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Fatty acid profile of *Chlorella* biomass obtained by fed batch heterotrophic cultivation

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Abstract

The cultivation of microalgae is of great interest due to the high yields and rapid growth rates that can be produced. The fatty acids from microalgal biomass may have therapeutic effects for humans and can be used for biodiesel production. The aim of this study was to evaluate the fatty acid profile of microalgae grown in a heterotrophic mode. Cultures that were carried out with BG11 medium supplemented with 10 $g.L^{-1}$ of glucose produced the highest cellular concentrations (1.62; 1.53; 1.14 $g.L^{-1}$) for *Chlorella* sp., *C. homosphaera* and *C. minutissima*, respectively, while the assays without glucose remained at a cellular concentration equal to that at the beginning of the experiments (0.15 $g.L^{-1}$). The microalga *C. homosphaera* grown in BG11 supplemented with 10 $g.L^{-1}$ of glucose had the highest concentration of lipids in dry biomass (22.4% w/w). The maximum concentration of total PUFA (35.25% w/w) and essential fatty acids (35.05% w/w) was found in *C. homosphaera* in Basal medium without glucose, which is the most suitable method for producing PUFAs and essential fatty acids.

Introduction

The cultivation of microalgae has been developed because of its many advantages: simple nutrients, the doubling of biomass in a short period of time and the possibility of manipulating the conditions, in order to increase the production of a specific metabolite, such as fatty acids (Costa *et al.*, 2006). Lipids from microalgae consist of different saturated and unsaturated fatty acids, some of them from the ω3 and ω6 families (Hu *et al*., 2008; Huang *et al*., 2010). The increased yields of biomass to obtain unsaturated fatty acids have attracted commercial interest, mainly as a source of ω3 (Bertoldi *et al*., 2008). The intake of ω3 obtained from microalgae is beneficial to neural development and also prevents coronary problems, cancer, hypertension, diabetes, cystic fibrosis, arthritis, asthma, depression and schizophrenia (Kris-Etherton *et al*., 2002; Adarme-Vega *et al*., 2012).

The fatty acids in microalgae correspond to the largest lipidic fraction and, in some species, polyunsaturated fatty acids (PUFA's) represent between 25 and 60% of total lipids (Becker, 2004). Compared to higher plants, microalgae have a greater photosynthetic efficiency and can be grown in simple saline medium (Olguín *et al*., 2001). Regarding the production of lipids, microalgae can produce at least fifteen times more than the palm *(Elaeis guineensis)*, one of the most productive vegetables. The estimated yield of lipids by microalgae varies from 15,000 to 30,000 L km^{-2} and extraction is simple: traditional methods can be used in the chemical industry, including solvent extraction (Radmann and Costa, 2008).

Microalgal fatty acids are mainly used to enrich feeds for fish, in biodiesel production and as a source of essential fatty acids in the human diet (Morais and Costa, 2008). The heterotrophic cultivation of microalgae uses organic compounds without light, and organic carbon is used as an energy source for the production of biomass (Chojnacka and Marquez-Rocha, 2004). The most commonly used organic sources are sugar substrates, especially glucose (Xu *et al*., 2006; Miao and Wu 2006; Liang *et al*., 2009; Heredia-Arroyo *et al*., 2010; Shen *et al*., 2010; O'Grady and Morgan, 2011), but other sources of organic carbon can be used, such as glycerol (Liang *et al.*, 2009; O'Grady and Morgan, 2011) and acetate (Liang *et al*., 2009; Heredia-Arroyo *et al*., 2010). This type of cultivation has several advantages over autotrophic cultivation, such as eliminating the variation of the natural light source, control over the process and the low cost of biomass recovery due to the high cellular density obtained (Xu *et al.*, 2006; Miao and Wu, 2006; Huang *et al.*, 2010).

According to Wu *et al.* (1994), some species

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of *Chlorella* are capable of heterotrophic and photoautotrophic growth in the environment, such as *Chlorella protothecoides*. Xu *et al*. (2006) cultivated microalgae that in both modes, autotrophic and heterotrophic, and 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 microalgae *Chlorella homosphaera*, *Chlorella* sp. and *Chlorella minutissima* Bank Biochemical Engineering Laboratory strains (LEB) of the Federal University of Rio Grande (FURG) to conduct the study. The aim of this study was to evaluate the fatty acid profile of the microalgae *Chlorella homosphaera, Chlorella* sp. and *Chlorella minutissima* grown under heterotrophic conditions.

Materials and Methods

Micro-organisms and culture media

The microalgae used in this study were *Chlorella homosphaera, Chlorella* sp. and *Chlorella minutissima* (Costa *et al*., 2006), all from the cultures collection of the Biochemical Engineering Laboratory, Federal University of Rio Grande (FURG). All microalgae were maintained and grown in BG11 medium (Rippka *et al*., 1979) containing ($g.L^{-1}$): $NaNO_3$ (1.50); K_2HPO_4,$3H_2O$ (0.04); $MgSO_4$,$7H_2O$ (0.075); $CaCl_2$,$2H_2O$ (0.036); C6H11FeNO7 (0.006); disodium EDTA (0.001); Na_2CO_3 (0.02); C6H8O7 (0.006); H_3BO_3 (2.86); $MnCl_2$,$4H_2O$ (1.81); $ZnSO_4$,$7H_2O$ (0.222); Na_2MoO_4,$2H_2O$ (0.39); $CuSO_4$,$5H_2O$ (0.079); $Co(NO_3)_2$,$6H_2O$ (0.0494); and in Basal medium (Xiong *et al*., 2008) containing ($g.L^{-1}$): KH_2PO_4 (0.7); K_2HPO_4 (0.3); $MgSO_4 \cdot 7H_2O$ (0.3); $FeSO_4 \cdot 7H_2O$ (0.003); glycine (0.1); vitamin B1 (0.00001) and 1 $mL.L^{-1}$ of A5 mineral solution.

Growth conditions

The cultures were carried out under heterotrophic conditions using glucose as the carbon and energy source. Basal and BG11 media were supplemented with 0; 5 and 10 $g.L^{-1}$ of glucose. There were three assays performed in duplicate for each microalga in both culture media, totaling 36 assays. Each day glucose was added to the cultures in fed batch mode, at a ratio of 1/10 of the total concentration (5 $g.L^{-1}$ and 10 $g.L^{-1}$) of glucose during the 10 days of the experiment. The cultures were carried out in shaker (INNOVA®44, USA) at 150 rpm and 30°C using 2L closed bioreactors with a working volume of 1.6 L (*C. homosphaera* and *C. minutissima*) and 1.4 L (*Chlorella* sp.). The initial biomass concentration was 0.15 $g.L^{-1}$. At the end of cultivation, the samples were centrifuged, dried at 40°C and ground.

Analytical determinations

Daily samples were aseptically collected for biomass determination, which was calculated using a 670 nm optical density in a spectrophotometer (Femto Plus 700, Brazil) with the help of a predetermined calibration curve. Were evaluated the maximum concentration of biomass (X_{max}, $g.L^{-1}$), the maximum productivity (P_{max}, $g.L^{-1}.d^{-1}$) calculated according to the equation $P = (X_t X_0)/(t\ t_0)$, where X_t is the biomass concentration ($g.L^{-1}$) at time t (d) and X_0 the biomass concentration ($g.L^{-1}$) at time t_0 (d) (Schmidell *et al*., 2001) and the maximum specific growth rate (μmax, d^{-1}) by exponential regression applied to the logarithmic growth phase (Bailey and Ollis, 1986).

The culture medium's glucose concentration was determined every 48 hours using the enzymatic colorimetric kit Glucose PAP Liquiform (Labtest, Brazil), with a reading absorbance of 505 nm and converted to the glucose concentration by using a calibration curve. The pH of the cultures was determined using a digital pH meter (Quimis Q400HM, Brazil) and at the end of cultivation, the samples were centrifuged, dried at 40°C for 60 h and ground.

Quantification of total lipids and fatty acids profile

For the quantification of total lipids, the methodology proposed by Folch *et al.* (1957) was used with a preliminary stage of breaking up the cell wall using an ultrasonic bath. The lipid fraction was esterified to obtain methyl esters of fatty acids, according to the adapted methodology of Metcalfe *et al.* (1966).

The fatty acid analysis was carried out in a 3400CX Varian Gas Chromatograph equipped with a flame ionization detector and ZB-WAX column that was 30 m in length and has an internal diameter of 0.32 mm and 0.25 μm of film. The carrier gas was hydrogen at 0.5 mL min^{-1}. The temperatures of the injector and detector were 250 and 300°C, respectively. The initial column temperature was 40°C increasing 6°C/min to 100°C for 1 min, then to 160°C for 5 min, and 230°C for 10 min. The fatty acids were identified by comparison of retention times with standards (Sigma Supelco; Belle-fonte, EUA) and were quantified by normalization of areas.

The standards of fatty acids (Supelco Sigma; Belle-fonte, USA) used were butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0) acid, 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 (C18:1n9c), elaidic acid (C18:1n9t), linoleic acid (C18:2n6c) linolelaidic acid (C18: 2n6t), alpha linolenic acid (C18:3n3), gamma-linolenic 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), henicosanoic acid (C21:0), behenic acid (C22:0), erucic 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).

Results and Discussion

Figure 1 shows the growth curves of heterotrophic cultures of the microalgae *Chlorella homosphaera*, *Chlorella* sp. and *Chlorella minutissima*. Only those cultures supplemented with glucose had an increased cellular concentration over 10 days of cultivation. The microalgae *C. homosphaera* and *Chlorella minutissima* presented a stationary phase after six days when grown in BG11 medium supplemented with 10 $g.L^{-1}$ glucose. Figure 1 shows that all three microalgae had greater logarithmic growth when cultivated with BG11 supplemented with 10 $g.L^{-1}$ glucose compared with the other assays. Figure 1a and 1c show that the microalgae *Chlorella homosphaera* and *Chlorella minutissima* had an exponential phase (1st to 6th day), but this was lower when the cultures were carried out with BG11 with 5 $g.L^{-1}$ of added glucose.

The greatest growth of the microalgae occurred when they were grown with the highest concentration of glucose (10 $g.L^{-1}$); this probably occurred because the cultivation was heterotrophic, so the microalgae used an organic source, in this case glucose, as the carbon and energy source, thus requiring more nutrient support to maintain their physiological functions and to grow.

Table 1 shows the values of cell maximum cellular concentration (X_{max}, $g.L^{-1}$), maximum productivity (P_{max}, $g.L^{-1}.d^{-1}$), maximum specific growth rate (μ_{max}, $1.d^{-1}$), lipid (% w/w), PUFA (% w/w) and ω6 + ω3 (% w/w) concentrations for tests with the three species of *Chlorella*. The microalgae *C. homosphaera* reached 1.53 $g.L^{-1}$ in 10 days when grown in the dark in BG11 medium supplemented with 10 $g.L^{-1}$ of glucose. When

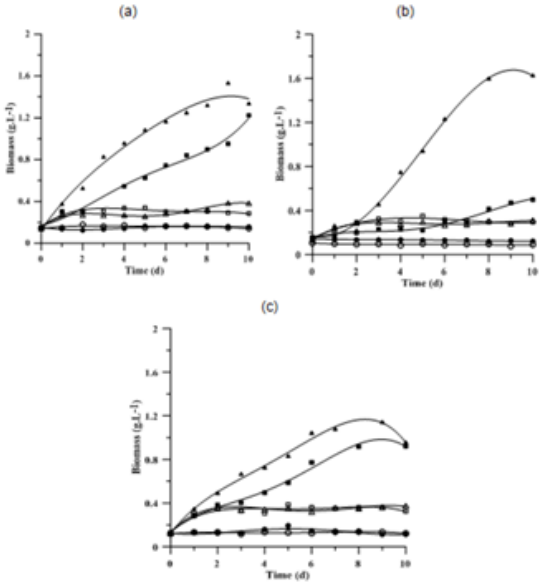

Figure 1. Growth curves of microalgae grown in BG11 and Basal media: (a) *Chlorella homosphaera* (b) *Chlorella* sp. and (c) *Chlorella minutissima*, grown in (●) BG11 + 0 $g.L^{-1}$ glucose; (■) BG11 + 5 $g.L^{-1}$ glucose; (▲) BG11 + 10 $g.L^{-1}$ glucose; (○) Basal + 0 $g.L^{-1}$ glucose; (□) Basal + 5 $g.L^{-1}$ glucose; (Δ) Basal + 10 $g.L^{-1}$ glucose.

cultivated with the addition of 5 $g.L^{-1}$ of glucose in the medium, *C. homosphaera* reached 1.22 $g.L^{-1}$. Lower results were found by Liang *et al.* (2009), when they cultivated *C. vulgaris* in the dark with 1% glucose, which achieved a maximum cellular concentration of 1.20 $g.L^{-1}$ and maximum productivity of 0.15 $g.L^{-1}.d^{-1}$. However, Xu *et al.* (2006) found higher values for the microalga *Chlorella protothecoides*, but under heterotrophic conditions. These authors observed that cellular growth reached 3.92 $g.L^{-1}$ and 3.74 $g.L^{-1}$ in 6 days of cultivation when they used hydrolyzed corn and glucose as the carbon source, respectively. Both cultivation were carried out in basal medium with limitation of the nitrogen source (glycine). Liu *et al.* (2011) cultivated *Chlorella zofingiensis* autotrophically and heterotrophically and found best results (μ= 0.769 d^{-1} and X_{max}= 9.7 $g.L^{-1}$) for the heterotrophic cultivation.

Some species of *Chlorella* have the ability to grow in an autotrophic and heterotrophic environment. Studies by Xu *et al*. (2006) and Liu *et al*. (2011) proved that this microalga has greater yields when grown in heterotrophic mode using an exogenous carbon source such as glucose. Comparing these authors' results with this study shows there is a difference in maximum cellular growth achieved by the microalgae. These data show that although the *Chlorellas* grew well in the absence of light, there are differences regarding the species (strain) studied.

The maximum cell concentration (X_{max}, Table 1)

Table 1. Maximum cellular concentration ($X_{max,}$ $g.L^{-1}$), maximum productivity (P_{max}, $g.L^{-1}.d^{-1}$), maximum specific velocity (μ_{max},$1.d^{-1}$), concentrations of lipids (% w/w), polyunsaturated fatty acids - PUFA (% w/w) and essential fatty acids ω3 + ω6 (% w/w) for assays the three species of *Chlorella.*

Medium	Glucose ($g.L^{-1}$)	X_{max} ($g.L^{-1}$)	P_{max} ($g.L^{-1}.d^{-1}$)	μ_{max} (d^{-1})	Lipids (% w/w)	PUFA (% w/w)	ω6+ω3 (% w/w)
			Chlorella homosphaera				
BG11	0	0.15	0.03	-	15.0±0	6.1±0	4.6±0
	5	1.22	0.30	0.23	11.1±0.64	18.6±0.85	19.1±1.06
	10	1.53	0.32	0.59	22.4±0	20.2±2.55	19.5±0
Basal	0	0.15	0.03	-	19.3±3.96	35.25±1.06	35.05±1.06
	5	0.34	0.16	-	13.8±1.20	20.45±9.97	19.85±9.69
	10	0.39	0.13	-	16.5±4.95	24.75±2.05	24.3±1.84
			Chlorella sp.				
BG11	0	0.15	0.01	-	11.4±0	10.6±0.9	10.0±0.71
	5	0.50	0.29	0.08	9.0±0.14	3.6±1.13	3.45±1.06
	10	1.62	0.38	0.35	12.9±0	15.75±0.64	15.35±0.92
Basal	0	0.15	0.02	-	7.2±0	24.35±0.21	24.05±0.21
	5	0.35	0.09	-	21.0±0	18.2±0	17.75±0
	10	0.31	0.10	-	11.9±2.55	19.5±1.13	19.4±0.99
			Chlorella minutissima				
BG11	0	0.15	0.04	-	14.4±0	16.8±0	16.1±0
	5	0.92	0.19	0.18	8.4±0.42	21.45±1.06	21.0±1.13
	10	1.14	0.26	0.22	8.7±0.71	18.55±1.34	17.95±0.92
Basal	0	0.15	0.03	-	15.3±2.47	19.6±0.14	18.9±0.28
	5	0.39	0.16	-	17.1±0	21.7±0	21.4±0
	10	0.38	0.20	-	21.5±1.13	25.65±8.84	24.95±7.99

for the three microalgae studied occurred in BG11 medium supplemented with 10 $g.L^{-1}$ of glucose. However, this is not observed for the production of lipids, PUFA and essential ω6 + ω3, where the maximum values varied according to the species of microalga. As a result, the differentiated quantities of bioactives produced by the microalgae may be associated with the metabolism of the carbon source of each species. According to Narang and Pilyugin (2005), the glucose concentration required for optimal metabolic growth may be related to the combination of factors specific to the microalgal species as a primary factor, and the environmental conditions and cultivation as a secondary factor. Consequently, each combination of factors can lead to different levels of consumption and production of bioproducts.

The maximum yields for the three microalgae were achieved in the tests carried out in BG11 medium supplemented with 10 $g.L^{-1}$ of glucose, which indicates a more rapid growth in the same time interval when compared with other assays in other culture conditions. This is probably due to the different nutrients that are present in the cultivation medium and the greater concentration of organic carbon.

The maximum specific growth rates achieved were 0.59 $1.d^{-1}$ and 0.35 $1.d^{-1}$ for the microalgae *C. homosphaera* and *Chlorella* sp. respectively, both cultivated in BG11 medium supplemented with 10 $g.L^{-1}$ of glucose. Were not considered μmax values for cultures performed with BG11 medium without addition of glucose and Basal Medium with because there is no exponential phase of cell growth.

The highest concentration of lipids (22.4% w/w) was obtained in the assay with the microalga *C. homosphaera* grown in BG11 medium supplemented with 10 $g.L^{-1}$ of glucose. Lower results were found by Costa *et al.* (2006), where *C. minutissima* obtained 7.98% w/w when grown at 30°C, 2500 Lux, $NaHCO_3$ and 0.5 $g.L^{-1}$ of NO_3. However, higher results were obtained by Xu *et al.* (2006): the cells of *Chlorella protothecoides* presented 55.2% w/w and 54.7% w/w

Table 2. Chromatographic profile (% w/w) of microalgae grown under different conditions

FA	*Chlorella homosphaera*						*Chlorella* sp.						*Chlorella minutissima*					
	BG11			Basal			BG11			Basal			BG11			Basal		
	Glucose ($g.L^{-1}$)						Glucose ($g.L^{-1}$)						Glucose ($g.L^{-1}$)					
	0	5	10	0	5	10	0	5	10	0	5	10	0	5	10	0	5	10
C12:0	4.1	2.5	4.9	1.9	19.4	1.6	0.2	6.9	4.5	4.9	3.5	2.6	*	3.5	3.5	*	3.6	*
C14:0	1.3	1.6	1.0	0.7	4.8	0.5	1.6	1.0	1.6	1.4	0.7	0.7	0.9	1.1	0.5	1.1	0.6	0.2
C14:1	1.0	0.9	0.9	0.2	4.0	0.3	0.3	0.6	0.8	0.7	0.7	0.7	0.7	0.7	1.3	0.7	1.2	0.5
C15:0	1.3	1.7	1.1	0.6	1.2	0.3	0.3	1.2	0.9	1.8	0.7	0.3	5.7	1.5	1.7	1.6	0.5	1.9
C15:1	0.3	0.8	0.8	0.2	0.2	0.1	2.4	0.5	1.3	3.1	0.5	0.6	2.3	1.1	0.3	0.9	0.3	2.7
C16:0	22.1	23.6	23.7	15.8	28.2	26.2	18.1	20.3	19.6	14.5	19.1	20.8	17.3	21.6	22.6	16.7	29.6	21.9
C16:1	15.1	8.1	8.5	11.9	3.7	6.4	26.4	11.9	13.8	16.1	7.5	4.9	14.2	8.6	6.4	18.4	5.9	3.1
C17:0	2.7	1.6	*	4.9	0.4	1.7	0.3	0.9	5.4	9.5	*	*	*	*	0.3	*	0.3	*
C17:1	*	*	*	0.3	*	0.5	0.6	12.6	7.2	0.4	*	*	*	*	*	*	*	*
C18:0	9.4	2.4	2.4	3.9	7.9	0.8	3.4	2.7	2.2	1.1	4.1	3.1	0.9	2.9	5.5	0.7	0.7	1.2
C18:1n9	28.7	22.4	22.3	10.4	7.3	30.0	33.9	32.1	19.9	10.5	29.9	35.1	9.0	22.1	33.7	7.0	27.1	24.2
C18:2n6	1.5	16.9	16.8	33.9	8.0	19.8	6.4	2.1	14.6	23.6	16.9	18.9	8.6	15.5	14.3	13.8	20.8	18.5
C20:0	7.1	14.6	11.4	13.7	0.9	6.4	1.0	3.7	6.3	12.3	12.9	10.1	30.8	13.0	4.8	32.7	6.8	17.3
C18:3n6	0.6	0.4	0.5	0.8	8.2	0.4	0.5	0.4	0.2	0.5	0.3	0.3	5.4	2.3	1.4	3.3	0.2	2.9
C18:3n3	0.6	0.3	0.6	0.1	0.8	2.2	2.0	0.5	0.2	*	0.2	0.3	0.2	*	0.3	0.2	0.2	2.3
C20:1n9	0.2	0.6	0.3	0.4	0.2	0.2	0.2	0.5	0.5	0.6	0.2	*	0.2	0.5	0.4	0.1	0.6	0.4
C20:5	0.5	*	0.3	*	0.4	0.2	*	*	0.5	0.6	0.4	0.2	*	*	0.4	0.6	0.4	0.4
C20:3n6	0.3	*	*	*	0.2	0.4	0.1	0.2	*	*	*	*	*	*	0.2	0.2	*	0.5
C20:3n3	*	*	*	*	0.4	0.1	*	*	*	*	*	*	*	1.6	1.0	0.2	*	0.2
C20:4n6	0.2	0.2	0.2	0.2	0.5	0.3	0.2	0.1	0.1	*	0.1	*	1.5	0.6	1.0	1.0	0.2	0.3
C22:2	1.0	0.2	0.3	0.2	0.3	0.3	0.5	0.2	0.2	*	0.5	*	0.6	0.4	0.5	0.2	*	0.7
C20:5n3	1.1	0.4	1.1	0.2	0.3	0.2	0.8	0.2	*	*	0.5	0.5	*	*	0.3	0.2	*	0.2
C24:0	0.3	0.2	1.3	0.2	0.6	0.3	0.8	1.0	2.7	0.7	1.5	1.2	1.0	1.6	0.7	0.4	0.4	0.6
C24:1n9	0.3	0.6	0.4	0.2	0.6	0.3	0.5	0.7	1.6	0.5	0.9	0.7	0.2	0.6	0.2	0.4	0.7	0.5
C22:6n3	0.4	0.3	0.3	0.1	2	1.1	0.6	0.2	0.6	*	0.1	*	0.2	0.8	0.7	0.4	*	0.3

FA:Fatty acids; t: *trans* fatty acids; c: cis fatty acids; n3: ω3 fatty acid; n6: ω6 fatty acid; n9: ω9 fatty acid; *: not detected

of lipids in heterotrophic cultivation using hydrolyzed corn and glucose, respectively, as a carbon source.

In Table 1, the highest concentrations of PUFA (35.25% w/w) and essential fatty acids (35.05% w/w) are obtained by microalga *C. homosphaera*, but when cultivated in Basal medium without added glucose. This result shows that the carbon source Basal culture medium was sufficient, within 10 days of cultivation, micoralga for maintaining their physiological functions and even produce lipids. Radmann and Costa (2008) cultivated *C. vulgaris* in 12% w/w CO_2, 60µL.L^{-1} SO_2, 100 µL.L^{-1} NO at 30°C, was obtained 10.1% w/w of polynstaurated fatty acids (ω6 + ω3).

Although the Basal medium resulted in the highest concentrations of lipids, PUFA and ω6 + ω3, Table 1 shows that the cellular growth of the three microalgae was lower in this medium when compared with culttivation with BG11 medium. This indicates that the microalga *Chlorella* withstands nitrogen deficiency and can alter its metabolic pathway, reducing protein synthesis and hence increasing lipid synthesis. These results are consistent with those of Illman *et al*. (2000), Macedo and Alegre (2001), Zhila *et al.* (2005), Xiong *et al.* (2008), Hu *et al.* (2008), Griffiths and Harrison (2009), Liang *et al.* (2009), Widjaja *et al.* (2009) and Shen *et al.* (2010) who found that high concentrations of nitrogen resulted in higher biomass concentrations, whereas higher concentrations of lipids are obtained at lower nitrogen concentrations. Wei *et al.* (2009) observed in the heterotrophic cultivation of the microalga *Chlorella protothecoides*, that the lowest amount of added glucose was accompanied by a higher content of lipids and lower cellular concentration. The addition of 10 $g.L^{-1}$ of glucose resulted in 34.5% w/w of lipids. Furthermore, when the microorganism cell

multiplication ceases and enters the stationary phase, lipid metabolism and more pronounced. Thus, it is possible to justify the synthesis of biomolecules, in this case of lipids by microalgae starts when it ceases the production of biomass.

Microalgae are photoautotrophic microorganisms and when subjected to stress conditions, they are stimulated to produce higher concentrations of a specific metabolite. The absence of light and nitrogen in the culture medium causes cellular stress, diverting the cellular metabolism of microalgae to lipid production. Therefore, the highest concentration of PUFA and essential fatty acids observed in the cultivation carried out with the Basal culture medium may be explained by the low concentration of glycine ($C_2H_5O_2N$ = 0.1 $g.L^{-1}$) available in the medium.

Perez-Garcia *et al*. (2011) reported that in the heterotrophic cultivation of microalgae, oxidative glucose assimilation begins with the phosphorylation of hexoses, yielding glucose-6-phosphate, which is readily available for storage, cellular synthesis and respiration. Among the pathways used by microorganisms for breakdown of glucose are glycolytic – EMP and pentose phosphate - PPP, which have been demonstrated in microalgae. Hong and Lee (2007) reported that probably the largest difference in the metabolism of glucose in microalgae grown heterotrophically, compared with the autotrophic metabolism of glucose or other non-carbohydrate organic substrates, is that under dark conditions, glucose is mainly metabolized by the PPP, while the EMP is the form preferred by cells under conditions of lighting. Both pathways occur in cytosol and are functional in microalgal cells.

Palmitic acid (C16:0), the precursor of saturated and unsaturated fatty acids (Nelson and Cox, 2011), ranged from 15.8 to 29.6% w/w in the different assays, and this saturated fatty acid appears in highest concentrations in the assays with added glucose (Table 2). Radmann and Costa (2008), obtained 4.6% w/w of palmitic acid in the cultivation of the microalga *C. vulgaris*; Costa *et al*. (2006), obtained values ranging from 4.55% w/w to 21.68% w/w for the same fatty acid in the cultivation of the microalga *C. minutissima*, while Wei *et al.* (2009), in the heterotrophic cultivation of microalga *C. protothecoides*, found values of 19.44% w/w to 27.27% w/w. This saturated fatty acid is very important for infant nutrition and is found in human milk at concentrations of 20 to 30% w/w (Willis *et al*., 1998). Furthermore, it is known that saturated fatty acids are mainly responsible for the production of energy; however, the spatial distribution of palmitic acid is also essential for maximizing calcium absorption (Agostini, 2003).

The variation of the cultivation medium, glucose concentration and strain influenced the yield of palmitoleic acid (C16:1), which appeared at higher concentration (26.4% w/w) in the culture *Chlorella* sp. (BG11 without addition of glucose). This fatty acid is an important part of the human diet, since it assists in preventing cardiovascular disease (Willis *et al*., 1998), balances levels of HDL and LDL cholesterol, reduces blood sugar, and encourages the break up of fat from tissues involved in the liver and heart (Wen and Chen, 2000).

In this study, the oleic fatty acid (C18:1n9) had the highest concentration (35.1% w/w) in the assay with *Chlorella* sp. (Basal supplemented with 10 $g.L^{-1}$ glucose). Isleten-Hosoglu *et al.* (2012) reported a similar composition of this fatty acid, achieving 32.2% w/w in the heterotrophic cultivation of *Chlorella saccharophila,* while Wang *et al.* (2012), obtained a maximum concentration of 20.96% w/w when heterotrophically cultivating the microalga *Chlorella kesleri.*

The ω3 family of fatty acids: α-linolenic (C18:3), EPA (C20:5) and DHA (C22:6) and the ω6 family: linoleic (C18:2), gamma-linolenic (C18:3) arachidonic (C20:4) are considered essential, since the human metabolism is unable to synthesize them and they should therefore be added to the diet to prevent the attenuation of a host of diseases. Unlike humans, microalgae are able to synthesize these fatty acids, especially in heterotrophic cultivation. According to Adarme-Vega et al. (2012), heterotrophic microalgae are excellent alternative sources of DHA, especially when added to infant formula.

The highest concentration of the essential fatty acids linoleic acid (C18:2n6) (33.9% w/w), gamma-linoleic acid (C18:3n6) (8.2% w/w) and α-linolenic (C18:3n3) (2.3% w/w) was obtained in the cultivation of microalgae *C. homosphaera* (Basal without added glucose), *C. homosphaera* (Basal supplemented with 5 $g.L^{-1}$ of glucose) and *C.minutissima* (Basal supplemented with 10 $g.L^{-1}$ glucose), respectively. O'Grady and Morgan (2011), Radmann and Costa (2008) and Costa *et al.* (2006) cultivated *Chlorella* strains under different conditions and found maximum values of 4.1% w/w; 3.1% w/w and 2.1% w/w for the fatty acids linoleic, gamma-linoleic and α-linolenic, respectively.

The highest concentration of arachidonic acid (C20:4n6) (1.5% w/w) was in the assay *C. minutissima* BG11 without added glucose. Radmann and Costa (2008) achieved a maximum concentration of 0.49% w/w of C20:4n6 when cultivating *C. vulgaris*. This essential fatty acid from the ω6 family is required by

infants for normal growth and functional development (Adarme-Vega *et al.*, 2012).

Polyunsaturated fatty acids with a very long chain, cis-5,8,11,14,17-eicosapentaenoic acid (C20:5n3) (EPA, C20:5n3) and cis-4,7,10,13,16,19-docosahexaenoic acid (C22:6n3) (DHA, C22:6n3) appeared in higher concentrations in the assays *C. homosphaera* BG11 without glucose addition, *C. homosphaera* BG11 supplemented with 10 $g.L^{-1}$ glucose and *Chlorella* sp. BG11 without added glucose, at concentrations of 1.1; 1.1 and 0.8% w/w, respectively, and in the assays *C. homosphaera* Basal supplemented with 5 $g.L^{-1}$ glucose and *C. homosphaera* Basal supplemented with 10 $g.L^{-1}$ glucose at concentrations of 2.0 and 1.1% w/w, respectively. This family of ω3 fatty acids not only prevents cardiovascular diseases due to a higher HDL/LDL ratio, but also provides benefits to the nervous system and brain functions. In pregnant women, adequate intake of EPA and DHA is crucial for healthy brain development (Adarme-Vega *et al*., 2012) and the vision of the fetus, therefore the PUFAS are included in a variety of baby food (Abad and Turon, 2012).

From the results of this study it can be concluded that BG11 medium used for the cultivation of *Chlorellas* favored the production of biomass, whereas Basal medium was efficient at obtaining PUFAs and essential fatty acids. The maximum cellular concentration obtained was 1.62 $g.L^{-1}$ for the microalga *Chlorella* sp. in BG11 medium supplemented with 10 $g.L^{-1}$ glucose. The highest lipid content (22.4% w/w) was obtained in the cultivation of the microalga *C. homosphaera* with BG11 medium supplemented with 10 $g.L^{-1}$ of glucose. The highest concentrations of total PUFA (35.25% w/w) and essential fatty acids ω6+ω3 (35.05% w/w) were also obtained by the same microalga *(C. homosphaera)*, but when grown in Basal medium without added glucose.

The fatty acid profile showed oleic acid (C18:1n9) was the most abundant (35.1% w/w) in the biomass of *Chlorella* sp. in Basal medium supplemented with 10 $g.L^{-1}$ of glucose. However, the saturated fatty acid that appeared at the highest concentration (32.7% w/w) was arachidic acid (C20:0) in the cultivation of *C. minutissima* in Basal medium without added glucose.

The heterotrophic cultivation of *Chlorellas* is effective for the production of polyunsaturated fatty acids, particularly the essential fatty acids. These can be used for food, since the human metabolism is unable to synthesize them. Also, they can contribute to the prevention and mitigation of heart problems, chronic diseases and help to control cholesterol.

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