, which allows an increase considerable dry biomass.

The assessment of fat content, produced by the microalga *Phaeodactylum tricornutum* in each of the treatments, and compared with the production of biomass has the important function of showing that the added glycerol is increased production of fat cells or if there is expenditure of nutrients.

The variation of lipid content for the four treatments in the experiment appears in Tab. 6, posted the best result appears. In Tab. 7 are the values of lipid productivity, by comparing the cultures mixotrophy with autotrophic observed a significant increase of lipids especially in the last two days of cultivation (19th and 20th).

Table 5. Content of lipids (mg/L).

Cultivation time	Treatment 1	Treatment 2	Treatment 3	Treatment 4
12nd day	58.89 ±2.070	102.66 ±3.760	101.79 ±12.870	78.08 ±9.190
14th day	76.55 ±3.380	131.94 ±3.070	136.60 ±12.280	110.91 ±10.220
16th day	104.94 ±5.520	146.72 ±4.890	156.30 ±1.760	117.71 ±4.900
18th day	136.26 ±7.250	194.81 ±7.350	208.27 ±10.410	167.49 ±11.840
20th day	220.07 ±12.730	279.51 ±16.200	**288.34 ±12.360**	253.06 ±4.080

Table 6. Productivity of lipids (mg/L).

Cultivation time	Treatment 1	Treatment 2	Treatment 3	Treatment 4
12nd day	4.91 ±0.016	8.56 ±0.250	8.48 ±1.000	6.51 ±0.750
14th day	5.47 ±0.214	9.42 ±0.214	9.76 ±0.857	7.92 ±0.714
16th day	6.56 ±0.312	9.17 ±0.250	9.77 ±0.062	7.36 ±0.250
18th day	7.57 ±0.388	10.82 ±0.388	11.57 ±0.555	9.31 ±0.657
20th day	11.00 ±0.600	13.98 ±0.800	14.42 ±0.600	12.65 ±0.200

When comparing the production of lipids for the different treatments, as shown in Fig 7, 8 and 9, it was found that the microalgae grown at a concentration of 0.15M glycerol lipids produced a lower rate when compared with the cultivation of concentration 0.1M, allowing to confirm that the high concentration in addition to not increase the production of lipids, resulted in a decrease in oil production, so with, generates waste of nutrients.

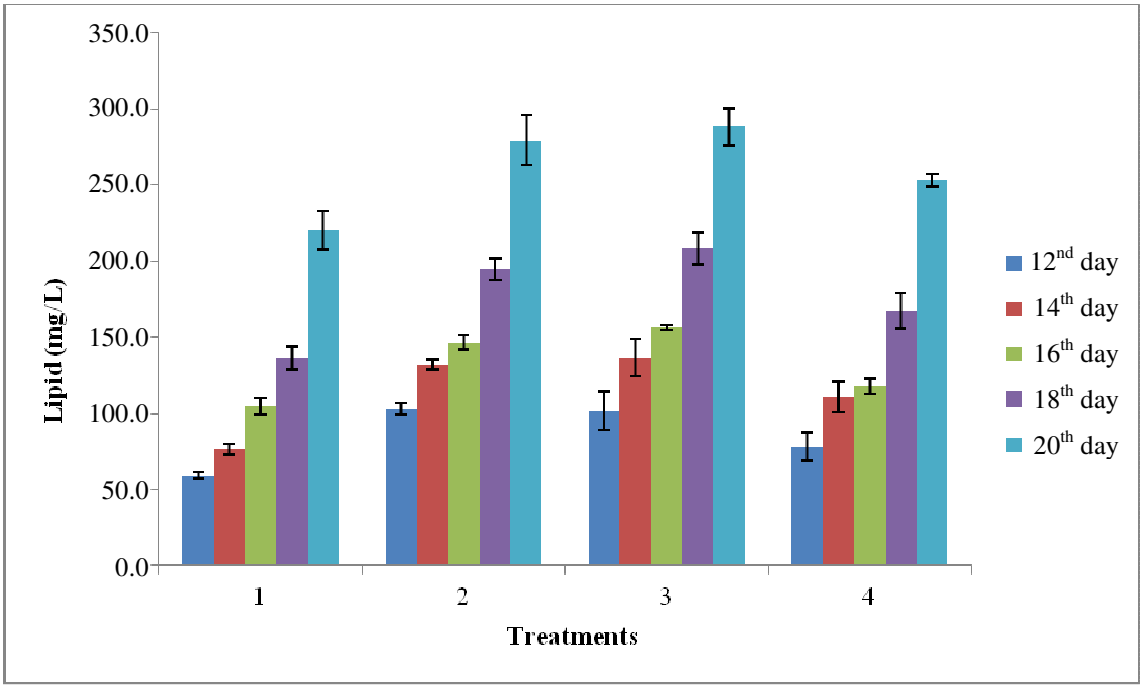

Figure 7. Content of lipids between 12nd and 20th days

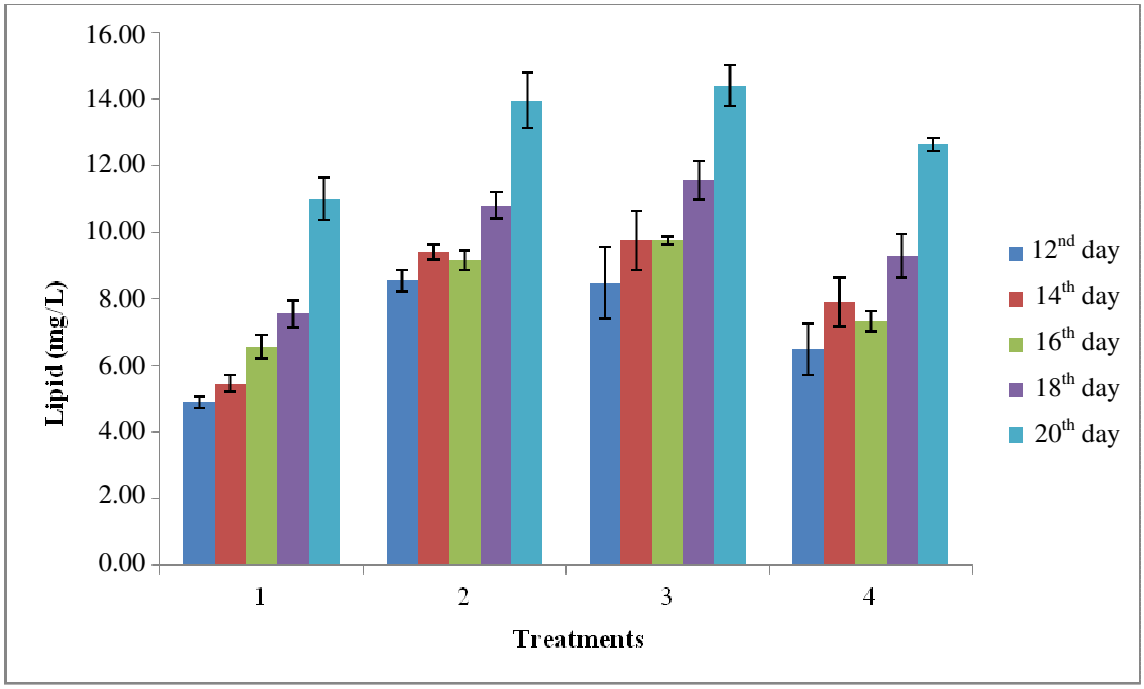

Figure 8. Productivity of lipids between 12nd and 20th days

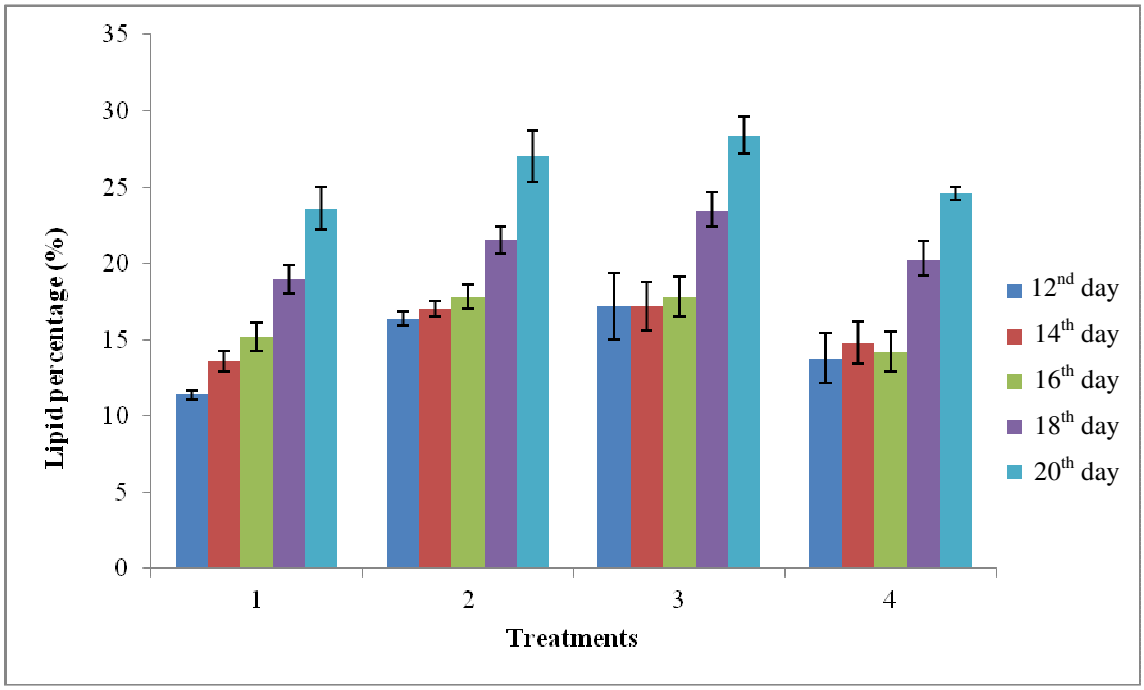

Figure 9. Percentage of lipids between 12nd and 20th days

Treatment 3, the concentration of 0.1M glycerol, showed the best results regarding the rate of lipids, in milligrams per liter the cultivation medium, despite that the best rate of biomass found was in treatment 4, a concentration of 0, 15M glycerol. Therefore, it was found that disbursement was actually the source of carbon in the treatments with higher concentrations (greater than 0.1M) due to the interest in these experiments is the fat cells

4. CONCLUSION

Through this research it was possible to assess the amount of glycerol that the microalga *Phaeodactylum tricornutum* can assimilate in such a way that contributes to the increase of lipid content produced by the cells.

The quest for increasing the production of fat cells through the addition of glycerol in the culture of microalgae, has shown very significant results when comparing the treatments without addition of glycerol with glycerol treatments obtained an increase of approximately 20% in the total rate of lipids produced by the cells.

I also found that the amount of lipid stored by cells (milligrams per grams of dry biomass) is not greater at higher concentration of glycerol, indicating that addition of glycerol at high concentrations may be detrimental to the microalgae during the stationary phase.

5. ACKNOWLEDGEMENTS

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