RESEARCH ARTICLE OPEN ACCESS

Increment of carbohydrate concentration of *Chlorella minutissima* microalgae for bioethanol production

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ABSTRACT
Microalgae, like any other microorganism react to changes in the external environment with changes in their intracellular environment. Thus, the manipulation of cultivation conditions, especially the presence or absence of certain nutrients, stimulates the biosynthesis of compounds of interest. Their carbohydrates can be used to produce bioethanol. The objective of this study was to evaluate the effect of the medium and the concentrations of nitrogen and phosphate components used in the culture medium of the microalgae *Chlorella minutissima* in the carbohydrate concentration of the same. Box-Behnken Planning was used, totaling 15 trials. The cultivations were carried out until early stationary phase of growth of the microalgae in closed 2 L reactors. At the end of the cultivation, the carbohydrate concentrations of dry biomass (%) and yield in carbohydrates ($g.L^{-1}.d^{-1}$) were determined. According to the analysis of effects, the microalgae *Chlorella minutissima* cultivated in Basal medium, with the addition of 0.125 gL^{-1} of the nitrogenized component (KNO_3) and without addition of phosphatized components (K_2HPO_4 and KH_2PO_4) had a higher yield in carbohydrates in the cultivation (0,030 ± 0.002 $g.L^{-1}.d^{-1}$).
Keywords: culture medium, microalgae, nutrients.

I. INTRODUCTION

The global change in climate, the rising price of oil and the rapid depletion of fossil fuel reserves forced governments, scientists and researchers to invest in alternative energy sources, including wind and solar energy and biofuels [1]. The production of biofuels from renewable sources can reduce dependence on fossil fuels and contribute to the maintenance of a healthy environment and economic sustainability [2]. The biomass currently used in biofuel production is also used for human consumption, and it is believed that this can cause food shortages and dissatisfaction throughout the world, especially in developing nations [3] [4]. Therefore, microalgae can be an alternative raw material for biofuel production due to their fast growth rate, ability to fix greenhouse effect and high production capacity of lipids and carbohydrates, and does not compete with the production food, because they can be grown on non-arable land [5] [6] [7].

Like any other microorganism, microalgae respond to changes in the external environment with changes in their intracellular environment. Thus, manipulation of the cultivation conditions, particularly the presence or absence of certain nutrients stimulates the biosynthesis of compounds of interest [8]. The cultivation of microalgae in medium with reduced nitrogen and phosphorus, important elements for the metabolism of microalgae, allows lipids and carbohydrates to be preferentially synthesized [9]. The increase in carbohydrate concentration of microalgal biomass favors its use as alternative raw material for bioethanol production [10].

The aim of this study was to evaluate the effect of the cultivation medium and the concentrations of nitrogenized and phosphatized components in the carbohydrate concentration of the microalgae *Chlorella minutissima*.

II. MATERIALS AND METHODS

The microalgae studied in this work was *Chlorella minutissima*. To assess the effects of the variables culture medium, the concentration of nitrogenized and phosphate components in the carbohydrate concentration of the microalgae *Chlorella minutissima*, Box-Behnken Planning was carried out totaling 15 experiments (Table 1).

Table 1 Actual and coded (in parentheses) levels of the variables concentration of nitrogenized (KNO_3) and phosphate (K2HPO4 and KH2PO4) components used in the cultivation of microalgae *C. minutissima*.

Assay	Culture Medium	Nitrogenized Components (KNO_3, $g.L^{-1}$)	Phosphatized Components (K_2HPO_4/ KH_2PO_4, $g.L^{-1}$)
1	Basal (-1)	0 (-1)	0.0375 / 0.0875 (0)
2	BG-11 (+1)	0 (-1)	0.0375 / 0.0875 (0)
3	Basal (-1)	0.250 (+1)	0.0375 / 0.0875 (0)
4	BG-11(+1)	0.250 (+1)	0.0375 / 0.0875 (0)
5	Basal (-1)	0.125 (0)	0 / 0 (-1)
6	BG-11 (-1)	0.125 (0)	0 / 0 (-1)
7	Basal (-1)	0.125 (0)	0.075 / 0.175 (+1)
8	BG-11 (-1)	0.125 (0)	0.075 / 0.175 (+1)
9	BMM (0)	0 (-1)	0 / 0 (-1)
10	BMM (0)	0.250 (+1)	0 / 0 (-1)
11	BMM (0)	0 (-1)	0.075 / 0.175 (+1)
12	BMM (0)	0.250 (+1)	0.075 / 0.175 (+1)
13	BMM (0)	0.125 (0)	0.0375 / 0.0875 (0)
14	BMM (0)	0.125 (0)	0.0375 / 0.0875 (0)
15	BMM (0)	0.125 (0)	0.0375 / 0.0875 (0)

The culture media used were Bristol`s Modified Medium (BMM) [11], BG-11 [12] and Basal [13]. The BMM consisted of ($g.L^{-1}$): $CaCl_2$, 0.01; $MgSO_4.7H_2O$, 0.075; NaCl, 0.025, $FeSO_4.7H_2O$, 0.02 and 1 $mL.L^{-1}$ solution of A_5 micronutrients. The BG-11 medium consisted of ($g\ L^{-1}$): $MgSO_4.7H_2O$, 0.075; $CaCl_2.2H_2O$, 0.036; Ferric ammonium citrate, 0.006; Disodium EDTA, 0.001; Na_2CO_3, 0.02, citric acid, 0.006 and $1mL.L^{-1}$solution of A_5+Co micronutrients. The Basal Medium consisted of ($g.L^{-1}$): $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.003 and 1 $mL.L^{-1}$ solution of A_5 micronutrients. The same nitrogen (KNO_3) and phosphorus (K_2HPO_4/KH_2PO_4) sources were used in the three different culture media, and the differences between these were only covered in the rest of the components.

The cultivations were carried out in closed 2 L photobioreactors, with continuous stirring by injection of sterile air, illuminance of 2500 lux and 12 h light/dark photoperiod, and initial inoculum concentration of 0.20 $g\ L^{-1}$.

2.2. Analytical determinations

2.2.1. Cell Growth

The biomass concentration was determined every 24 h by measuring optical density in a spectrophotometer at 670 nm [14], using a pre-established relation between the dry biomass weight and absorbance. Cultures were maintained until the early stationary phase of growth.

The parameters final biomass concentration (X_f, $g.L^{-1}$); maximum yield (P_{max}, $g.L^{-1}.d^{-1}$) were evaluated, calculated according to Equation1 and maximum specific growth rate (μ_{max}, d^{-1}) obtained by exponential regression applied to the logarithmic growth phase [15].

$$P(g.L^{-1}d^{-1}) = \frac{X - X_0}{t - t_0} \quad (1)$$

Where:

X = biomass concentration ($g.L^{-1}$) at time t (d)

X_0 = biomass concentration ($g.L^{-1}$) at time t_0 (d)

2.2.2. Determination of carbohydrates

The carbohydrate concentration of dry biomass (% w/w) was evaluated at the end of the cultivation of the microalgae *C. minutissima*. The method used was an adaptation of DNS with prior acid hydrolysis of the polysaccharide with the addition of 1.5 N HCl. For this, 20 ml of HCl (1.5 M) was added in 0.2 g of dry microalgae biomass and the solution was heated at 121 °C for 20 min. The mixture was then cooled to room temperature, neutralized with NaOH (40% and 10%) and transferred to a volumetric flask (50 mL), in which 1 mL of 15% potassium ferrocyanide and 1 ml of sulphate or 30% zinc acetate were added. To homogenize, the volume was completed with distilled water. The solution was allowed to stand for 15 min forsubsequent decantation and filtration of the precipitate. In 25 ml of the filtrate, 0.5 ml of ethanol (99.5%) and 2 ml of acetate buffer pH 7.0 were addedand homogenized for 10 min on a magnetic stirrer.The solution then remained in a water bath at 70 °C for 20 min. Later, 0.2 ml of sodium tungstate (12%) was added and the mixture filtered through a paper filter, discarding the first 10 drops, and then removing 1 ml from the filtrate to determine reducing sugars by the method proposed by Miller [16].

The yield in carbohydrates in the cultures ($g.L^{-1}.d^{-1}$) was obtained according to Equation 2:

$$\text{Yield in carbohydrates } (g.L^{-1}.d^{-1}) = \frac{X_f \times CHO}{100 \times t_c} \quad (2)$$

Where:

X_f= final biomass concentration ($g.L^{-1}$);

CHO = carbohydrate concentration (%);

t_c= cultivation time (d).

III. RESULTS AND DISCUSSION

The microalgae *C. minutissima* presented different behavior for the assays performed (Fig. 1) (Table 2). This can be explained by the differences in the proportions of nitrogenized and phosphatized components, as well as the different culture media used.

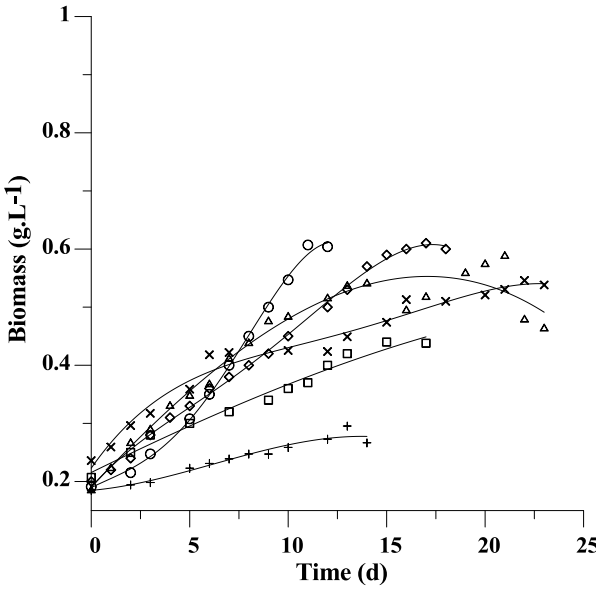
The cultures reached the stationary phase at different times, with the lowest times being reached in assays 9 and 11, (5 d and 6 d, respectively). These cultivations were carried out in the BMM medium, and in the assay 9 there was no addition of nitrogenized and phosphatized components, and the assay 11 was performed without the addition of a nitrogen source, but the phosphatized components were added in higher planning concentrations (0,075 g L^{-1} and 0.175 g L^{-1} KH_2PO_4 and K_2HPO_4, respectively). The low concentrations of nitrogen and phosphorus may have limited the cultivation time, resulting in low cell concentrations, since these components are essential for cell growth [17]. Nitrogen and phosphorus play an important role in cellular metabolism, since they are present in many biochemical processes. Nitrogen is responsible for the formation of proteins, amino acids and nucleic acids, while the phosphorus is a component of nucleic acids and phospholipids [8], acting also as a carrier of substrates or chemical energy, since it is as part of the membrane [18].

In assay 12, conducted in BMM medium at higher concentrations levels of KNO_3 (0.250 $g.L^{-1}$) and K_2HPO_4 / KH_2PO_4 (0.075 $g.L^{-1}$ and 0.175 $g.L^{-1}$, respectively) the highest values of X_f (± 0.826 0.008 $g.L^{-1}$) and μ_{max} (0.112 ± 0.002 $g.L^{-1}$) were obtained, indicating that the use of higher nitrogen and phosphorus in crops results in increased cell growth.

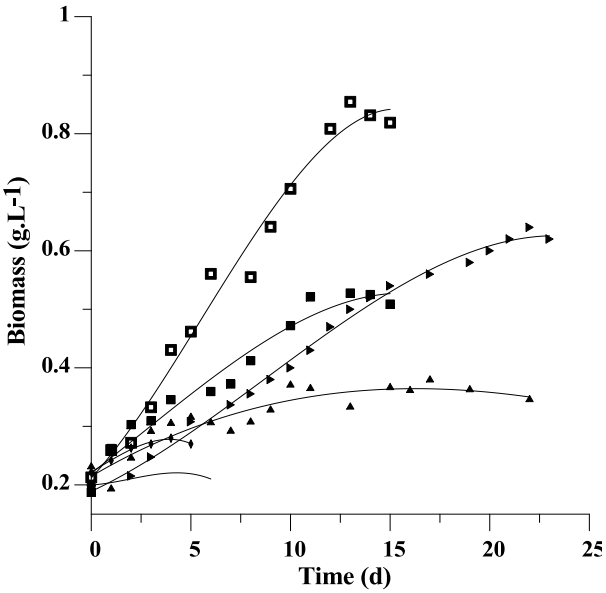
The interactions of the culture medium with nitrogenized component, the culture medium with phosphatized component and nitrogenized component with phosphatized component showed a significant effect on the concentration of carbohydrates in cultures (Table 3).

According to the assessment of the effects of the interactions when the microalgae *Chlorella minutissima* was grown in a Basal medium, with the addition of 0.125 $g.L^{-1}$ nitrogenized component (KNO_3) and without addition of phosphatized components (K_2HPO_4 and KH_2PO_4) the yield in carbohydrates in cultures was higher (0.030 ± 0.002 $g.L^{-1}.d^{-1}$).

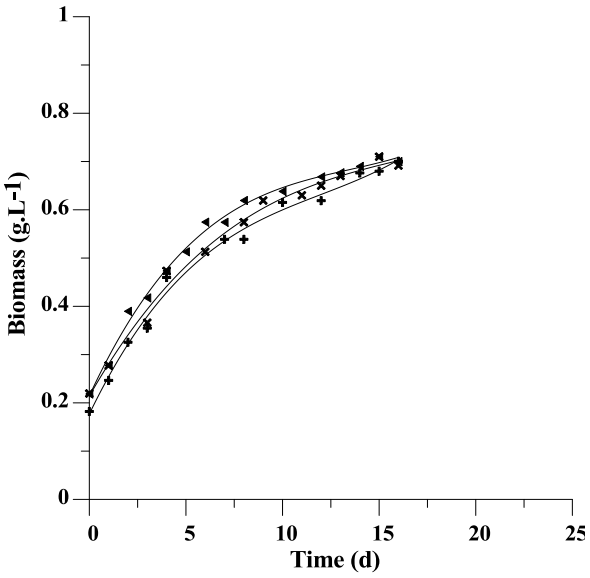
It can be seen from Table 2 that in assay 5, where the highest concentration of carbohydrates was found, the highest final biomass concentration (0.604 ± 0.015 g L^{-1}) was not verified. According to Dismukes et al. [10] and Lourenço [19], low concentrations of phosphorus and nitrogen in the culture medium may limit the growth of some species of microalgae, but the lack of these nutrients produces an increase in carbohydrate content of microalgal biomass.



(a)



(b)



(c)

Figure 1 - Growth curves of the Box-Behnken Planning assays for *Chlorella minutissima*: **(a)** 1 (┼); 2 (△); 3 (◊); 4 (□); 5 (○); 6 (x); **(b)** 7 (►); 8 (▲); 9 (◆); 10 (■), 11 (●);12 (◘) e **(c)** 13 (◄); 14 (✚);15 (✖).

In the literature, there are reports of assays in which the concentration of nitrogen and phosphorus influenced the carbohydrate concentration of microalgae. Markou [20] reported that the decrease of 8 mg g^{-1} to 2 mg $g^{-1}$of intracellular phosphorus in *Spirulina platensis* caused an increase of 55% carbohydrates. Santos, Macedo and Alegre [21] observed the effect of the concentration of nitrogenized component in carbohydrate content of microalgae. Halving the concentration of KNO_3, there was a decrease of 28.84% in protein content and increase of 30.34% in the carbohydrate content of *Spirulina máxima*.

Table 2 - Concentration of final biomass (X_f), maximum cell yield (P_{max}), maximum specific growth rate (μ_{max}), time interval of the exponential phase, concentrations of carbohydrates in the dry biomass and carbohydrates yield of *Chlorella minutissima*.

Assay	Final biomass concentration ($g.L^{-1}$)	P_{max} ($g.L^{-1}.d^{-1}$)	μ_{max} (d^{-1})	Exponential phase Interval (d)	Dry biomass carbohydrate Concentration (%)	Yield in carbohydrates ($g.L^{-1}.d^{-1}$)
1	0.270 ± 0.006^{b}	0.012 ± 0.003^{ab}	0.032 ± 0.004^{c}	3-12	61.33 ± 1.31^{g}	O.012±0.001
2	0.465 ± 0.012^{d}	0.043 ± 0.003^{f}	0.055 ± 0.003^{e}	4-13	55.99 ± 1.34^{ef}	0.011±0.001
3	0.603 ± 0.009^{f}	0.041 ± 0.002^{ef}	0.058 ± 0.002^{e}	4-15	52.17 ± 1.54^{e}	0.017±0.002
4	0.438 ± 0.008^{d}	0.032 ± 0.006^{cd}	0.039 ± 0.002^{d}	3-15	21.52 ± 0.97^{b}	0.006±0.001
5	0.604 ± 0.015^{f}	0.039 ± 0.004^{def}	0.058 ± 0.003^{e}	5-12	69.20 ± 0.67^{h}	0.030±0.002
6	0.538 ± 0.004^{e}	0.060 ± 0.003^{g}	0.017 ± 0.002^{b}	6-21	24.54 ± 1.13^{b}	0.006±0.001
7	0.599 ± 0.005^{f}	0.029 ± 0.004^{bc}	0.060 ± 0.002^{e}	5-15	50.57 ± 2.03^{fg}	0.016±0.002
8	0.343 ± 0.029^{c}	0.045 ± 0.002^{f}	0.017 ± 0.002^{b}	3-16	15.28 ± 1.27^{a}	0.003±0.001
9	0.248 ± 0.007^{b}	0.020 ± 0.003^{bc}	0.008 ± 0.001^{a}	2-5	43.49 ± 1.98^{d}	0.022±0.002
10	0.511 ± 0.006^{e}	0.075 ± 0.003^{h}	0.055 ± 0.001^{e}	2-10	23.60 ± 2.70^{b}	0.008±0.001
11	0.211 ± 0.003^{a}	0.009 ± 0.001^{a}	0.016 ± 0.001^{b}	1-4	30.51 ± 1.85^{c}	0.011±0.003
12	0.826 ± 0.008^{h}	0.098 ± 0.004^{i}	0.112 ± 0.002^{g}	2-10	46.95 ± 1.03^{d}	0.026±0.003
13	0.682 ± 0.012^{g}	0.112 ± 0.002^{ij}	0.079 ± 0.001^{f}	2-10	45.42 ± 2.24^{d}	0.019±0.002
14	0.707 ± 0.005^{g}	0.105 ± 0.001^{ij}	0.077 ± 0.002^{f}	2-10	44.19 ± 1.19^{d}	0.020±0.002
15	0.708 ± 0.005^{g}	0.107 ± 0.002^{j}	0.075 ± 0.002^{f}	2-10	44.21 ± 2.12^{d}	0.020±0.001

Mean values of analysis performed in triplicate ± standard deviation. Same letters in the same column indicate that there was no significant difference at 95% confidence level ($p> 0.05$).

Table 3 - Estimates of linear (L) and quadratic effects (Q) of the culture medium, nitrogenized component and phosphatized components in carbohydrates yield in cultures of *Chlorella minutissima* at the 90% level of confidence.

Factor	**Effects**	**Standard error**	**t**	**p**
Mean	0.0138	<0.001	448.330	<0.001
X_1 - culture medium (L)	-0.0125	<0.001	-165.224	<0.001
X_1 - culture medium (Q)	0.0055	<0.001	100.082	<0.001
X_2 - nitrogenized component (KNO_3) (L)	0.0004	<0.001	5.635	0.030
X_2 - nitrogenized component (KNO_3) (Q)	-0.0024	<0.001	-43.059	<0.001
X_3 - phosphatized components (K_2HPO_4/ KH_2PO_4) (L)	-0.0026	<0.001	-35.372	<0.001
X_3 - phosphatized components (K_2HPO_4/ KH_2PO_4) (Q)	0.0004	<0.001	8.968	0.012
X_1 x X_2	-0.0055	<0.001	-53.076	<0.001
X_1 x X_3	0.0055	<0.001	51.726	<0.001
X_2 x X_3	0.0144	<0.001	134.628	<0.001

In this study, when comparing the assay in which the microalgae had the highest biomass carbohydrate content (assay 5 - 69.20 ± 0.67%), held in Basal culture medium with addition of intermediate concentration of nitrogen source (0.125 $g.L^{-1}$) and without a phosphorus source, with the assay which showed the lowest concentration of carbohydrates (assay 8 - 15.28 ± 1.27%), held in

BG-11 medium with addition of 0.125 g L^{-1} KNO_3, 0.075 $g.L^{-1}$ K_2HPO_4 and 0.175 $g.L^{-1}$ KH_2PO_4, the increase in carbohydrate content was approximately 353%.

When the culture medium has a deficiency in nitrogen or phosphorus, as can be seen in the estationary phase of the culture or when the culture medium has reduced levels of these nutrients, the cells exhibit an increase in the rate of uptake of the limiting nutrient. As the nutrient runs out in the medium, it becomes harder for the cell to use it. The fact that the cell has enhanced the uptake of its systems to maintain its growth rate, without nutrient availability for this to occur, generates a physiological stress which increases as the concentration of nutrients decreases. This stress changes the microalgal metabolism, directing the metabolic processes to the production of carbohydrate or lipid reserves to prepare the cell for the period of deprivation [19] [22].

Compared to other growth media used, the basal medium has a higher concentration of magnesium sulfate (0.3 gL^{-1}) against 0.075 gL^{-1} in the BG-11 and BMM media. This component may have influenced the concentration of carbohydrates in crops, since magnesium plays an essential role in microalgae for being a constituent of the chlorophyll molecule [19], what may result in an increase in photosynthetic efficiency of these. Besides its importance in photosynthesis, magnesium has other functions essential to the cell such as aggregation of ribosomes in functional units and formation of catalase [23].

For the concentrations of nitrogen and phosphorus, it has been found that it is necessary that at least one of these components be added, as both affect cell growth and, consequently, the carbohydrate content of cultures.

In the analysis of the culture media, different conditions to obtain higher yields in carbohydrates were found. For the BG-11 medium, higher carbohydrate yield was observed in Assay 2 (0.011 ± 0.001 $g.L^{-1}.d^{-1}$), wherein the microalgae was cultured without the addition of nitrogenized component and in the intermediate level of the phosphatized components (0.0375 $g.L^{-1}$ K_2HPO_4 and 0.0875 $g.L^{-1}$ KH_2PO_4). This concentration of carbohydrates is 72% lower when compared to carbohydrate concentrations obtained in assay 5, conducted in the Basal medium.

In cultures performed in BMM, the highest yield in carbohydrates was obtained in assay 12 (0.026 ± 0.003 $g.L^{-1}.d^{-1}$), conducted with the addition of nitrogenized and phosphatized components at higher levels (0.250 $g.L^{-1}$ KNO_3, 0.075 $g.L^{-1}$ K_2HPO_4 and 0.175 $g.L^{-1}$ KH_2PO_4) and assay 12 (0.388 ± 0.010 $g.L^{-1}$), a cultivation that also had the highest final cell concentration (0.826 ± 0.008 $g.L^{-1}$). In Experiment 5, in which the highest carbohydrates yield was detected (0.030 ± 0.002 $g.L^{-1}.d^{-1}$), an average increase of 15% was obtained compared to assay 12.

In the Basal medium, the highest carbohydrates yield in cultures was observed in assay 5 (0.030 ± 0.002 $g.L^{-1}.d^{-1}$), with the addition of 0.125 g L^{-1} KNO_3 and without a phosphorus source, and 3 (0.017 ± 0.002 $g.L^{-1}.d^{-1}$), conducted with the highest concentration of nitrogen (0.250 $g.L^{-1}$ KNO_3) and intermediate levels of phosphorus sources (0.0375 $g.L^{-1}$ of K_2HPO_4 and 0.0875 $g.L^{-1}$ of KH_2PO_4). Assay 5 showed 76% increase in productivity in carbohydrates compared to assay 3.

Microalgae produce a wide range of carbohydrates such as starch, which are mostly reserve products [24] [25]. Changes in biochemical constitution of microalgae are important in achieving high levels of carbohydrates and these can be used as alternative raw material for bioethanol production. In this study, high carbohydrates concentrations were obtained for *Chlorella minutissima*, and these carbohydrates can be used to produce bioethanol.

IV. CONCLUSIONS

The changes made to the concentrations of nitrogenized and phosphatized components, as well as assays in different culture media, allowed the verification of the bestconditionsfor obtaining an increase in the carbohydrate content of the *Chlorella minutissima*.

The highest yield in carbohydrates was obtained when *Chlorella minutissima* was cultured in Basal Medium, with the addition of 0.125 $g.L^{-1}$ of nitrogenized component (KNO_3) and without the addition of phosphatized components (K_2HPO_4 and KH_2PO_4).

The high yields verified in carbohydrates in *Chlorella minutissima* show that it can be used as an alternative raw material to the current energy source, reducing the current problems, such as the use of food and arable land for biofuel production.

V. ACKNOWLEDGEMENTS

The authors are greatful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support which made this research possible.

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