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Influence on cultivation conditions in the heterotrophic lipid production of the microalga *Chlorella minutissima*

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Abstract

Chlorella, among microalgae, has possibly been the most used for feeding, as well as the most researched in heterotrophic growing lately due to the highest lipid yield. The objective of this work was to study the effect of carbon and nitrogen concentration for lipid productivity and biomass production of microalga *Chlorella minutissima*, cultivated in absence of light. The complete factorial design used was type 2^2 with three repetitions at the central point, having as glucose concentration factor (1, 5 e 9 $g.L^{-1}$) and as sodium nitrate ($NaNO_3$) (0.2; 0.75 and 1.3 $g.L^{-1}$), and total lipid and biomass concentration as responses, with a total number of 7 experiments. The cultivation carried out with 9 $g.L^{-1}$ of glucose and 0.2 $g.L^{-1}$ of $NaNO_3$ was the one that presented better lipid concentration (29.38% w/w), better cell growth (0.89 $g.L^{-1}$) and a lipid profile comprising saturated, monounsaturated and polyunsaturated fatty acids in 26.6% w/w, 62.4% w/w and 11% w/w concentrations, respectively.

Introduction

Microalgae are a source of many products with high added value for human beings, which vary from carbohydrates, essential fatty acids, pigments, food supplements, fertilizers, pharmaceutical products and biofuels (Hemaiswarya, 2011). The denomination microalgae includes organisms with two types of cellular structure: prokaryotic and eukaryotic. The prokaryotic organisms have representatives in the divisions *Cyanophyta* (cyanobacteria) and *Prochlorophyta.* Whereas the eukaryotic have representatives in the divisions *Chlorophyta, Euglenophyta, Rhodophyta, Haptophyta (Prymnesiophyta), Heterokontophyta* (*Bacillariophyceae, Chrysophyceae, Xantophyceae* etc.), *Cryptophytae Dinophyta* (Derner *et al*., 2006).

Chlorella is an eukaryotic unicellular spherical microalga, its diameter varies from 5-10 µm depending on the specie (Illman *et al*., 2000), it is a microalga division *Chlorophyta* and order *Chlorococcales.* This microalga has 53% of proteins, 23% of carbohydrates, 9% of lipids and 5% of minerals (Henrikson, 1994). *Chlorella minutissima* is a microscopic, fresh water and green alga that has a hard cell wall. It contains the biggest chlorophyll concentration when compared to other algae (Morais, 2006). The economic interest for its cultivation has grown considerably, based on some of its characteristics, such as easy cultivation, high protein content, that can reach 65% in dry matter, depending on the environment (Venkataraman and Becker, 1985).

The level of lipids in microalgae is an important parameter to biofuel production and polyunsaturated fatty acids acquisition. However, some algae only start lipid production, when they face some environmental stress (for example, nutrient deprivation, high light intensity or absence of light) (Rodolfi *et al*., 2009; Tang *et al.*, 2011). Because of this, first, it is interesting to cultivate the microalgae, in order to have a high biomass concentration, and then, it is necessary to induce the lipid accumulation under nutrient stress (Jiang *et al*., 2011).

Heterotrophic and mixotrophic cultivation are very well known because they provide very fast algae growth and high lipid accumulation material (fatty acids) inside microalgae cells when compared to autotrophic cultivation (Mitra *et al.*, 2012). However, these kind of cultivation require an exogenous source organic carbon such as glucose (Heredia-Arroyo *et al.*, 2010; Shen *et al*., 2010), acetate (Heredia-Arroyo *et al*., 2010), glycerol (Liang *et al*., 2009, Heredia-Arroyo *et al*., 2010; O'Grady and Morgan, 2011), hydrolyzed corn (Xu *et al*., 2006), among others.

According to Wu *et al.* (1994), some species of *Chlorella* are capable growing heterotrophic and photoautotrophic in environment, such as *Chlorella protothecoides*. Xu *et al.* (2006) cultivated microalga this in two ways, autotrophic and heterotrophic, and

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Table 1. Factorial 2^2 planning matrix with codified variables and as maximum biomass (x_{max}, $g.L^{-1}$) responses to concentrations and total concentration of lipids (% w/w).

Experiment	Codified variables		*Chlorella minutissima*		
	X_1	X_2	X_{max} ($g.L^{-1}$)	Lipids (% w/w)	Lipid productivity ($mg.L^{-1}.d^{-1}$)
1	-1 (1)	-1 (0.2)	0.25	8.02±0.60	1.12
2	+1 (9)	-1 (0.2)	0.89	29.38±1.88	25.27
3	-1 (1)	+1 (1.3)	0.27	5.11±0.84	0.50
4	+1 (9)	+1 (1.3)	0.64	21.79±1.12	13.06
5	0 (5)	0 (0.75)	0.83	21.60±1.45	17.05
6	0 (5)	0 (0.75)	0.88	21.62±2.40	18.14
7	0 (5)	0 (0.75)	0.81	20.94±2.54	16.08

X_1: glucose concentration ($g.L^{-1}$); X_2: $NaNO_3$ concentration ($g.L^{-1}$)

lipid content was 14.57% and 55.20%, respectively. Knowing that the microalgae *Chlorella* genus is excellent producer of lipids when it comes to cultivation in heterotrophic so it was decided to choose the microalga *Chlorella minutissima* of strains bank of Biochemical Engineering Laboratory (LEB) of Universidade Federal do Rio Grande (FURG) to conduct the study. The objective of this work was to study the effect of carbon and nitrogen concentration in lipid productivity and biomass production of *Chlorella minutissima*, cultivated in absence of light.

Material and Methods

Microorganism and culture media

The microalga used in this study was *Chlorella* minutissima (Costa *et al.*, 2006), it belongs to the Culture Collection from Biochemical Engineering Laboratory of Universidade Federal do Rio Grande (FURG). The microalga was kept and cultivated in Culture Medium BG11 (Rippka *et al.*, 1979) containing ($g.L^{-1}$): sodium nitrate ($NaNO_3$) (1.50); potassium phosphate dibasic trihydrate ($K_2HPO_4.3H_2O$) (0.04); magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (0.075); calcium chloride dihydrate ($CaCl_2.2H_2O$) (0.036); cmmonium ferric citrate ($C_6H_{11}FeNO_7$) (0.006); ethylenediamine tetraacetic acid (EDTA) disodium (0.001); sodium carbonate (Na_2CO_3) (0.02); citric acid ($C_6H_8O_7$) (0.006); boric acid (H_3BO_3) (2.86); manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$) (1.81); zinc sulfate heptahydrate ($ZnSO4.7H_2O$) (0.222); sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) (0.39); copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) (0.079), cobalt (II) nitrate hexahydrate ($Co(NO_3)_2.6H_2O$) (0.0494).

Growth cultivation conditions

Before the beginning of the experiments, the inoculum of microalga *Chlorella minutissima* was put in a thermostatically controlled greenhouse at 30ºC with 12 h of light/dark photoperiod, illuminance of 2500 lux supplied by 40 W fluorescent bulbs similar to day light and sterile air circulation, in order to increase cellular concentration. After that, a microscope follow up was conducted, in order to perceive if there was only the microalga that was interesting for cultivation. Next, the microalga was adapted to the experiment conditions. The final adaptation moment happened when the microalga totally consumed the glucose, this varied according to the carbon source concentration. The assay adaptation conducted with 1 and 5 $g.L^{-1}$ of glucose ended within 1 day, but the assay with 9 $g.L^{-1}$ ended in 2 days.

The cultivation was carried out in a heterotrophic way, using glucose as carbon and energy source. The addition of glucose to the cultivation happened on a batch mode daily, it was fed in 1/10 proportion for the total glucose concentration (according to experimental design, Table 1), during the 10 days of the experiment. The cultivation took place in an orbital shaker (INNOVA®44, USA) at 150 rpm and 30ºC using closed bioreactors of 2 L with storage volume 1.6 L. The initial biomass concentration was 0.15 $g.L^{-1}$. At the end of the cultivation, the samples were centrifuged and subsequently, freeze-dried.

Analytical determinations

The cultivation samples were made every 24 h. The samples were aseptically collected in order to determine their cellular concentration, which was obtained through optical density analyses at 670 nm with spectrophotometer (Femto Plus 700, Brazil) with the help of a pre-determinate calibration curve.

The glucose concentration was determined through culture medium every 24 h using colorimetric enzymatic kit (Doles, Brazil), with

absorbency measurement at 510 nm and conversion to glucose through calibration curve. The cultures pH was daily measured using pHmetro digital (Quimis Q400HM, Brazil). The nitrogen concentration was daily determined for the culture medium using the colorimetric method proposed by Cataldo *et al.* (1975).

Total lipid quantification and fatty acids profile

In order to quantify the lipid total, the methodology proposed by Folch *et al.* (1957) was used with the previous stage of cellular wall disruption through ultrasonic dip. The lipid fraction was esterified to obtain ester methyl from fatty acids, according to the methodology adapted by Metcalfe *et al.* (1966).

The fatty acids determination was determined with Gas Chromatograph Varian 3400CX, equipped with flame ionization detector and column ZB-WAX with 30 m length, internal diameter of 0.32 mm and footage of 0.25 µm. The carrier gas was hydrogen at 0.5 mL.min^{-1}. The injector and detector temperatures were 250 and 300°C, respectively. The column initial temperature was 40°C increasing 6°C.min^{-1} until 100°C keeping 1 min, after at 160°C keeping 5 min and at 230°C keeping 10 min. The fatty acids were identified by the retention time comparison with standards and quantified by standardization of areas. The fatty acids standard used were (Sigma Supelco; Belle-fonte, EUA) butyric acid (C4:0), capric acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), undecanoic acid (C11:0), lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), *cis*-10-pentadecenoic acid (C15:1), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), *cis*-10-heptadecenoic acid (C17:1), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2n6), alpha-linoleic acid (C18:3n3), γ-linoleic acid (C18:3n6), arachidic acid (C20:0), *cis*-11-eicosenoic acid (C20:1n9), *cis*-11,14-eicosadienoic acid (C20:2), *cis*-11,14,17-eicosatrienoic acid (C20:3n3), *cis*-8,11,14-eicosatrienoic acid (C20:3n6), arachidonic acid (C20:4n6), *cis*-5,8,11,14,17-eicosapentaenoic acid (C20:5n3), heneicosanoic acid (C21:0), behenic acid (C22:0), erucid acid (C22:1n9), *cis*-13,16-docosadienoic acid (C22:2), *cis*-4,7,10,13,16,19-docosahexaenoic acid (C22:6n3), tricosanoic acid (C23:0), lignoceric acid (C24:0), nervonic acid (C24:1n9).

Experimental design

Factorial Design 2^2 with com triplicate at the central point was carried out for this study, in which the studied variables were glucose concentration and $NaNO_3$ concentration. The analyzed responses were total lipid concentration and maximum cellular concentration (X_{max}). The levels proposed for each variable were based on previous tests (the data is not shown). The effect of the variables studied upon the responses was statistically analyzed with significance level of 95% ($p \leq 0.05$).

Lipid productivity

The lipid productivity for different tests was calculated taking into consideration the cellular concentration of each experiment, lipid concentration from the inoculum where the experiments came from and after 10 days of cultivation and the cultivation tome.

The lipid productivity was calculated through Equation 1.

$$P = \frac{X_f - X_0}{t_f - t_0} \quad (1)$$

Where:

X_f= final cellular concentration . initial lipid concentration (mg_{lip}. L^{-1})

X_0= initial cellular concentration . final lipid concentration (mg_{lip}. L^{-1})

t_f= final time (d^{-1})

t_0= initial time (d^{-1})

Conversion factors

The conversion factors calculation $Y_{P/S1}$ (glucose into lipid), $Y_{P/S2}$ (nitrogen into lipid), $Y_{X/S1}$ (glucose into biomass) e $Y_{X/S2}$ (nitrogen into biomass) was made based on the Equations 2 and 3, respectively.

$$Y_{P/S} = \frac{P - P_0}{S_0 - S} \quad (2)$$

$$Y_{X/S} = \frac{X - X_0}{S_0 - S} \quad (3)$$

Where:

P= product, in this case, lipids

S= substrate

S_1= glucose

S_2= nitrogen

X= biomass

The lipid initial cellular concentration (P_0), was considered the one obtained from the *Chlorella minutissima* inoculum, because the experimental design experiments came from it. The inoculum total lipid quantification was made according to the methodology proposed by Folch *et al*. (1957), the value obtained was 5.87% w/w.

The initial glucose concentration (S_0) was considered equal to the total glucose concentration added to the cultivation (1, 5 or 9 g.L^{-1}), even if the glucose addition had happened in batch mode and fed in the 1/10 proportion from total concentration.

Results and Discussion

Figure 1 shows the microalga *Chlorella minutissima* growth curves according to concentrations of nitrogen and glucose that were consumed throughout 7 and 10 day of growth, in the different experiments conducted (Table 1). When the results of Figure 1 are analyzed, it is perceived that laboratory tests 2 and 4 were concluded 3 days before the others. This happened because it was verified, through the reduction of pH value, the existence of another microorganism competing for the nutrient in the media with the microalga *C. minutissima*. From this moment on, we could not say which would be producing the bioproducts important for the study and for this reason, it was decided to stop with these cultivations.

In microalgae growth, under heterotrophic conditions, the energy necessary for the growth is supplied by breathing using an organic carbon source as substrate (Chojnacka and Zielinska, 2012). Furthermore, in this type of cultivation, the organic carbon has an effective role in cellular growth, lipids and fatty acids accumulation in microalgae cells (Xiong *et al*., 2008; Heredia-Arroyo *et al*., 2011; Wang *et al.*, 2012).

In this cultivation conditions it is necessary to reduce the source of nitrogen in order to achieve to redirect the metabolic route from protein production to lipid production. According to Liu *et al.* (2011) the microalgae only accumulate high lipid concentration under stress (specially with nutrients restriction) that also limit the cellular growth, and for this reason, it is difficult to balance cellular growth and lipid production simultaneously in microalgae. An alternative to obtain high biomass and lipid concentration is to carry out the growth in batch fed from carbon and nitrogen source, according to study made by Wang *et al*. (2012).

Besides that, it is possible to carry out the growth in two stages: the first with standard nitrogen concentration in the media, in order to obtain high biomass production. And, the second, to centrifuge this biomass, resuspend it and cultivate it in the absence of nitrogen having as objective to increase the biomass lipid level (Jiang *et al*., 2011), but in a heterotrophic manner.

When the experimental tests 1, 3, 5, 6 and 7, at

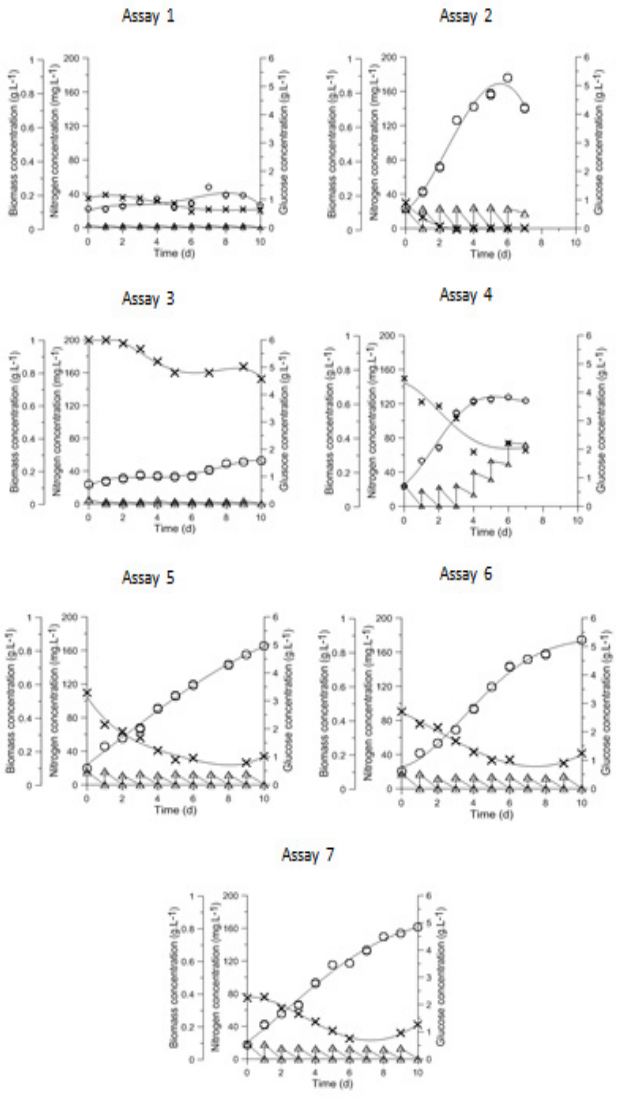

Figure 1. Cell growth curves in function of the consumption of the glucose and nitrogen. O (biomass concentration), Δ (glucose concentration) e × (nitrogen concentration).

Figure 1, were analyzed, it was possible to verify that the green alga *C. minutissima* totally consumed the glucose that was daily added. The microalga cellular growth was proportional to the glucose concentration added; it was already expected to happen since the carbon source contributes to the biomass production. The experimental test 1, carried out with less $NaNO_3$ (0.2 g.L^{-1}) concentration, resulted in 8.02% w/w of lipid, a 13.36% smaller value when compared to the lipid production achieved in experimental tests 5, 6 and 7, that were carried out with 0.75 g.L^{-1} of $NaNO_3$. When the nitrogen source is limited during cultivation, the objective is to increase lipid bioproduct. However, this was not observed, and the result can be justified by the influence of glucose concentration available for the different experimental tests.

Experimental tests 1 and 2 were carried out with

the same $NaNO_3$ (0.2 $g.L^{-1}$) concentration. When they are compared, it is possible to note that in test 1 the microalga grew only 0.1 $g.L^{-1}$, consumed approximately 40% of nitrogen and produced 21.36% less lipid than in test 2. When the curves in test 2 are observed, it is possible to note an evident exponential phase until the sixth cultivation day (X_{max}= 0.89 $g.L^{-1}$), this moment corresponds to the complete consumption of the glucose that was provided as carbon and energy source so that the microalga could perform all its nuclear functions and multiply. Furthermore, it is possible to verify that the microalga *C. minutissima* consumed all the nitrogen available in the culture media (0.2 $g.L^{-1}$ of $NaNO_3$), and that it already presented a reduced concentration of $NaNO_3$. It is possible to observe that from the second cultivation day on, when the nitrogen concentration in the media ceased, the microalga entered in an exponential phase, which was not expected. Due to the depletion of nitrogen concentration in the media, *C. minutissima* produced more lipid concentration. According to Lourenço (2006), when the available nitrogen concentrations for microalgae are low, it is possible to perceive an evident decrease in the cell division rate, besides the reduction in protein and chlorophyll concentrations. Thus, the lipid concentration can increase very much in relation to the cell protein total.

At experimental test 3, it was possible to verify that the microalga produced only 0.12 $g.L^{-1}$ of biomass and consumed 25% of the available nitrogen present in the culture media, producing the lowest lipid concentration (5.11% w/w). The graphic for experimental test 4 shows the absolute glucose consumption by the microalga until the third growth day. Subsequently, the carbon source concentration was gradually increasing, being 2.17 $g.L^{-1}$ available in the media during the seventh growth day. At the same time, the microalga multiplied and reached 0.64 $g.L^{-1}$ of maximum cell concentration. Besides that, a 60% decrease in nitrogen and a 300% increase in cellular concentration occurred until the fourth day of growth. From the fifth day on, the microalga started a stationary phase and *C. minutissima* did not consume nitrogen anymore, 43% were available to the cultivation media.

The experimental tests 5, 6 and 7, which corresponded to the central point of the experimental design, presented similar behavior. At the three experiments, the microalga consumed all the culture media glucose. Besides that, a decline in the nitrogen curve was observed until the sixth cultivation day and subsequently the concentration of the nitrogen source remained constant, there was not any consumption by the microalga anymore. The microalga cellular growth at exponential phase occurred until the last cultivation day, reaching approximately 0.8 $g.L^{-1}$ of biomass concentration. The lipid total concentration was 21.34% w/w in average, a much similar value to the one found in experimental test 4 (21.79% w/w).

The microalgae of the genus *Chlorella* are green algae that can grow both as autotrophic manner (CO_2 as carbon and energy source) or as heterotrophic (exogenous organic carbon as carbon and energy source). However, studies prove that when *Chlorella zofingiensis* (Liu *et al*., 2011), *Chlorella protothecoides* (Xu *et al*., 2006; Huang *et al.*, 2010), *Chlorella vulgaris* (Liu *et al*., 2008), *Chlorella* sp. (Hsieh and Wu, 2009) and *Chlorella saccharophila* (Isleten-Hosoglu *et al.*, 2012) were cultivated in an heterotrophic manner their lipid levels increased considerably.

Table 1 presents the experimental planning matrix, its real study variables codified, and responses analyzed. It was possible to verify that the lipid concentration varied from 5.11 to 29.38% w/w and that the maximum cellular concentration varied from 0.20 to 0.89 $g.L^{-1}$ when the Table 1 data was analyzed. It is noted that when the microalga *C. minutissima* was cultivated with 9 $g.L^{-1}$ of glucose and 0.2 $g.L^{-1}$ of $NaNO_3$ (Test 2) the biggest concentrations of lipid (29.38% w/w) and biomass (0.89 $g.L^{-1}$) were obtained. These results justify that the biggest organic carbon concentrations provides the biggest biomass production and that the nitrogen limitation in the culture media results in an increase for lipid levels in the biomass. The same results were found by other researchers in a study with microalga from the species *Chlorella* (Perez-Garcia *et al.*, 2011; Isleten-Hosoglu *et al*., 2012; Wang *et al*., 2012).

The factorial planning for test 2 was concluded on the seventh cultivation day and even so, the lipid productivity (25.27 $mg.L^{-1}.d^{-1}$) in this experiment was bigger than the one obtained in the other tests, which were carried out in 7 and 10 days of cultivation (Table 1). So, it is possible to verify that the microalga was very efficient in biomass production with high lipid content when it used the biggest glucose concentration (9 $g.L^{-1}$) and smallest $NaNO_3$ (0.2 $g.L^{-1}$) concentration.

Table 1 also shows that the smallest microalga growth (0.20 and 0.27 $g.L^{-1}$) was achieved in the tests 1 and 3, respectively. They were conducted with the smallest glucose concentration (1 $g.L^{-1}$). This result was already expected since the organic carbon source in heterotrophic cultivation is the one that provides the energy necessary for cellular growth as well as the development on some important cellular functions.

Table 2. Conversion factors calculated for different factorial planning experiments.

Experiment	Conversion Factors					
	$Y_{P/S1}$	$Y_{P/S2}$	$Y_{X/S1}$	$Y_{X/S2}$		
1	0.0112	0.9333	0.10	8.33		
2	0.0298	8.4200	0.09	24.67		
3	0.0049	0.0049	0.12	2.50		
4	0.0191	1.5547	0.07	5.83		
5	0.0340	2.2421	0.14	8.95		
6	0.0362	3.7020	0.15	14.90		
7	0.0321	4.8727	0.13	20.00		
Experiment	$S_{1\,final}$ ($g.L^{-1}$)	$S_{1\,initial}$ ($g.L^{-1}$)	$S_{2\,final}$ ($g.L^{-1}$)	$S_{2\,initial}$ ($g.L^{-1}$)	Lip. $_{final}$ ($g.L^{-1}$)	X_{max} ($g.L^{-1}$)
1	0.00	1	0.021	0.033	0.020	0.25
2	0.53	9	0.000	0.030	0.2614	0.89
3	0.00	1	0.152	0.200	0.0137	0.27
4	2.17	9	0.066	0.150	0.1394	0.64
5	0.00	5	0.034	0.110	0.1792	0.83
6	0.00	5	0.042	0.091	0.1902	0.88
7	0.00	5	0.042	0.075	0.1696	0.81

$Y_{P/S1}$: conversion factor for glucose into lipid; $Y_{P/S2}$: conversion factor for nitrogen into lipid; $Y_{X/S1}$: conversion factor for glucose into biomass; $Y_{X/S2}$: conversion factor for nitrogen into biomass; S_1: glucose concentration ($g.L^{-1}$); S_2: nitrogen concentration ($g.L^{-1}$); Lip.: Lipids; X_{max}: maximum cellular concentration.

Regarding lipid content, at test 1 the microalga accumulated 2.91% w/w intracellularly more than in test 3, due to the depletion of nitrogen.

Tests 2 and 4 from the factorial planning, which were carried out with the biggest glucose concentration (9 $g.L^{-1}$), it is possible to make the same relations as for tests 1 and 3, concerning the lipid levels. That is, the restriction for nitrogen source in the culture media caused cellular stress, redirecting the metabolic route from protein production to lipid production; this resulted in a higher oil concentration in the biomass of microalga *C. minutissima*. Therefore, as to the biomass maximum concentration, it was expected that it reached the highest value at test 4, since it was conducted in the factorial planning maximum conditions.

The tests in the central point of the experimental planning (5, 6 and 7) presented lipid concentration values very similar to the ones from test 4. These results point out that it is more economically advantageous to carry out the heterotrophic growth of the microalga *Chlorella minutissima* using lower glucose concentration (5 $g.L^{-1}$) and $NaNO_3$ (0.75 $g.L^{-1}$) concentration.

When the values for lipid productivity in this study are compared in relation to the matter (heterotrophic), with the autotrophic cultivation of various microalgae genus (Song *et al.*, 2013), it is possible to verify that the heterotrophic cultivation provided higher lipid productivity. The values varied from 1.12 to 25.27 $mg.L^{-1}.d^{-1}$ in 10 days of cultivation, while the autotrophic cultivation, carried out during 15 days, these values ranged from 0.84 to 7.96 $mg.L^{-1}.d^{-1}$. However, the autotrophic study carried out with the microalga *Chlorella sorokiniana* (Chen *et al.*, 2013), some values for lipid productivity were higher, because they varied from 10.3 to 140.8 $mg.L^{-1}.d^{-1}$. This results points out that not only the cultivation manner (autotrophic or heterotrophic), but also the strain studied, the nutrient concentration in the culture media, the nutrient sources, the temperature, the shaking, among other parameters will define the type and the concentration of the bioproduct obtained.

Table 2 presents the conversion factors for substrate into product ($Y_{P/S}$) and for substrate into cell ($Y_{X/S}$). The substrates considered are glucose (S1) and nitrogen (S2), product (P) is the lipid concentration and cell (X) is the biomass. The biggest conversion factors $Y_{P/S1} = 0.0362$ and $Y_{X/S1} = 0.15$ were achieved at experiment 6. Being so, 1 g of glucose produces 0.0362 g of lipids and 0.15 g of biomass. In test 2 there was the biggest conversion of nitrogen into lipids ($Y_{P/S2} = 8.4200$) and of nitrogen into biomass ($Y_{X/S2} = 24.67$), this indicates that 1 g of nitrogen originates 8.4200 g of lipids and 24.67 g of biomass.

The data in Table 2 show that not always the glucose substrate (tests 2 and 4) nor the nitrogen substrate (1, 3, 4, 5, 6 and 7) were completely

Table 3. Microalga *Chlorella minutissima* chromatographic profile (% w/w) cultivated with 9 g.L^{-1} of glucose and 0.2 g.L^{-1} of $NaNO_3$

FA	Concentration (% w/w)	FA	Concentration (% w/w)
C12:0	0.4	C20:0	2.9
C14:0	0.3	C20:1n9	2.3
C14:1	1.0	C20:3n6	2.9
C15:0	0.6	C20:3n3	0.2
C15:1	0.1	C20:4n6	*
C16:0	10.3	C20:5n3	*
C16:1	2.6	C22:0	3.4
C17:0	*	C22:1n9	0.2
C17:1	0.2	C22:2	3.2
C18:0	6.0	C22:6n3	0.6
C18:1n9	55.1	C23:0	*
C18:2n6	2.2	C24:0	2.7
C18:3n6	1.4	C24:1n9	0.9
C18:3n3	0.5		
SFA (%)		26.6	
MUFA (%)		62.4	
PUFA (%)		11.0	
ω6+ω3 (%)		7.8	

FA: Fatty acids; n3: ω3 fatty acid; n6: ω6 fatty acid; n9: ω9 fatty acid; *: not detected; SFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids.

depleted when the cellular concentration presented its maximum value, it was still possible to exist residual concentration for glucose or nitrogen in the culture media, at the end of the cultivation. According to Schmidell *et al.* (2001), this happens because as far as the microorganism reproduces itself, products that inhibit cellular growth are formed, not to mention the own substrate, that can hinder the bacterial activity. Moreover, these authors mention that a specific substrate will not always produce a proportional biomass increase ($Y_{X/S1}$ and $Y_{X/S2}$), being that a part of the energy, resulting from that consumption, is intended to the maintenance of the microorganism vital functions.

Table 3 presents the microalga *C. minutissima* chromatographic profile when cultivated at the highest carbon source concentration condition (9 g.L^{-1}) and the lowest nitrogen source concentration (0.2 g.L^{-1}) in the culture media, this resulted in the highest total lipid concentration (29.38% w/w). The culture media composition, specially carbon and nitrogen sources, is a critical factor to the microalgae growth, which can grow in an autotrophic or heterotrophic manner (Shen *et al*., 2009; O'Grady e Morgan, 2011; Perez-Garcia *et al*., 2011).

In heterotrophic conditions, the lipid content in some *Chlorella* species can vary an average from 10 to 55% in dry weight (Liang *et al*., 2009; Shen *et al.*, 2009; Perez-Garcia *et al.*, 2010; O'Grady and Morgan, 2011; Liu *et al*., 2011), and the fatty acid composition has been reported among C14:0 and C20:0 (Petkov and Garcia, 2007; O'Grady and Morgan, 2011; Liu *et al*., 2011; Isleten-Hosoglu *et al*., 2012; Wang *et al*., 2012).

C. minutissima microalga, of the study in question, produced high oleic fatty acid concentration (C18:1, 55.1% w/w), and this was superior to the one produced by the microalga *Chlorella protothecoides* in which the concentration C18:1 varied from 36 to 43% w/w (O'Grady and Morgan, 2011) and *Chlorella saccharophila* when the concentration of C18:1 varied from 21.1 to 32.2% w/w (Isleten-Hosoglu *et al*., 2012). However, the strain of *Chlorella protothecoides* (O'Grady and Morgan, 2011) and *Chlorella saccharophila* (Isleten-Hosoglu *et al.*, 2012) produced highest concentration of linoleic fatty acid (C18:2) with values ranging from 4.7 to 2.1% w/w and 22.7 to 31.1% w/w, respectively.

SFA concentrations (26.6%) and MUFA (62.4%) were also superior in the study with microalga *Chlorella minutissima* when compared to studies carried out with microalga *Chlorella zofingiensis* (SFA = 23.6%, MUFA = 37.4%) (Liu *et al*., 2011) and *Chlorella kessleri* (SFA varying from 34.75 to 41.34% and MUFA from 17.51 to 24.07% (Wang *et al*., 2012). The PUFA concentration was superior for these two microalgae species with values from 39.1% to *Chlorella zofingiensis* (Liu *et al.*, 2011) and from 40 to 44.72% to the microalga *Chlorella kessleri* (Wang *et al*., 2012).

In the analysis of variance (ANOVA) of the results obtained for total lipid concentration the parameters statistically non-significant were incorporated to the residues. The statistic analysis results showed that just

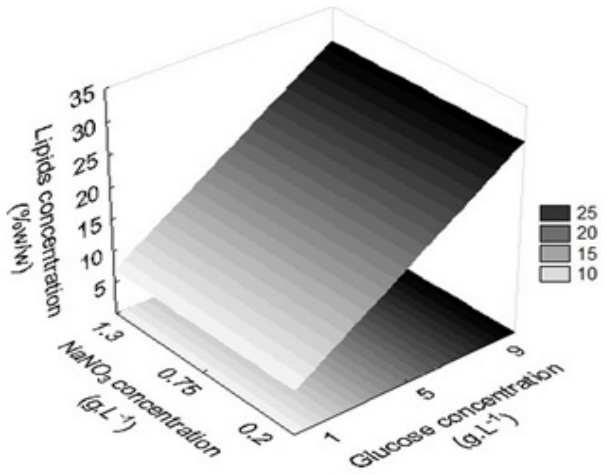

Figure 2. Response surface concentration of total lipids in relation to variables glucose concentration and $NaNO_3$ concentration.

the glucose concentration variable was significant to a 95% confidence level. The value F calculated (22.14) for regression was higher than the F tabulated (6.60). The model has an explained variation percentage of 89% (R^2). Equation 4 represents the obtained codified model that describes the response surface of total lipid concentration, where X_1 = glucose concentration.

$$\text{Total lipids} = 18.35 + 9.51.X_1 \quad (4)$$

Based on the model obtained, it was possible to achieve the response surface in order to establish the best conditions for glucose concentration and $NaNO_3$ concentration that provide the highest total lipid level. The response surface is presented in Figure 2. The results achieved in Table 1 are proved through the response surface (Figure 2), since it is possible to verify that the highest lipid concentration, with values around 30% w/w was achieved when the microalga *Chlorella minutissima* was cultivated with 9 $g.L^{-1}$ of glucose and 0.2 $g.L^{-1}$ of $NaNO_3$.

Moreover, it is possible to check in Figure 2 that when we pass the level of glucose concentration variable from -1 (1 $g.L^{-1}$) to the level +1 (9 $g.L^{-1}$) we observe a positive effect of 19% in the response variable total lipid concentration. Through the data of maximum cellular concentration response, achieved in the experimental planning, was verified that there was not any significant effect ($p \leq 0.05$) for the variables studied on the response X_{max}, it is not possible to generate a significant and predictive statistic model for this experiment.

The microalga *Chlorella minutissima* produced the highest lipid concentration (29.38% w/w) using the lowest nitrogen source (0.2 $g.L^{-1}$ of $NaNO_3$), it results in nutrient saving in the culture media generating lower costs in the biomass and lipid production, which can be used to elaborate functional foods and even to biodiesel production. Furthermore, the high concentration of oleic acid (C18:1, 55.1% w/w) achieved is very profitable because this fatty acid is the precursor in the ω9 fatty acid family and vital for construction, membrane protection, protection and barrier against dehydration, because it is present in the epidermis (skin most external layer). Besides, it is very important for hormone formation.

Conclusion

When the microalga *Chlorella minutissima* was cultivated with 9 $g.L^{-1}$ of glucose and 0.2 $g.L^{-1}$ of $NaNO_3$ (test 2), it presented the highest lipid productivity (2.27 $mg.L^{-1}.d^{-1}$), with predominance of oleic fatty acid (C18:1 – 55.1% w/w) and the highest biomass production (0.89 $g.L^{-1}$). The glucose concentration significantly influenced ($p \leq 0.05$) just the total lipid concentration. Moreover, both study variables (glucose and $NaNO_3$ concentrations) do not have a significant effect ($p \leq 0.05$) in the maximum biomass concentration.

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