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Original Research Paper

Evaluation of the cultivation conditions of marine microalgae *Chlorella* sp. to be used as feedstock in ultrasound-assisted ethanolysis

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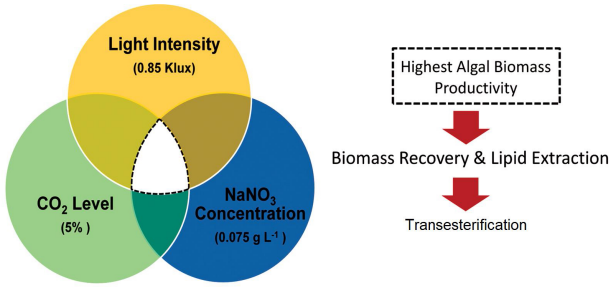
HIGHLIGHTS

- *Culture conditions of Chorella sp. were simultaneously investigated employing a Taguchi design experiment.*
- *Highest lipid content of 19.8% was obtained.*
- *Main fatty acids were C16 and C18 which are suitable for the production of high-quality biodiesel.*
- *Ultrasound-assisted biodiesel synthesis led to 78.4% conversion rate.*

GRAPHICAL ABSTRACT



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ABSTRACT

A total of 8 assays was conducted to study the influence of different variables namely, light intensity, CO_2 level, $NaNO_3$ concentration and aeration rate, on the cultivation of the marine microalgae *Chlorella* sp. to enhance the biomass feedstock availability for biodiesel. The experiments were designed using a Taguchi L8 experimental array set at two levels of operation, having light intensity (0.85 and 14.5 klux), CO_2 (5 and 10%), $NaNO_3$ (0.025 and 0.075 g L^{-1}) and aeration rate (3:33 and 1.67 vvm) as independent variables and considering biomass productivity and lipid content as response variables. All the experiments were performed in six photobioreactor vessels connected in series with a total volume of 8.4 L and working volumes of 2 L and 4 L, depending on the conditions assessed. The highest biomass productivity was 210.9 mg $L^{-1}day^{-1}$, corresponding to a lipid content of 8.2%. Such results were attained when the culture conditions were set at 0.85 klux light intensity, 5% CO_2 and 0.075 g L^{-1} $NaNO_3$. The aeration rate showed no significant influence on the biomass productivity. On the other hand, the highest lipid content was achieved when the cultures were grown using the lowest concentration of $NaNO_3$ (0.025 g L^{-1}) and an aeration rate of 1.67 vvm, while the other factors had no statistical significance. Under these conditions, the lipid content obtained was 19.8%, at the expense of reducing the biomass productivity to 85.9 mg $L^{-1}day^{-1}$.The fatty acid profile of the lipid material characterized by gas chromatography identified fourteen fatty acids with carbon chain ranging from C8 to C20 in which most of the fatty acids present were saturated (58.7 %) and monounsaturated (36.1%) fatty acids. Those obtained at higher proportions were the oleic (22.8%), palmitic (20.7%) and lauric (17.7 %) acids, indicating a suitable composition for fatty acid ethyl esters (FAEE) synthesis. This was confirmed by acid catalysis performed under ultrasound irradiations reaching a conversion rate of 78.4% within only 4 h.

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1. Introduction

An increase in greenhouse gas emissions, followed by a simultaneous depreciation on fossil fuel reservoirs have incited many studies to aim at finding environmentally-sustainable alternative feedstocks for biofuel production (Beetul et al., 2014; Girard et al., 2014; Zhu, 2015). In this context, microalgae have been highlighted as promising raw materials for biofuel production owing to its numerous advantages, such as being able to grow on low-value agricultural land, great capacity of capturing atmospheric CO_2, as well as lipid accumulation (Koller et al., 2014). As a complimentary source to the limited fossil fuels, lipids extracted from microalgae can be processed into biodiesel by catalyzed transesterification reactions (D'oca et al., 2011a; Teo et al., 2014).

Microalgae are photosynthetic microorganisms (endowed with chlorophyll-a) that can grow quickly and be found throughout the most diverse ecosystems e.g. on the surface of certain types of soils, most abundantly in aquatic environments, or grouped in the form of linked linear segments of cells (Chisti, 2007; Mata et al., 2010).

Many studies have been conducted with numerous species of microalgae to assess their potential for lipid accumulation, such as *Isochrysis* sp. (25-33% lipids), *Nannochloris* sp. (20-35% lipids), *Nannochloropsis* sp. (31-68% lipids), *Neochlorisoleo abundans* (35-54% lipids) and *Tetraselmessueica* (15-23% lipids) (Mata et al, 2010). According to Huntley and Redalje (2007), the *Chlorella* species are considered as a promising candidate for commercial lipid production due to its rapid growth, and also because it is highlighted as one of the most robust species for cultivation in open ponds. However, factors associated with microalgae cultivation, including nutrient requirements (nitrogen, phosphorus, metals, among others), pH, temperature, light intensity and carbon source (glucose, glycerol, lactose, CO_2), could influence both the cell growth and the accumulation of lipids (Millán-Oropeza et al., 2015; Nascimento et al., 2015). The conventional optimization method involves the variation of one parameter at a time while keeping the others constant. This is a time-consuming procedure and often does not identify the interaction effect among various parameters as compared to statistical methods. Taguchi method uses orthogonal arrays allowing the investigation of various factors with a reduced number of experiments, while is able to identify the significant parameters of an ideal process with multiple qualitative aspects (Ross, 1995).

This statistical tool involves the calculation of the signal to noise ratio (S/N) throughout experimental replicates, representing a magnitude relation between sensitivity and variability of a particular output measurement, and the analysis of variance (ANOVA) to evaluate the significance of the variables studied on the process (Ross, 1995; Sharma et al., 2005).

The present study was aimed at establishing the cultivation conditions of the marine microalgae *Chlorella* sp. to obtain high cell productivity and lipid content. To achieve this, a Taguchi's L8 array with four independent variables at two levels was performed. The feasibility of using the resulting lipids as feedstock for biodiesel synthesis was also assessed. Owing to the high free fatty acids levels generally found in microbial oils (Da Rós et al, 2013), homogeneous acid catalysis was attempted.

2. Materials and methods

2.1. Microalgae strain

All experiments were performed with the Chlor-CF strain of the marine microalgae *Chlorella* sp., isolated in Cabo Frio (Rio de Janeiro-Brazil), obtained from the Seaweed Culture Collection of the Oceanographic Institute at the University of São Paulo (São Paulo, SP, Brazil).

2.2. Photobioreactors and microalgae cultivation

The experimental set up comprised of six closed photobioreactor vessels (14 × 20 × 30 cm) connected in series with a total volume of 8.4 L, as shown in **Figure 1**. The photobioreactor was run on modified Guillard f/2 medium (Guillard, 1975), supplemented with $NaNO_3$ (0.025 and 0.075 g L^{-1}) and CO_2 (5 and 10%) and was stirred with sterile air (filtered with 0.22-µm filter). The photobioreactor vessels were inoculated by adjusting the initial cell concentration to 3×10^6 mL^{-1}. The reactor vessels were sparged with sterile air at different rates (3.33 and 1.67 vvm) and maintained at 27±1ºC under photoperiods of 12:12 h (light: dark) at different light intensities (0.85 and 14.5 Klux) using halogen lamps placed above the photobioreactor vessels.

2.3. Experimental design and data treatment

The experimental design was performed using Taguchi L8 orthogonal array being operated according to the factors and levels displayed in **Table 1**. All runs were carried out in triplicates. Biomass productivity and lipid content were taken as response variables. The statistical analysis was performed using the Statistica software (version 12.0) by analyzing the graph effects and tables of the analysis of variance (ANOVA). S/N ratio was calculated for larger-the-better response using the **Equation 1**:

$$S/N = -10 \log 1/n \, \Sigma(1/Y_i^2) \quad \text{(Eq.1)}$$

Table 1.
Factors evaluated in the experimental design and their respective coding levels.

Code	Factor	Level	
		1	2
A	Light intensity (klux)	0.85	14.50
B	CO_2 (%)	5	10
C	$NaNO_3$ (g/L)	0.025	0.075
D	Aeration rate (vvm)	3.33	1.67

2.4. Harvesting and biomass determination

The cell growth was monitored using a counting Neubauer hemocytometer and by measuring the optical density at 570 nm. The resulting biomass at the end of the batch run was recovered by coagulation using 1 $mol.L^{-1}$ sodium hydroxide. The supernatant was then removed and the cells were washed with a 0.6 $mol.L^{-1}$ ammonium formate solution in order to remove sea salt. The cells were subsequently lyophilized. After drying, the biomass was weighed to determine the biomass productivity (mass of dried biomass per culture volume per day of cultivation, mg L^{-1} day^{-1}).

2.5. Lipid extraction

Lipids were extracted from the lyophilized biomass according to a modified Bligh's and Dyer's method (Bligh and Dyer, 1959), using a mixture of chloroform, methanol and water as extracting solvent, in the following respective ratio: 1: 2: 0.8 (v/v/v). The lipid extracted was dried in a rotary evaporator to remove solvent residues and was subsequently dried at 60 °C to constant weight. The total lipids were measured gravimetrically, and then lipids contents and yields were calculated (Da Rós et al., 2013).

2.6. Lipid feedstock characterization

The AOCS's method (American Oil Chemist's Society, 2004) was used for the determination of total free fatty acids (FFA), which was expressed in terms of free oleic acid (%). The fatty acid composition analysis was performed in a capillary gas chromatography (CGC Agilent 6850 Series GC System) according to the methodology described by Silva et al., (2014).

2.7. Biodiesel synthesis

The biodiesel synthesis reaction from the oil extracted from the *Chlorella* sp. biomass with ethanol (molar ratio 30:1 alcohol/lipid material) was performed in a cylindrical reactor (2×10 cm) in the presence of 10% H_2SO_4 (98%, Synth-SP, Brazil) as catalyst, using an ultrasonic bath (Unique Model USC 1800) to control the stirring and keep the temperature at 60 °C. The reactions were conducted for 4 h.

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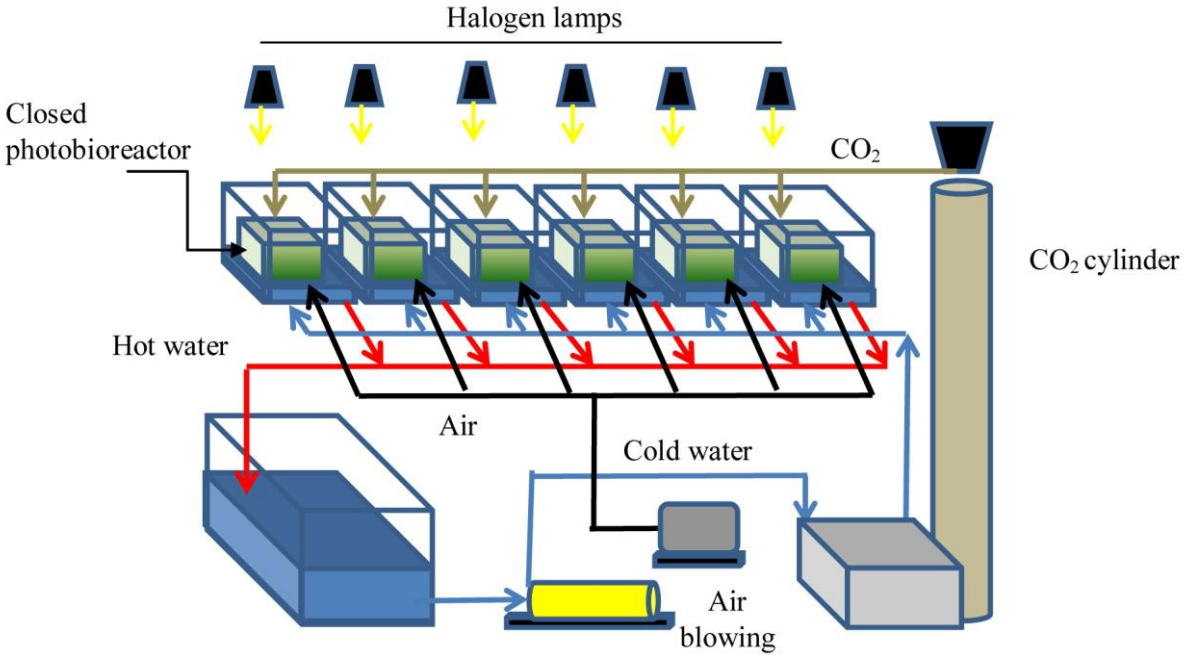

Fig.1. Schematic representation of the photobioreactor system·

2.8. Downstream procedure

Once the reaction was complete, the reaction medium was subjected to natural cooling for approximately 1 h. The sample containing ethyl esters was purified according to the method described by Da Rós et al., (2013), and was further subjected to analysis.

2.9. Ester content quantification

The conversion into ethyl esters was evaluated by proton nuclear magnetic resonance spectrometry (^{1}H NMR) in a Mercury 300 MHz-Varian spectrometer, with 5 mm glass tubes, using $CDCl_3$ as solvent and 0.3% tetramethylsilane (TMS) as internal standard. The calculations involving the conversion of esters were performed using an equation according to the methodology described by Paiva et al., (2013). It allowed the identification of ester molecules produced during transesterificationthrough the peaks in the region of 4.05 to 4.35 ppm.

3. Results and discussion

3.1. Statistical analysis

Screening experiments were conducted to identify the factors influencing the biomass concentration and lipid contents during the cultivation of marine *Chorella* sp. and to verify if any changes to their settings should be made to improve the process. The effects of different experimental variables on this bioprocess were simultaneously investigated, employing a Taguchi design experiment. Four variables (A: Light Intensity, B: CO_2, C: $NaNO_3$, and D: aeration rate) were taken into consideration. The experimental matrix and the results are shown in **Table 2**. Each line in the table represents an experimental run and each column stands for an independent variable (factor), along with the mean values and S/N ratios of the response variables obtained for each condition.

The biomass productivity values ranged from 54.3 to 210.9 mg $L^{-1}day^{-1}$, while the lipid content varied between 8.2 and 19.8%. The highest biomass productivity was achieved for the experiment 2, in which the employed conditions were: 0.85 klux of light intensity; CO_2 at 5%, $NaNO_3$ at 0.075 g L^{-1}, and aeration rate at 1.67 vvm. Under these conditions, the lipid content was 8.2%. On the other hand, the experiment 3 yielded higher levels of lipids (19.8%), at the expense of reducing the biomass productivity to 85.9 mg L^{-1} day^{-1}. In this experiment the following conditions were set: 0.85 klux of light intensity; CO_2 at 10%; $NaNO_3$ at 0.025 g L^{-1}, and aeration rate of 1.67 vvm.

In general, the results obtained showed that the most important variable was the $NaNO_3$ concentration, although conditions that maximized the biomass productivity were different from those attained for the lipid contents.

3.1.1. Effect of factors on biomass productivity

Among the factors studied (i.e. A: Light Intensity, B: CO_2, C : $NaNO_3$, and D: aeration rate), factor C showed less variability and greater significance in the process due to the highest value of F statistics and lowest p-value, followed by factor B. Factor A showed moderate significance, while factor D (aeration rate) was not significant on biomass productivity under the used conditions (Supplementary Data, **Table S1**).

The graphical analysis (**Fig. 2**) in relation with the effect of the factors on biomass productivity showed that factor C at level 2 ($NaNO_3$ at 0.075 g L^{-1}) was the most significant factor, followed by factor B at level 1 (CO_2 at 5%), and factor A at level 1 (0.85 Klux of light intensity). The adjustment of the variables to optimize the process concerning this response variable, i.e. maximizing biomass productivity, suggest that factors A, B, and C (light intensity, CO_2% and $NaNO_3$ concentration, respectively) should be set at levels 1 (0.85 KLux), 1 (5 %) and 2 (0.075 g L^{-1}), respectively.

Light intensity, CO_2 and nitrogen concentration in the culture medium are reported as important factors by a number of studies (Gushina and Harwood, 2006; Mata et al., 2010; Suali and Sarbatly, 2012). Light intensity is directly related to the photosynthesis process and has different effects on microalgae species. Different species require different levels of light energy to conduct the process; however excessive luminosity can cause photo inhibition and cell death.

CO_2 concentration in the culture medium could significantly affect the photosynthesis process, but each species responds differently to different CO_2 concentrations (Wang et al., 2013a). Nitrogen is a key component to obtain three classes of structural substances of cells: proteins, nucleic acids and photosynthetic pigments. If nitrogen supply is abundant in the culture, the production rates of proteins and chlorophyll in the cells increase. On the other hand, when the available nitrogen concentration is

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Table 2.
Experimental matrix and results obtained for biomass productivity and lipid content according to experimental design from growth of the Chorella sp.

Experiment	Independent variable				Response variable								
	Factor				Biomass productivity ($mg.L^{-1}.day^{-1}$)					Lipids (%)			
	A	B	C	D	Triplicate			Average	S/N	Triplicate			Average	S/N
1	1	1	1	1	64.4	50.0	68.8	**61.0**	**35.5**	13.5	13.7	13.9	**13.7**	**22.7**
2	1	1	2	2	210.0	214.7	208.2	**210.9**	**46.5**	8.6	7.8	8.2	**8.2**	**18.3**
3	1	2	1	2	78.5	100.0	79.2	**85.9**	**38.5**	19.7	20.0	19.5	**19.7**	**25.9**
4	1	2	2	1	158.8	66.3	91.3	**105.4**	**39.1**	9.5	8.7	7.7	**8.6**	**18.6**
5	2	1	1	2	64.3	64.3	34.3	**54.3**	**35.5**	15.9	17.6	15.2	**16.2**	**24.2**
6	2	1	2	1	199.2	193.1	198.5	**196.9**	**45.9**	10.4	12.6	10.7	**11.2**	**20.9**
7	2	2	1	1	98.3	38.3	65.0	**67.2**	**34.7**	10.7	13.4	12.8	**12.3**	**21.7**
8	2	2	2	2	78.6	71.4	68.6	**72.9**	**37.2**	7.3	9.9	10.2	**9.1**	**18.9**

A: Light intensity (klux) C:$NaNO_3$ (g/L)
B: CO_2 (%) D: Aeration rate (vvm)

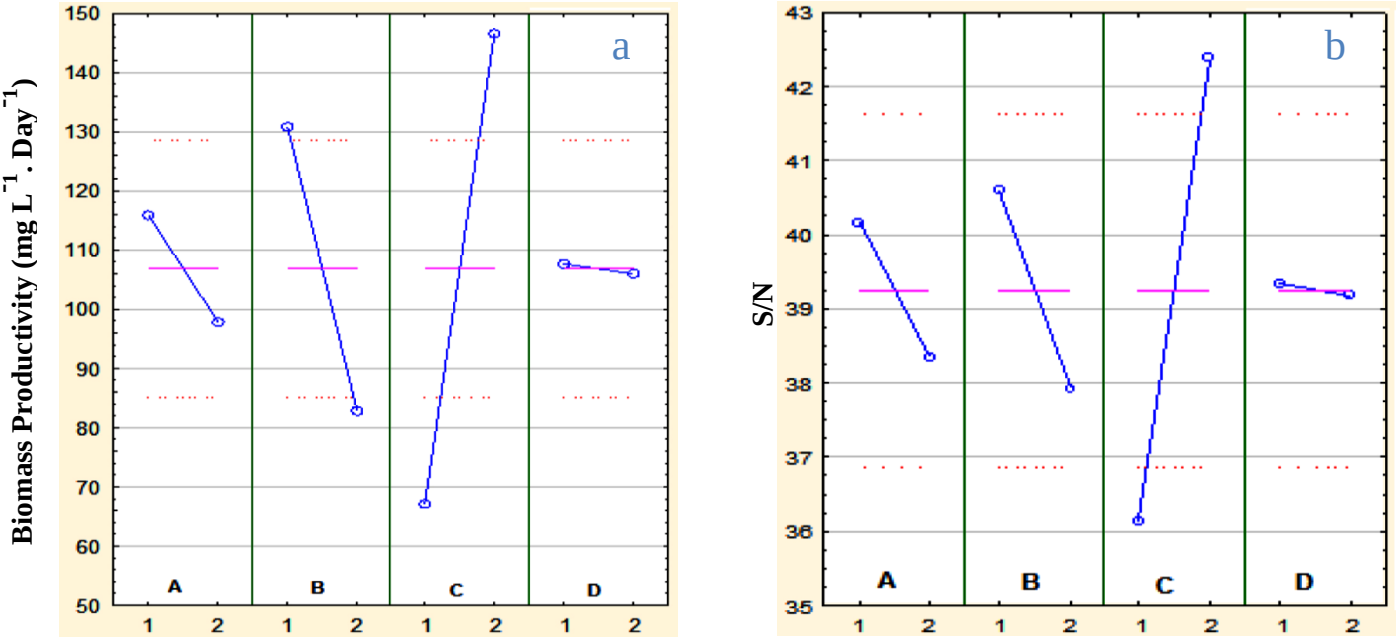

Fig.2. Influence of factors on the biomass productivity (a) on the average, (b) on the signal /noise ratio. Factors: A - Light Intensity (klux), B - Carbon Dioxide (%), C - concentration of NaNO3 (g / L), D – Aeration rate (vvm).

low, there is a significant decrease in the biomass productivity (Mata et al., 2010; Praveenkumar et al., 2012).

3.1.2. Effect of factors on lipid content

The statistical analysis of the results obtained for the variable related to the lipid content during the cultivation of the marine microalgae *Chlorella* sp. showed that the factors which presented a significant influence were the concentration of $NaNO_3$ (factor C) and aeration rate (factor D) (Supplementary Data, **Table S2**).

Through the analysis of variance (ANOVA) of the mean value and the S/N ratio shown in **Figure 3**, it could be observed that factor C at level 1 ($NaNO_3$ at 0.025 g L^{-1}) followed by factor C at level 2 (1.67 vvm) led to the least variability and the greatest significance on the process. This could be ascribed to the fact that these factors at the mentioned levels yielded the highest S/N values and the lowest p-values. The other factors (light intensity and CO_2) were not statistically significant for this response variable within the range studied.

Thus, the process adjustment proposal aiming at maximizing the response variable (lipid content) suggests that factors C and D (concentration of $NaNO_3$ and vvm) should be set at levels 1 (0.025 g L^{-1}) and 2 (1.67 vvm), respectively.

It is reported that microalgae cultivation performed under stress conditions, such as low homogenization of the culture medium, and nutrient deprivation, e.g. nitrogen, tends to stimulate the synthesis and accumulation of lipids (Mata et al., 2010; Wang et al., 2013b). The nitrogen limitation can generate a physiological stress which increases as the concentration of nutrients decreases. This stress changes the cell's metabolism, directing the metabolic processes to the production of lipid

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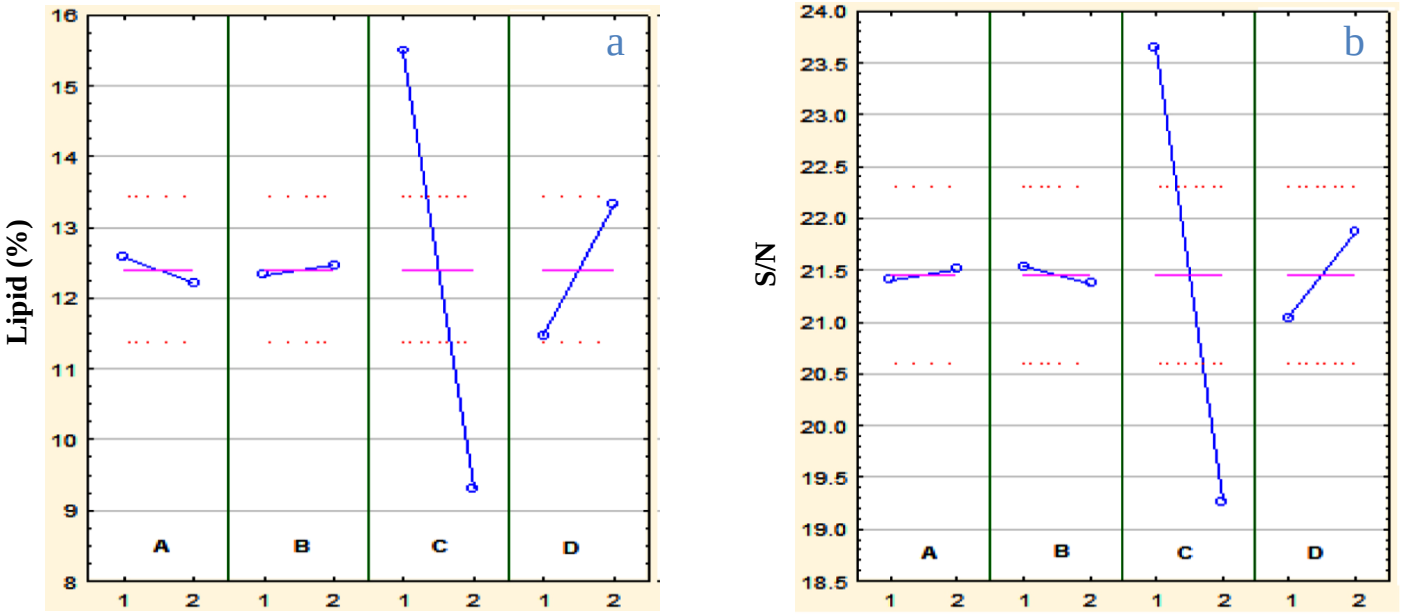

Fig. 3. Influence of factors on the lipid levels (a) on the average, (b) on the signal / noise ratio. Factors: A - Light Intensity (klux), B - Carbon Dioxide (%), C - concentration of NaNO3 (g / L), D - Aeration rate (vvm).

reservoirs, thus preparing the cell for a period of deprivation (Sanchez et at., 1996).

3.2. Fatty acid profile

Table 3 shows the fatty acid profile of the lipid material recovered from the cultivation of marine microalgae *Chlorella* sp. along with the profile of other *Chlorella* strains described in the literature. The lipid feedstock from the *Chlorella* sp. strain studied in the present work presented a composition of 57.8% of saturated fatty acids, 31.6% of monounsaturated fatty acids, and 8.3% of polyunsaturated fatty acids. Those obtained at higher proportions were the oleic (27.8%), palmitic (20.7%) and lauric (17.7%) acids. From the composition of fatty acids, the average molecular weight was found to be 743.5 g mol^{-1}.

From a comparative point of view, the resulting lipid profile proved to be distinct from other strains of the same species, particularly in regard to the degree of unsaturation (DU) which can be determined by taking into account, the amount of monounsaturated and polyunsaturated fatty acids (wt.%) present in a feedstock (Da Rós et al., 2013).

While the microalgal oils obtained through the cultivation of *Chlorella* strains reported in different studies (D'oca et al., 2011b; Lam and Lee, 2013; Wang et al., 2013a) have DU values higher than 87.8%, the microalgal oil obtained from the *Chorella* sp. in the present study had only 48.1% as shown in **Table 3.**

It is known that the amount of each fatty acid present in the triglyceride molecule used in the transesterification reaction, as well as the chain length and unsaturation number are important factors for determining the physical characteristics of both the feedstock and the resulting biodiesel (Ramos et al., 2009). One of the critical parameters for biodiesel commercialization is its oxidative stability which is closely related to the amount of polyunsaturated fatty acids, especially the linoleic (C18: 2) and linolenic (C18: 3) acids present in the raw materials to be used in the process (Knothe, 2005; Francisco et al., 2010).

Thus, the fatty acid profile of the marine microalgae *Chlorella* sp.

Table 3.
Comparison of the fatty acid profile of the lipid material from different strains of the *Chlorella* sp.

Strain			*Chlorella* sp.	*Chlorella vulgaris*	*Chlorella vulgaris*	*Chlorella pyrenoidosa*
Saturated FA (%wt)						
C8:0	Caprilic		8.2	0.0	0.0	0.0
C10:0	Capric		3.9	0.0	0.0	0.0
C12:0	Lauric		17.7	0.0	0.0	0.0
C14:0	Myristic		6.3	0.0	0.0	0.7
C16:0	Palmitic		20.7	14.0	16.6	17.3
C17:0	Margaric		0.0	0.3	0.0	0.0
C18:0	Stearic		1.0	2.0	4.0	1.2
		Total	**57.8**	**16.3**	**20.6**	**19.2**
Monounsaturated FA						
C16:1	Palmitoleic		4.2	0.0	2.4	0.8
C18:1	Oleic		27.2	70.8	53.6	3.3
C20:1	Gadoleic		0.3	0.0	0.0	0.0
		Total	**31.6**	**70.8**	**56.0**	**4.1**
Polyunsaturated FA						
C16:2	Hexadecadienoic		0.0	0.0	4.2	7.0
C16:3	Hexadecatrienoic		0.0	0.0	0.0	9.3
C18:2	Linoleic		3.9	10.5	11.8	18.5
C18:3	Linolenic		4.3	0.8	4.1	41.8
		Total	**8.3**	**11.3**	**20.1**	**76.6**
Others			2.3	1.6	3.3	0.0
Degree of unsaturation* (DU)			48.1	93.4	87.8	150.3
Reference			This work	Wang et al. (2013B)	Lam and Lee, (2013)	D'oca et al. (2011)

* Calculated according to the equation DU = (wt.% monounsaturated)+2.(wt.% polyunsaturated) as described by Francisco et al. (2010) and Da Rós et al. (2013).

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reported in this study shows advantages over the other strains of the same species, favoring its use as lipid feedstock for biodiesel synthesis. In contrast to the other studies that reported, respectively, 11.3%, 20.1% polyunsaturated acids for *Chlorella vulgaris* (Lam and Lee, 2013; Wang et al., 2013a), 76.6% for *Chlorella pyrenoidosa* (D'oca et al., 2011b), the resulting lipid material from the microalgae *Chlorella* sp. investigated herein contained only 8.3% of these acids. It should be noted that polyunsaturated fatty acids present in high amounts in the lipid material may result in a biodiesel which is prone to undergo polymerization when subjected to intense heat, causing engine deposits (Francisco et al., 2010; Ehimen et al., 2010).

3.3. Biodiesel synthesis

The acid value of the microalgal oil was determined to be 32.7 mg KOH/g, with a high FFA content of 18.7%. Lipids with FFA content over 5 wt % are not suitable for alkaline-catalyzed transesterification, as the FFA will tend to consume the catalyst and form soap, leading to serious separation problems. Hence, acid-catalyzed transesterification was employed in the present study but to overcome the slow rate of the reaction, it was conducted under ultrasound irradiations (Meng et al., 2009).

The ^{1}H-NMR spectrum of the ethyl esters obtained through the transesterification reaction using lipids from the marine microalgae *Chlorella* sp. and ethanol by acid catalysis showed a quartet signal at 4.1 ppm, referring to the ethylene hydrogens of the alcoholic portion of the ester [CH_3-CH_2-OC(=O)-R] (Supplementary Data, **Fig. S1**). Based on the yield calculation involved in this technique, an estimated conversion value of 78.4% was achieved. Similar results were achieved by Ehimen et al. (2010) who used an acid catalyzed transesterification of lipid materials from the microalgae *Chlorella* sp. at 90 °C and reported conversion rates of between 70 and 92%.

4. Conclusion

The conditions for enhancing *Chlorella* sp biomass yield were successfully established. This work demonstrated that the factors studied in the cultivation of microalgae *Chlorella* sp. could influence each response variable differently (biomass productivity and lipid content). The concentration of $NaNO_3$ played a key role in optimizing the total biomass yield *vs.* lipid content in cultivations designed to harvest lipids for downstream transesterification into biodiesel. For enhancing biomass productivity, concentrations of CO_2 and $NaNO_3$were found to be statistically significant and should be kept at 5% and 0.075 g $L^{-1,}$ respectively. The factors that maximized the accumulation of lipids were the concentration of $NaNO_3$ (0.025 gL^{-1}) and aeration rate (1.67 vvm). Moreover, the lipid from *Chlorella* sp had suitable composition for biodiesel production, yielding high amounts of C16-C18 fatty acids required for biodiesel synthesis. The transesterification of the lipid material employing acid catalysis led to a conversion rate of 78.4%.

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Supplementary Data

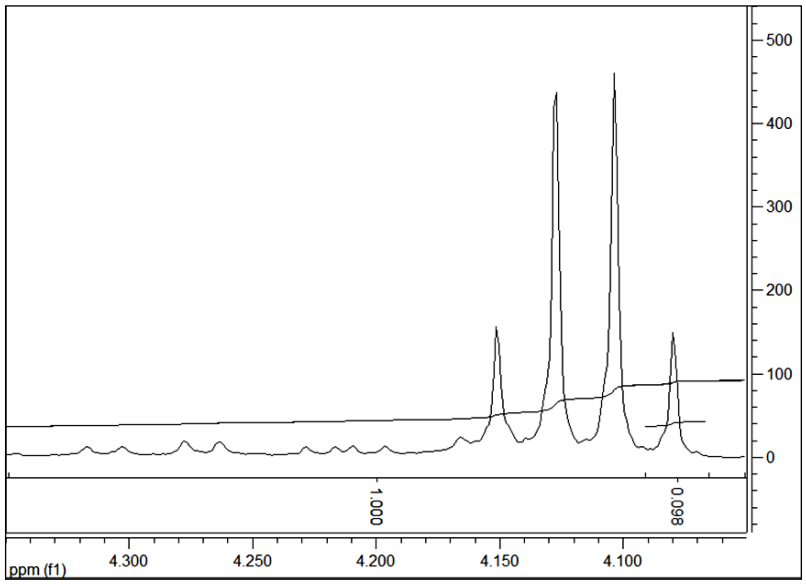

Fig.S.1. Nuclear magnetic resonance of protons of the ethyl esters obtained from the lipid feedstock of the microalgae *Chlorella* sp.

Table S.1.
ANOVA analysis, mean value, and signal to noise ratio (S/N) for biomass productivity of the microalgae *Chlorella* sp. grown under different conditions.

	Factor	SS	DF	MS	F	*p*
Mean	**A**	1949.40	1	1949.40	4.1168	0.0594
	B	13800.01	1	13800.01	29.1435	0.0001
	C	37865.87	1	37865.87	79.9669	0.0000
	D	16.50	1	16.50	0.0349	0.8543
	Residual error	7576.31	16	473.52	--	--
S/N	**A**	19.65	1	19.65	3.4562	0.0815
	B	42.77	1	42.77	7.5242	0.0144
	C	233.65	1	233.65	41.1025	0.0000
	D	0.16	1	0.16	0.0273	0.8708
	Residual error	90.95	16	5.68	--	--

SS: sum of squares DF: degree of freedom MS: Mean sum of squares F:F-test p: significance value.

Table S.2.
ANOVA analysis, mean value, and signal to noise ratio (S/N) for lipid content of the microalgae *Chlorella* sp. grown under different conditions.

	Factor	SS	DF	MS	F	*p*
Mean	**A**	0.76	1	0.76	0.7132	0.4108
	B	0.10	1	0.10	0.0932	0.7641
	C	231.01	1	231.01	217.900	0.0000
	D	20.72	1	20.72	19.545	0.0004
	Residual error	16.96	16	1.06	-	-
S/N	**A**	0.06	1	0.06	0.0893	0.7689
	B	0.16	1	0.16	0.2205	0.6450
	C	115.63	1	115.63	160.08	0.0000
	D	4.12	1	4.12	5.7096	0.0295
	Residual error	11.56	16	0.72	-	-

SS: sum of squares DF: degree of freedom MS: Mean sum of squares F: F- test p: significance value

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