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EXPERIMENTAL ANALYZES FOR GROWTH IN BIOMASS PRODUCTION OF THE MICROALGAE *TETRADESMUS OBLIQUUS* IN HETEROTROPHIC MEDIUM

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Abstract. *Microalgae have aroused great interest in the scientific community due to their potential as a renewable source of energy and as a raw material for the production of food, pharmaceuticals and cosmetics. The production of microalgae is a promising technique, as these organisms have high productivity, so that large amounts of biomass can be generated in relatively short periods of time when compared to other sources of biomass. Among the main applications of microalgae, its use to generate biofuels is one of the most desired, since they can be grown in desert areas or in non-potable waters, minimizing competition with food crops. In addition, microalgae production can be carried out in a sustainable way, with low environmental impact, as algae can use carbon dioxide and other pollutants as a source of nutrients. Microalgae can be autotrophic, capable of converting solar energy into biomass very efficiently, reaching conversion rates of up to 50%. They can also be grown heterotrophically, making these organisms extremely versatile for different experimental analyses, as these techniques can be combined to further optimize the productivity of their biomass. However, much remains to be explored in terms of technology and scalability to maximize the potential of these organisms. Therefore, the objective of this present work was to use experimental analytical tools to cultivate microalgae of the Tetradesmus obliquus species, initially in a heterotrophic medium, aiming to reduce the initial cultivation time and, subsequently, to finalize the production of microalgae in airlifts-type bioreactors in an autotrophic medium, decreasing the proportion of possible contaminations commonly related to scale-up. The results show that the combined use of heterotrophic media followed by autotrophic media significantly decrease the total microalgae cultivation time when compared to the exclusive use of autotrophic media, used in most cases.*

Keywords: *microalgae, biofuels, biomass, photobioreactor, renewable energy.*

1. Introduction

Microalgae are characterized as unicellular or colonial photosynthetic microorganisms. have high ecological importance as a primary production, since they fix carbon dioxide (CO2) and use nitrate and sulfur in their protein synthesis. Its industrial and commercial applications occur mainly in animal and human food, used as dyes and antioxidants (LOURENÇO, 2006). There is also applicability in other areas, such as the treatment of effluents and domestic sewage and even the production of biofuels, such as aviation kerosene and green biodiesel, from lipid extraction using hot organic solvents (FRANCO, 2013). Therefore, the importance of extracting the biomass produced from these microalgae is visible (LOURENÇO, 2006).

Green biofuels can be produced from the extraction of triacylglycerides from biomass, which reacts with an organic solvent such as ethanol (FRANCO, 2013). One of the ways to enhance energy metabolism to increase lipid production is the cultivation of microalgae in a heterotrophic medium, enriched with glucose. In order to increase the productivity of biomass and lipid extraction from the microalgae *Tetradesmus obliquus*, experimental analyzes were carried out in heterotrophic medium with the addition of glucose and yeast.

1.1 **Microalgae**

Microalgae are diverse groups of unicellular or colonial microorganisms of great importance to the ecosystem, being the greatest photosynthesis performers on the planet, along with macroalgae. These microorganisms have different types of growth, such as heterotrophic, autotrophic, among others (CUELLAR-BERMUDES, 2015).

Its most common growth is autotrophic, where microalgae use nutrients from the environment and sunlight to obtain energy and carry out photosynthesis. They use carbon dioxide, nitrogen and sulfur for their cell growth and can therefore reduce concentrations of air pollutants (MARTINEZ, 2018).

In heterotrophic medium, other carbon sources besides CO2 can be added, such as acetate and glucose, in order to increase cell concentration and biomass production (FRANCO, 2013). Along with glucose, you can also add yeasts that with their breathing release CO2, which will be consumed by the microalgae, in addition to also being a source of nutrients such as vitamins and hormones (CALDEIRA, 2011).

Emphasizing that its growth in environmental conditions can undergo changes such as the incidence of sunlight at different times of the year, temperature and pH. In an artificial culture, these factors that alter its growth in cell number can be controlled (Corrêa, 2015).

The cellular growth of microalgae can be influenced by the factors mentioned above along with light intensity, including photoperiod. In addition to changing the number of cells in the medium, the application of photoperiod combined with other factors that alter the metabolism of microalgae can result in increased production of lipids, carbohydrates, pigments and proteins (BRASIL et al., 2014).

It is known that the total lipid of microalgae is composed of neutral lipids, NLs, which are mainly composed of triacylglycerols, important compounds for the production of biodiesel based on microalgae, glycolipids, GLs, and phospholipids, PLs. It is known that the synthesis of NLs depends on factors such as nutrient concentration, light intensity and photoperiod (Jacob-Lopes et al., 2009).

1.2 **Culture Mediums**

Microalgae are autotrophic under natural environmental conditions, their cultivation in synthetic medium can be carried out with the same nutrients available in nature, or in heterotrophic medium, with the addition of other organic sources of carbon, such as sugar and mineral salts.

The nutrients used by microalgae are, in general, nitrogen, carbon, phosphorus and light energy. For this work, the microalgae *Tetradesmus obliquus* was cultivated in a synthetic medium, the CHU medium (CHU, 1942), with the addition of yeast and glucose in different volumes. On a laboratory scale, the culture was incubated in a room with artificial light and controlled temperature and aeration. For comparative purposes, a culture was also applied using only the synthetic medium mentioned above without the addition of another carbon source other than photosynthesis carried out by microalgae.

In other culture media, it is possible to use pig manure, as it contains a high level of available nitrogen and phosphorus (FURLAN, 2020).

Figure 1. Erlenmeyer flasks with microalgae inoculum, cultivation room. Source: the author, 2023.

1.3 Culture systems

On a large scale, microalgae can be cultivated in open and closed systems. In open systems, a tank is used to form an artificial lake of low depth, 15 cm to 50 cm, and these measures help in the distribution of sunlight over a larger area of the lake, while agitation is carried out by a system of shovels. Closed systems, known as photobioreactors, are formed by a solar collector with a transparent surface, for greater absorption of solar incidence, a column for gas exchange, aeration system, circulation pumps and a system for input and collection of culture medium. Photobioreactors are divided into several models, such as vertical, horizontal or inclined tubular (RAMPI, 2021).

Figure 2. Open cultivation system. Source: the author, 2023.

When compared to the open system, photobioreactors generate a more stable cultivation through the uniform distribution of sunlight, control of contaminants, such as protozoa, and evaporation control, promoting greater biomass extraction (CORREA, 2015).

Figure 3. Closed cultivation system. Source: the author, 2022.

1.4 **Commercial uses**

Biomass extracted from microalgae can have several applications in industrial, commercial, pharmaceutical, food and agricultural environments. Its extraction can be redirected to food dyes, antioxidants, cosmetics production, wastewater treatment, biostimulants and others (LOURENÇO, 2006). In addition, the extraction of lipid compounds from microalgae can also be applied for the production of green biofuels, such as aviation kerosene, this application has gained great importance for new sources of renewable energy in order to reduce the pollution caused by other fuels such as ethanol, in addition to the cultivation of microalgae, contribute to the removal of polluting gases such as CO2. (FRANCO, 2013).

2. MATERIALS AND METHODS

The species of seaweed cultivated at the NPDEAS (Núcleo de Pesquisa e Desenvolvimento de Energia Autossustentável) is *Tetradesmus obliquus*. First, the cultivation was carried out on a laboratory scale, cultivated in Erlenmeyer in a culture room with artificial light, aeration and a temperature of 22°C. For microalgae to grow in artificial medium, Chu medium was used, due to the presence of macro and micronutrients such as potassium nitrate and potassium iodide, for example, different volumes of glucose and yeast were also incorporated for growth analysis in heterotrophic medium.

Laboratory cultivations were carried out in 1000 milliliter Erlenmeyers in three groups in triplicates with different concentrations of glucose and addition of yeast. The inoculum volume for the three groups of Tetradesmus sp. was 50ml, together with 51,2 ml of CHU.

For the volume of glucose in the cultivation of group A, the concentration was already added in its total volume with 10g/L, in group B the initial concentration was 6g/L with addition of 1,33g/L daily, in group C, the initial concentration was 2g/L with the addition of 2,66g/L daily. After 4 days of inoculation, the biomass concentration of groups A, B and C was measured using the vacuum process with a membrane filter and then drying and weighing.

As a negative control, a culture was applied only with the synthetic Chu medium, 51.2 ml, also in a 1000 milliliter Erlenmeyer flask, with the addition of 50 ml inoculum of the same microalgae. Therefore, a comparison between both growths will be carried out, with the aim of demonstrating that more efficient growth occurs when other carbon sources are added, such as glucose.

2.1 **Cell counting, weighing of membranes with dry biomass and colorimetric enzymatic analysis**

The daily cell count, one of the ways to monitor the cell growth of microalgae, is performed in a Neubauer chamber and counted under an optical microscope with a 40x magnification. The 4 outer quadrants and the middle one of the two parts of the chamber were counted, and then an average was taken between them.

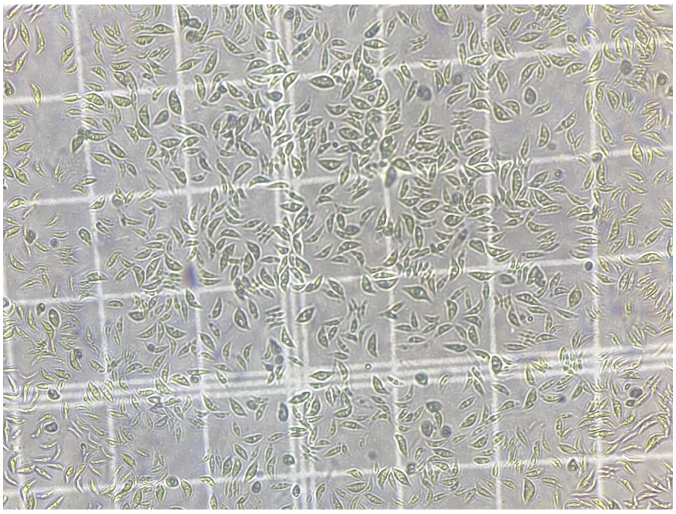

Figure 4. Cells under optical microscope 40x magnification.

Monitoring of membranes with dry biomass is carried out through vacuum filtration of a sample of 15 ml from cultures with glucose and 2 ml for cultures with Chu medium only, followed by drying at 80°C in an oven, finally, weighing the material with dry biomass. This weighing is carried out to obtain the concentration of biomass present in these crops and was carried out in triplicates.

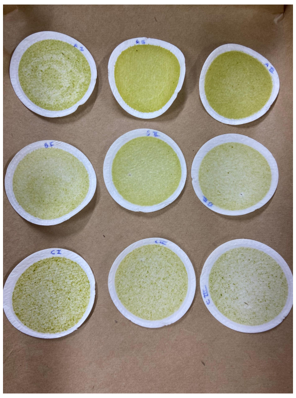

Figure 5 - Membrane with biomass.

Another method to monitor the sugar consumption of microalgae is by enzymatic colorimetric analysis, carried out in a spectrophotometer. This experiment consists of separating the three samples from group A, B and C in marked test tubes, adding 1ml of the enzyme glucose oxidase, then placed in an oven at 40°C for 10 minutes and then the dosage is done to analyze the consumption of glucose through the spectrophotometer. What is expected is that the glucose concentration decreases as the microalgae increase their cell number.

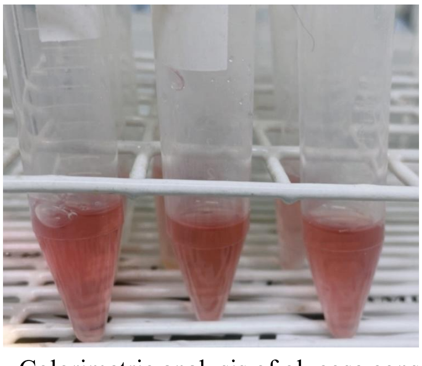

Figure 6 - Colorimetric analysis of glucose consumption.

2.2 Information about cultivation

The cultivation was carried out in the laboratory NPDEAS (Self-Sustainable Energy Research and Development Center), with microalgae native to Curitiba, *Tetradesmus obliquus*. The medium in which these microalgae were inoculated was CHU with the following proportions of inorganic salts, micro and macronutrients (Table 1), in a 1000ml Erlenmeyer flask. For the analysis experiment for the increase of microalgae biomass in heterotrophic medium, the cultures were divided into three groups with triplicates in each one, group A (I, II and III), group B (I, II and III) and group C (I, II and III) totaling 9 Erlenmeyer flasks, with different glucose concentrations in each group (Table 2). In group A, sugar was added only once, 10g/L, at the beginning of the experiment; in group B, the initial concentration was 6g/L with the addition of 1,33g/L daily, in group C, the initial concentration was of 2g/L with the addition of 2,66/L daily. The cultivation was maintained for 5 days, after this period of time, the biomass was extracted.

Components	Final concentration g L^{-1}
Sodium Nitrate	0,25
Calcium chloride dihydrate	0,025
Magnesium sulfate heptahydrate	0,075
Dibasic potassium phosphate	0,075
Potassium phosphate monobasic	0,175
Sodium chloride	0,025
EDTA	0,05
Sodium hydroxide	0,031
Ferrous sulfate heptahydrate	0,005
Boric acid	0,01142
Zinc sulfate heptahydrate	$8,82 \times 10^{-5}$
Manganese chloride tetrahydrate	$1,44 \times 10^{-5}$
Sodium molybdate dihydrate	$7,1 \times 10^{-6}$
Copper sulfate pentahydrate	$1,57 \times 10^{-5}$
Cobalt nitrate hexahydrate	$4,9 \times 10^{-6}$

Table 1 - CHU medium.

Group	Initial glucose g/L	Addition of daily glucose g/L
A	10 g/L	0 g/L
B	6 g/L	1,33 g/L
B	2 g/L	2,66 g/L

Table 2 - Glucose concentration distribution.

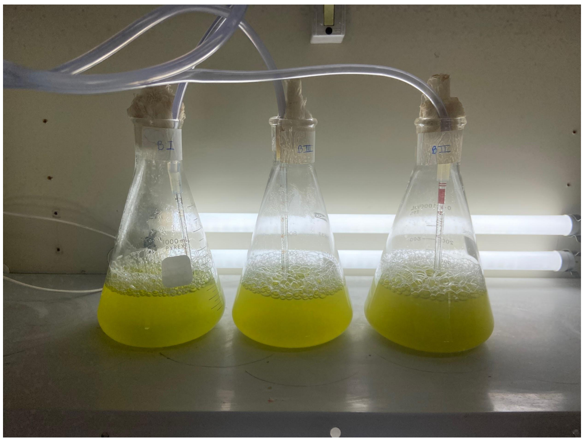

Figure 7 - Cultures in Erlenmeyer.

2.3 Culture growth evaluation

As mentioned previously, samples from these 9 cultures were collected over 5 days to obtain cell counts in a Neubauer chamber, with an optical microscope at 40x magnification. Cell counts were also performed for negative control cultivation without supplementation, samples were collected for 12 days to monitor the evolution of the cultivation.

The measurement for the determination of dry biomass was performed on the last day of cultivation, with a membrane filter through vacuum filtration, which were subsequently left in ovens at 40°C and weighed on a Shimadzu scale with a precision of 0.0001g.

To determine the glucose concentration for crops supplemented with these, an enzymatic analysis was carried out with the enzyme glucose oxidase, to establish the daily consumption of this monosaccharide by microalgae, these analyzes were carried out using a spectrophotometer.

2.4 Extraction of the biomass

After 5 days, the 9 cultures in the Erlenmeyer flasks were removed from the culture room, placed in open containers, each with its own group, and the flocculation process for biomass extraction began. The flocculation process consists of adding the TANFLOC organic polymer (1g/L), stirring until the organic compound dissolves in the culture and waiting for the product to divide into two parts, the precipitate, the biomass, and the supernatant, the liquid that will be discarded. After this process, it was necessary to filter the supernatant biomass in a filter fabric, the biomass in this fabric is then sent to an oven at 80°C, where it dries. After drying, the biomass is ground to a powder.

2.5 Biomass applications

With the extracted biomass, it can be redirected to various industrial areas, such as in the human food industry as antioxidants, anti-inflammatories, or as a supplement in feed for dogs and other animals, in agriculture as biostimulants for vegetables such as lettuce. As the medium cultivated in this experiment was the heterotrophic one with the addition of glucose, there is an increase in the production of lipids by the microalgae, and it can also be directed to the production of biokerosene from the lipid ingestion, the latter being of great importance today, as there is a need to find less aggressive fuels for the environment.

3. RESULTS AND DISCUSSION

After cell growth analyses, colorimetric diagnosis of the glucose concentration in the culture medium together with obtaining the final biomass concentration, results were developed, represented in the following graphs, which will clarify the importance of heterotrophic cultures in increasing cell production, final biomass concentration per g/l, in addition to explaining the possible and expected lipid increase in cells. Furthermore, in a study conducted by Liang, Sarkany and Cui (2009), where they investigated the cultivation of Chlorella vulgaris under mixotrophic conditions, it was observed that the addition of different concentrations of glucose to the cultivation medium had significant impacts on the growth of the microalgae. Specifically, they found that the inclusion of 1% and 2% glucose resulted in superior growth performance when compared to the higher concentrations of 5% and 10%. These results suggest that the optimal concentration of 1% glucose is the most suitable for achieving high productivity of biomass and lipids by microalgae, which has important implications for effective cultivation and obtaining products derived from microalgae with economic potential. This research contributes to understanding the importance of optimizing cultivation conditions in the production of microalgae with applications in several sectors, including the biofuels and food products industry. Thus, the cell growth analyzes carried out in this study aim to corroborate what has been proposed in previous research involving different species of microalgae. These researches indicate that the use of mixotrophic media with the addition of glucose can significantly increase both the productivity of microalgae growth and the concentration of biomass. These results have substantial relevance for studies involving Tetradesmus obliquus, highlighting the potential impact of this approach on research with this specific species.

Figure 8 represents cell growth as a function of time in group A, which contained triplicates of the same culture, which was initially inoculated with 10g/l of glucose. It is noticed that with the passage of time there is an increase in the cell number, as the microalgae consume macro and micronutrients from the CHU medium and glucose. On the first day of inoculation the cell number was at 1572 and on its last day of inoculation the cell concentration was at 5483.

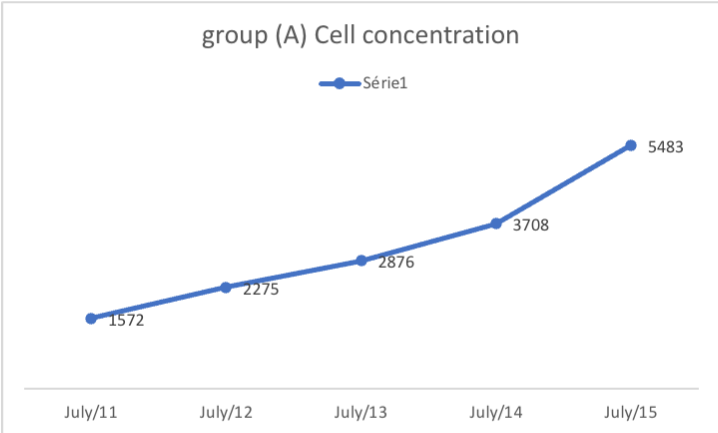

Figure 8. Group A - Cell density of *T. obliquus* cultured in CHU medium with added glucose.

Figure 9 represents cell growth as a function of time in group B, which contained a triplicate of the same culture, which was initially inoculated with 6g/l of glucose and supplemented with 1,33g/l for 3 days (July 12th, 13th and 14th). It is noticed that with the passage of time there is an increase in the cell number, as the microalgae consume macro and micronutrients from the CHU medium and glucose. On the first day of inoculation, the number of cells was at 1572 and on its last day of inoculation the cell concentration was at 4642.

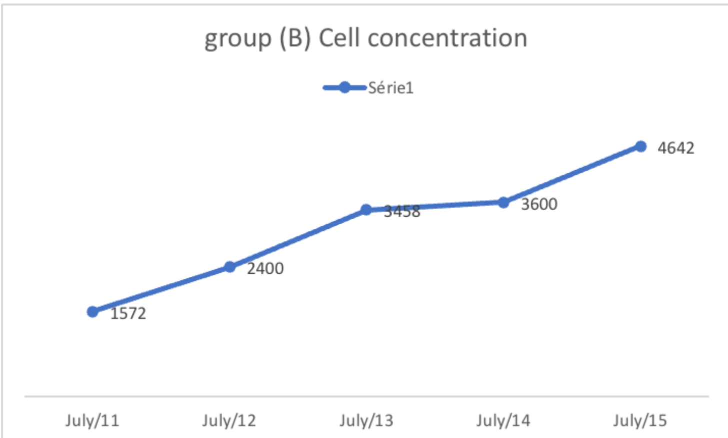

Figure 9. Group B - Cell density of *T. obliquus* cultured in CHU medium with added glucose.

Figure 10 represents cell growth as a function of time in group C, which contained a triplicate of the same culture, which was initially inoculated with 2g/l of glucose and supplemented with 2,66g/l for 3 days (July 12th, 13th and 14th). It is noticed that with the passage of time there is an increase in the cell number, as the microalgae consume macro and micronutrients from the CHU medium and glucose. On the first day of inoculation the cell number was at 1572 and on its last day of inoculation the cell concentration was at 3208.

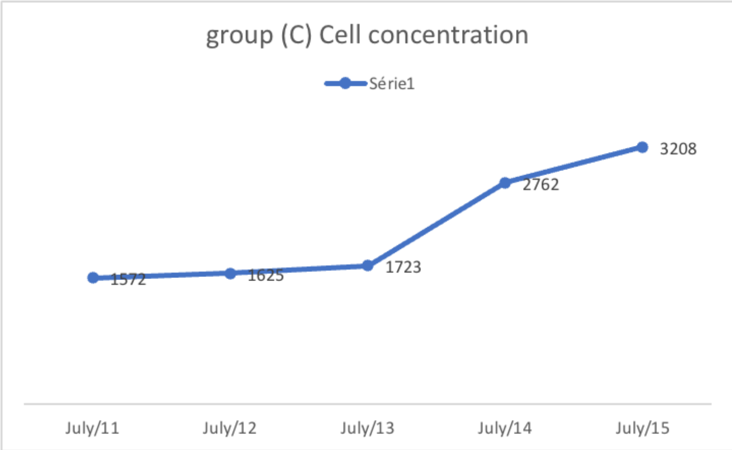

Figure 10. Group C - Cell density of *T. obliquus* cultured in CHU medium with added glucose.

According to the three graphs of the three groups presented above, it can be concluded that the best result in terms of cell growth was given by group A, which had its total glucose concentration initially placed in the culture (10g/l). The result of a lower cell number was for group C, which had its initial glucose concentration lower compared to the other two groups (2g/l), and higher supplementation (2.66g/l daily) compared to group B (1, 33g/l daily).

Figure 11 represents the colorimetric analysis of the glucose concentration of group A. Triplicate A was cultivated after adding the total initial glucose concentration, 10g/l, and according to the diagnosis represented by the graph, it shows that over the days Tetradesmus obliquus consumed the monosaccharide as the cell concentration increased. On July 12, the culture had a glucose concentration of 5,502 g/l, when the number of cells was 2275, and on July 15, the amount of dextrose in the culture medium was 1,171 g/l while the cells were 5483.

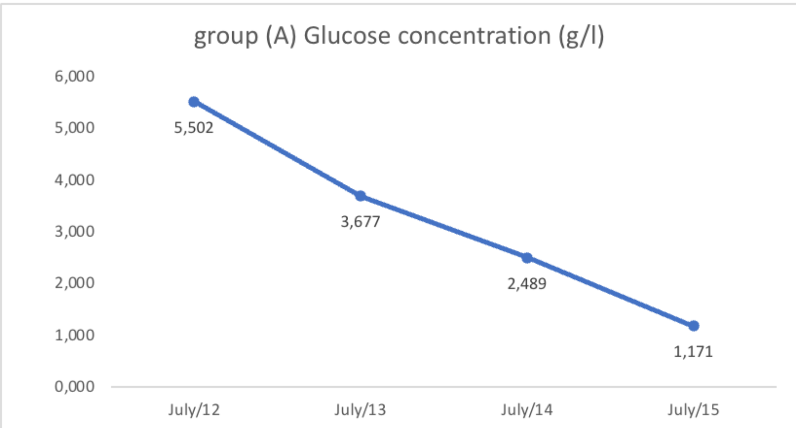

Figure 11. Group A - Glucose concentration (g/l) over time.

Figure 12 represents the colorimetric analysis of the glucose concentration of group B. Triplicate B was cultivated with an initial glucose concentration of 6g/l and 1.33g/l was added daily as a supplement. According to the diagnosis represented by the graph, it shows that initially T. obliquus consumed the glucose, in the second to the third day of cultivation the microalgae increased by more than 1000 (July 12th - July 13th), as the glucose was consumed. After this period, on the third day of supplementation, the microalgae accumulated glucose, possibly changing from heterotrophic to autotrophic cultivation and continuing its cell growth, and probably accumulating lipid, which is extremely important for biodiesel production.

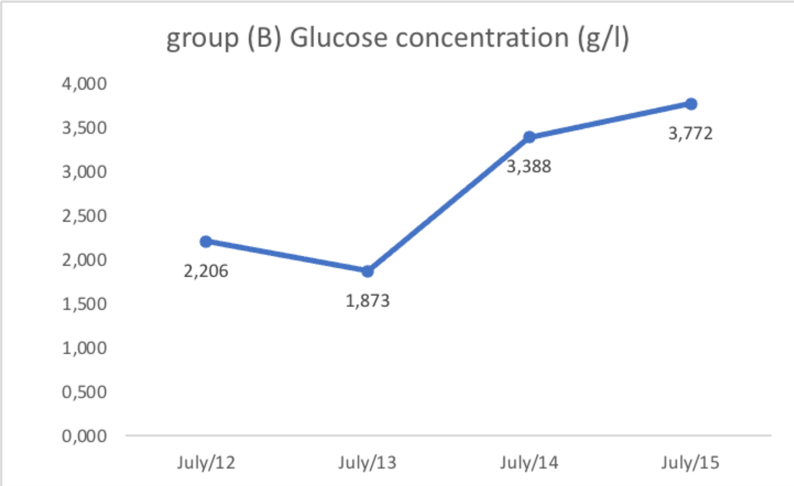

Figure 12. Group B - Glucose concentration (g/l) over time.

Figure 13 represents the colorimetric analysis of the glucose concentration of group C. Triplicate C was cultivated adding a glucose concentration of 2g/l, with daily supplementation of 2,66g/l. According to the reading of the graph, it shows that in the first days of cultivation, the microalgae consumed the glucose that was made available, but its cell growth was slow compared to the other groups. From the third day of supplementation, triplicate C began to accumulate glucose in the medium, possibly the cells moved from the heterotrophic medium to the autotrophic medium, and probably the cells began to accumulate lipids.

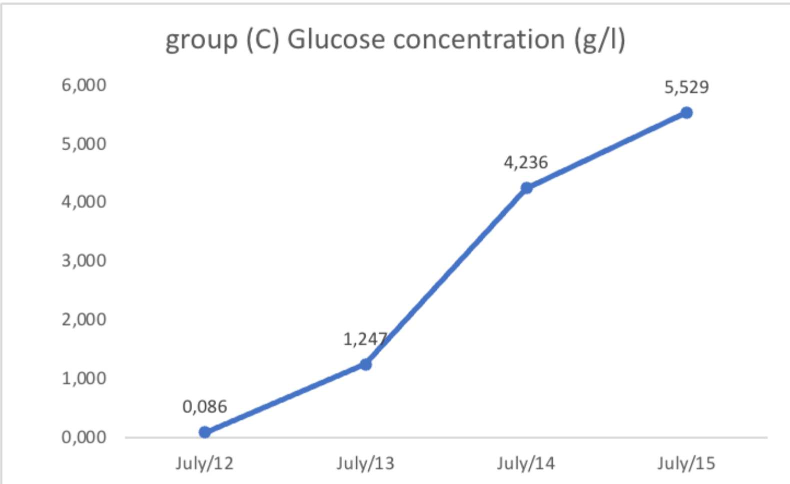

Figure 13. Group C - Glucose concentration (g/l) over time.

And finally, after the enzymatic colorimetric analysis to measure the glucose concentration, the biomass concentration per g/l was verified. Reading Figure 14 represents the concentration of the three triplicates, it can be seen that group A showed a little more advantage compared to groups B and C. This result can be compared with the cell and glucose concentration, since triplicate A also had a higher cell concentration and higher glucose consumption compared to the other groups. However, the results of the three biomass concentrations per g/l were satisfactory.

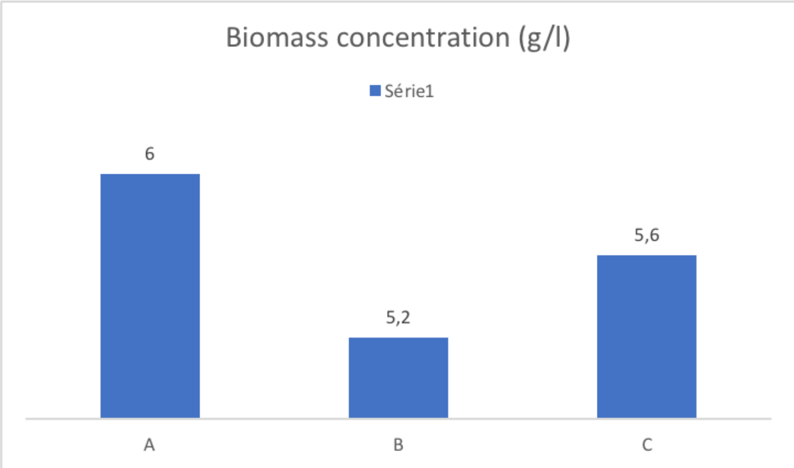

Figure 14. Comparison of biomass concentration of groups A, B and C.

To highlight the effectiveness of adding glucose as a carbon source in the microalgae culture medium, we conducted analyzes of cell growth and biomass concentration in the control group, which used only the synthetic Chu medium. This provided comparative data.

Figure 15 illustrates the growth of cell concentration in the Chu medium. It is evident that, compared to the cultures that received the addition of glucose, the control group showed slower cell growth, requiring 9 days after inoculation to reach a cell concentration of 3,375. In contrast, media containing glucose already reached a similar cell density on their third or fourth day, compared to the ninth day of cultures using only Chu medium. This confirms the greater effectiveness of glucose cultures in promoting cell growth.

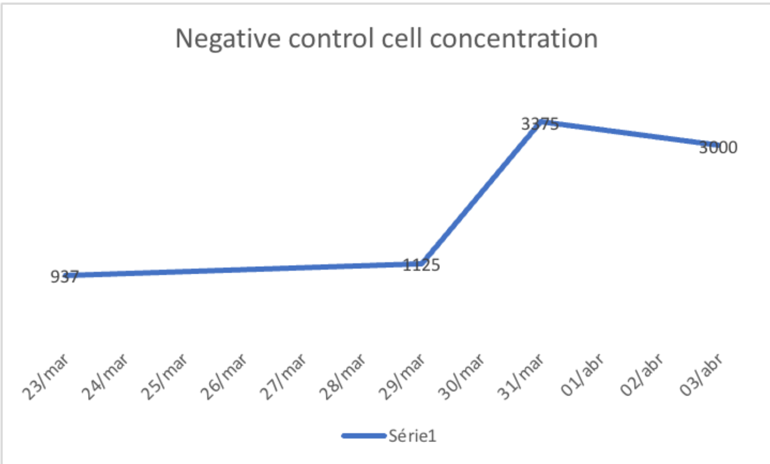

Figure 15. Negative control cell concentration.

Analyzes were carried out to quantify the concentration of dry biomass per gram per liter (g/L) of the negative control. These results will later be compared with the amounts of biomass found in other cultures that received glucose.

To determine the biomass concentration, a 2 ml sample was taken in triplicate, called B1, B2 and B3 from the negative control with Chu medium, thus obtaining the results represented in figure 16, and when analyzing them it is clear that the biomass concentration reduces practically by half in Chu medium cultures when compared to those with glucose.

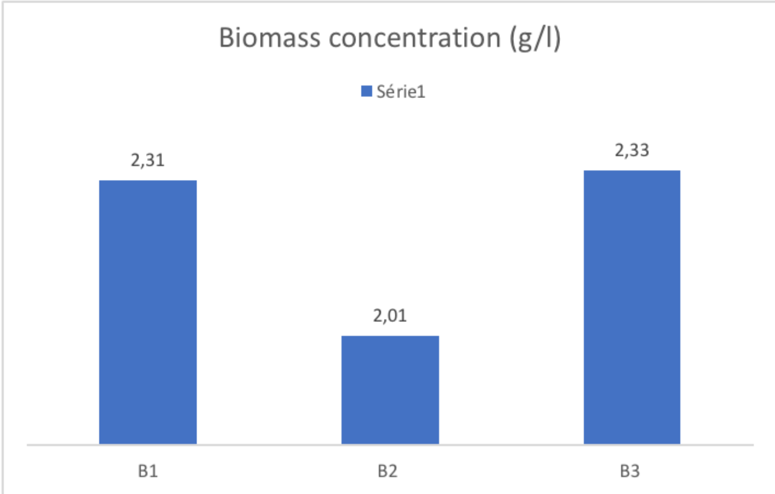

Figure 16. Triplicate the biomass concentration of the negative control.

Therefore, in addition to cultures with glucose showing better growth in cell density, they also demonstrate a higher concentration of biomass per g/L. With this, it is possible to define and emphasize the importance of adding glucose to culture media as a carbon source to improve growth and reduce cultivation time.

4. Conclusions

The results obtained from the analysis of the increase in cell productivity with the addition of glucose were highly satisfactory. These results not only validate the effectiveness of this approach, but also open the door for additional investigations, such as the analysis of lipids in the three biomasses achieved. This lipid analysis is crucial, as it will identify which of the triacylglycerol concentrations stands out as the most promising.

Based on the results that indicate the optimal concentration of triacylglycerol, efforts could be directed towards large-scale production. This implies the possibility of starting the production of biodiesel and green fuel, exploiting the full potential of these discoveries to contribute significantly to the production of sustainable fuels. This development represents an important step forward on the path towards more environmentally friendly and sustainable solutions in the energy sector.

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