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A N I N T E R N A T I O N A L J O U R N A L

Evaluation of CO_2 Biofixation and Biodiesel Production by *Spirulina* (*Arthospira*) Cultivated In Air-Lift Photobioreactor

Etiele Greque de Morais[1], Ana Paula Aguiar Cassuriaga[1], Nicolás Callejas[2], Natalia Martinez[2], Ignacio Vieitez [2], Iván Jachmanián[2], Lucielen Oliveira Santos[1], Michele Greque de Morais[1], Jorge Alberto Vieira Costa[1*]

[1]Universidade Federal do Rio Grande - Escola de Química e Alimentos - Rio Grande, Rio Grande do Sul, Brazil;
[2] Facultad de Química, Universidad de la República, Montevideo, Uruguay.

ABSTRACT

Spirulina is a microalgal genre that has the capacity to produce various bioproducts with applications in several areas including the energy sector. The study aimed to assess the ability of CO_2 biofixation, biodiesel and other biocompounds production by Spirulina sp LEB 18 cultured in air-lift photobioreactor. The microalgae presented a rich macronutrient composition: protein (47.3%), carbohydrates (13.4%) and a high lipid content (32.7%) in a media with nitrogen reduction, CO_2 using air-lift photobiorector. Furthermore, 160 mg.L.d^{-1} of CO_2 was biofixed, generating a maximum biomass yield of 0.02 g.L.d^{-1}. The lipids evaluated for biodiesel production presented a theoretical yield of 19.8% for in situ transesterification and 47.9% for conventional transesterification. The microalgal biomass has potential for producing biodiesel that can be applied instead or in mixture with traditional diesel fuel. The study of obtaining energy associated with the production of other high value-added biocompounds from the microalgal biomass is of high importance because in this way, the viability of biofuel production by this microorganism can be increased.

Keywords: biofuels, carbon dioxide, microalgae, photobiorefinary, biocompounds.

[*] Author for correspondence: jorgealbertovc@terra.com.br

INTRODUCTION

The planet's energy needs are met primarily by using fossil fuels such as coal, petroleum and natural gas. The reserves of these sources are finite, and their application and operation cause environmental problems, such as increased atmospheric CO_2 generated by burning these materials. The search for alternative renewable sources has intensified to meet the rising global energy and raw material demands [1]. One of the potential sources to comply with this challenge is the microalgal biomass that joins the production of sustainable energy and CO_2, the main greenhouse gas, biofixation.

The concept of microalgal photobiorefinary integrates the production of biofuels and bioproducts with the use of alternative sources of nutrients, making the process of obtaining energy economically viable. *Spirulina* is a cyanobacteria that has production capacity of several biocompounds, and it has been studied for application as the basis of photobiorefinaries. From its biomass macronutrients such as proteins, carbohydrates and lipids, can be obtained with applicability in the production of biofuels and compounds such as pigments and fatty acids [2].

Among the advantages of microalgae applications for biofuel production, such as biodiesel, is that there is no competition with food crops, and these microorganisms do not require agricultural land to be cultured. Microalgal cultures do not require large amounts of water and have high productivity with the possibility of a daily harvest, making this an attractive raw material for power generation. Microalgae are able to alter their intracellular composition with changes in nutrient medium and environmental factors. In crops with reduced nitrogen sources, for example, lipid accumulation can be increased in addition to the gradual change in the composition of free fatty acids and triacylglycerols[3]. Recent studies have demonstrated that the application of these microorganisms for energy production, has a higher viability coupled into a biorefinery system [4].

Spirulina has GRAS (Generally Recognized as Safe) certification, which allows both the biomass and the compounds derived from it to be marketed and consumed as food and applied in the cosmetics industry since production complies within good manufacturing practice, besides biofuels obtaining. This microalga have ability to produce high amounts of protein in biomass, about 70%, and lipid production is around 5 to 11%. [5]. The novelty of the work is the application of *Spirulina* microalga for lipids production along the CO_2 biofixation in an air-lift bioreactor combined with the reduction of nitrogen. These factors may lead to changes in the lipid content mainly, highlighting the biodiesel production through the biomass generated in the crop. In this way, the study aimed to assess the ability of CO_2 biofixation, biodiesel and other biocompounds production by *Spirulina* sp LEB 18 cultured in air-lift photobioreactor.

MATERIAL AND METHODS

Microorganism and inoculum preparation

Spirulina sp. LEB 18, isolated from Mangueira Lagoon in southern Rio Grande do Sul [6], was held in a Zarrouk [7] medium without a carbon source ($NaHCO_3$) and with 50% of the nitrogen source ($NaNO_3$) in a thermostatically controlled oven (30 °C) at an illuminance of 40.5 $\mu mol.m^2.s^{-1}$ and a 12 h light/dark photoperiod. The microalgae were maintained under these conditions until reaching the log phase of cell growth from the experiment's beginning.

Culture conditions

The medium used in this study was maintained the same as the *Spirulina* sp LEB 18 inoculum [7], wherein the carbon source ($NaHCO_3$) was replaced by 10% CO_2 (v/v) injected in a flow of 6.5 $L_{ar}.min^{-1}$ every 40 min for 5 min in the photochemical phase. The nitrogen source of the medium ($NaNO_3$) was reduced by 50%. The microalgae was cultivated in a horizontal tubular photobioreactor equipped with an air-lift (horizontal FBRT) (Figure 1) with a working volume of 130 L. The experiment took place in a thermostatic oven at 30 °C, with a 12 h light/dark photoperiod and 40.5 $\mu mol.m^2.s^{-1}$ provided by fluorescent illuminance of 40 W. The initial concentration of the culture was 0.2 $g.L^{-1}$. The experiment was conducted until the stationary growth phase was reached.

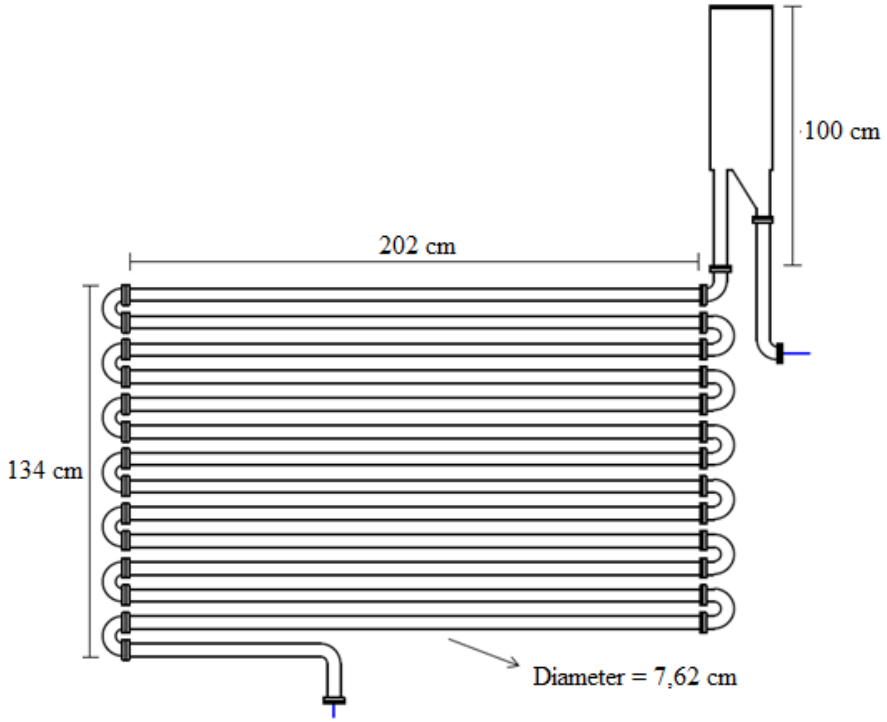

Figure 1 Horizontal tubular photobioreactor equipped with air-lift

Cell growth and kinetic parameters evaluation

Microalgae cell growth monitoring was performed daily by measuring the cellular concentration in triplicate, determined by the optical density of the culture at 670 nm in a spectrophotometer (QUIMIS Q798DRM). The cell concentration was obtained through a standard curve relating dry weight and optical density (X_{max}) [8]. From these data, the maximum productivity (P_{max}), maximum specific growth rate ($\mu_{máx}$) and generation time (t_g) were calculated.

The productivity (P, $g.L.d^{-1}$) was calculated according to Equation 1, where X ($g.L^{-1}$) represents the final cell concentration, X_0 ($g.L^{-1}$) represents the concentration of initial biomass cultivation, t (d) represents the final time and t^0 represents the initial time of cultivation.

$$P = \frac{(X - X_0)}{(t - t_0)} \quad (1)$$

The maximum specific growth rate ($\mu_{máx}$) was obtained by exponential regression on the log phase of cell multiplication, and generation time (tg) was obtained from Equation 2 [9].

$$tg = \frac{\ln 2}{\mu_{máx}} \quad (2)$$

Every 24 h, the pH of the culture was measured with a digital pH meter (QUIMIS Q.400H).

Biomass Characterization

The biomass obtained in the experiments was centrifuged at 15,000 g for 15 min and was lyophilized to perform the characterization analysis. To compare the results, the same measurements were carried out in *Spirulina* sp LEB 18 that was commercially produced by the company Olson microalgae (Camaquã, RS-Brazil) through cultures carried in raceway photobioreactors, outdoor conditions in a transparent film greenhouse.

Carbohydrate, protein and lipid composition

The lyophilized biomass was rehydrated and subjected to an ultrasonic probe (COLE PARMER CPX 130 - Illinois - USA) for 10 min in 10 s pulses by obtaining the microalgal extracts. From this extract, analysis of carbohydrates was performed using the Dubois et al[9] method (standard glucose curve) and protein content, with the method proposed by Lowry et al.[10] (albumin standard curve). The lipids were quantified from lyophilized biomass using the method used by Folch et al. [11]. The moisture content was determined according to the AOAC [12] . Analyses were performed in triplicate.

Determination of methyl esters and fatty acid composition

The determination of methyl esters was performed in triplicate in two ways. First, the lipid esterification method was applied directly to the microalgal biomass (*in situ*). In the second, the same esterification method was applied as that to the lipid extract obtained by Folch et al. (1957) (conventional). For esterification, approximately 30 mg of the sample (dry or lipid extract biomass) were treated with 0.05 M of sodium methoxide (MeONa) in methanol (MeOH) for 10 min at 100 °C and then with methyl chloride (MeCl) in MeOH for 10 min at the same temperature [13]. Then, the esterified fatty acids passed by purification on a 100 mg sílica column (Supelco, USA) to remove impurities that could adversely affect the chromatographic analysis. Thin layer chromatography (TLC) was used to monitor the reaction.
After purification, 1μL of the sample was analyzed by a gas chromatograph (GC-2014 - Shimadzu - Japan) in the presence of an internal standard, methyl heptadecanoate for methyl ester quantification. The conditions for analysis by GC were: ionizing flame detector (FID) 340 °C, Supelco SP 2330 column (Supelco, USA), 30 m x 0.25 mm x 0,2μm and N_2 carrier gas. The fatty acid peaks were identified through a chromatogram of the standard mixture of fatty acid methyl esters (FAME mix RMS; Supelco, USA).

Biodiesel Yield

Biodiesel yield was calculated in triplicate by referring to the quantity of total lipids from the biomass in accordance with the methyl ester content obtained from direct extraction (made from the dry biomass), indirect extraction (performed from the lipid extract) through Equation 3, where $R_{Tbiodiesel}$ is the theoretical biodiesel yield:

$$R_{Tbiodisel} = \frac{\text{methyl esters (g)/g dry biomass}}{\text{total lipids (g)/ g dry biomass}} . 100 \quad (3)$$

CO_2 biofixation

Through an elemental analyzer CHNS/O (Perkin Elmer 2400 - Series II) calibrated with certified acetanilide reference material, the amount of carbon in the microalgal biomass was obtained. From these data, the fixed CO_2 accumulation, the amount of CO_2 fixed daily and the CO_2 biofixation in $mg.L^{-1}.d^{-1}$ in relation to the daily fixed value to bioreactor volume were calculated.

The accumulation of fixed CO_2 (FA, g CO_2) was calculated in triplicate according to Equation 4 where X_t ($g.L^{-1}$) is the cell concentration at time t (d), X_0 ($g.L^{-1}$) is the concentration at time t_0, m_{CBM} ($g_C.g_{amostra}^{-1}$) is the weight fraction of carbon determined in the microalgal biomass $V_{biorreactor}$ (L) is the volume of medium in the bioreactor, M_{CO2} (g. mol^{-1}) and M_C are the molecular masses of CO_2 and carbon.

$$FA = (X_t - X_0)\, m_{cbm} V_{biorreactor} \left(\frac{M_{CO2}}{M_c}\right) \quad (4)$$

The daily fixing of CO_2 (FD, $g_{\text{fixed CO2}}.\, g_{\text{injected CO2}}^{-1}.d^{-1}$) from Equation 5 was also calculated, where $FA_{(t+1)}$ is the accumulation of CO_2 fixed at time t + 1 (d), FA_t is the CO_2 accumulation in time t (d), and m_{id} (g_{CO2}) is the mass of CO_2 injected daily. The maximum daily fixation (FD_{max}) is the maximum daily fixing that was reached [14].

$$FD = \frac{(FA_{(t+1)} - FA_t)}{m_{id}} \quad (5)$$

RESULTS AND DISCUSSION

The *Spirulina* sp LEB 18 experiment using Zarrouk medium with a reduction in nitrogen source and the replacement of the carbon source by CO_2 shows an adaptation phase of approximately 7 d, as shown in Figure 2. After this period, the cell growth rate gradually increased until the point where it remained constant in the exponential growth phase between 12 d to 40 d, reaching the maximum cell concentration. At this point, there was a slow down until the stationary phase, identified by the point where the cell concentration was stabilized. The entry into the stationary phase for microalgal cultures occurs resulting from nutrient limitation or cellular shading [15]. Throughout the experiments, there was little pH change because of the CO_2 injections; pH levels were between 7.75 and 8.83 (Table 1).

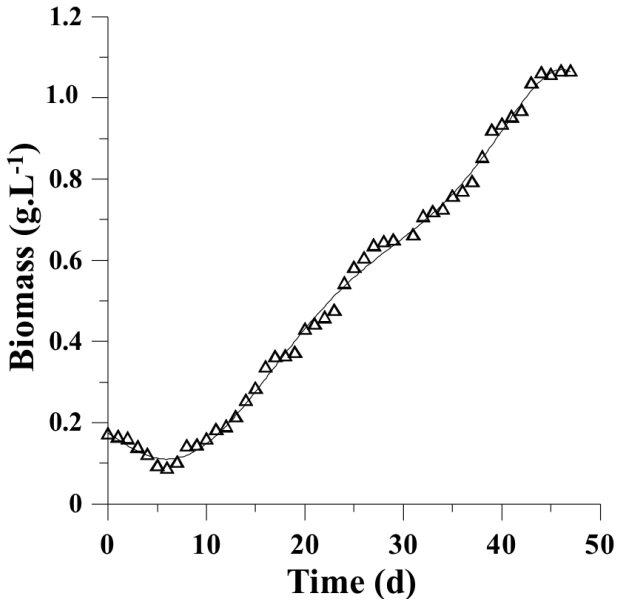

Figure 2 Growth curve of *Spirulina* sp LEB 18

Spirulina sp LEB 18 utilized the carbon source (CO_2) for cell growth, producing biomass, even with the reduction of the nitrogen source. The cultivation carried out in a horizontal tubular bioreactor presented biofixation of 160 $mg.L^{-1}.d^{-1}$ (Table 1). The microalgae CO_2 biofixation mechanism is based on the ability of these microorganisms to perform photosynthesis. Changes in nutritional sources generally do not affect the photosynthetic rate; however, the change in the concentration of nutrients such as nitrogen and phosphorus can cause a reduction in CO_2 absorption in microalgal culture [16]. Other factors such as illuminance, CO_2 injection rate on crops, pH, cell density and temperature can also determine increases or decreases in biofixation.

Table 1 Kinetic parameters and biomass composition of *Spirulina* sp LEB 18 cultured in tubular PTBR with CO_2 (laboratory scale) and commercially in raceway ponds (commercial scale).

Parameter	Laboratory scale	Commercial scale
Maximum biomass ($g.L^{-1}$)	1.07 ± 0.01	-
Maximum productivity ($g.L^{-1}.d^{-1}$)	0.02 ± 0.01	-
Specific growth rate (d^{-1})	0.12 ± 0.01	-
Generation time (d)	5.85 ± 1.05	-
Biofixation rate ($mg.L^{-1}.d^{-1}$)	160 ± 3.00	-
pH variation	7.75 - 8.83	-
Proteins (%, $w.w^{-1}$)	47.30 ± 1.10	44.70 ± 0.20
Lipids (%, $w.w^{-1}$)	32.70 ± 1.50	5.70 ± 0.50
Carbohidrates (%, $w.w^{-1}$)	13.40 ± 0.50	21.70 ± 0.10

Means ± standard deviations

In the experiment with *Spirulina* sp LEB 18, the kinetic parameters obtained at the end of the 47 d experiment were expected because of changes in the culture medium. A reduction in the nitrogen source is an effective way to increase the accumulation of lipids and/or carbohydrates in the microalgal biomass; however, the increase of these components or productivity is always proportional to cell concentration, maximum specific growth rate and generation time [17]. Radmann et al.[18]cultivated *Spirulina* sp LEB 18 in Zarrouk medium with 10% CO_2 as a carbon source, for 15 d in a vertical tubular

bioreactor achieved a maximum cell concentration of 1.53 $g.L^{-1}$ and maximum specific growth rate of 0.22 d^{-1}; these results were superior to those obtained in this study. The reduction of the nitrogen source may have adversely influenced the development of the microorganism, reducing the productivity (0.02 $g.L.d^{-1}$), maximum specific growth rate (0.12 d^{-1}) and maximum cell concentration (1.07 $g.L^{-1}$) and increasing the generation time (5.85 d). Nitrogen is an element used by cells primarily for the formation of proteins and nucleic acids and is essential for cellular development [19].

Spirulina sp LEB 18 biomass is rich in macronutrients such as carbohydrates, proteins and lipids that can generate high value-added bioproducts. The composition of microalgae, when grown both on a laboratory and a commercial scale, the protein was the macronutrient that was present in a larger quantity (47.3% and 44.7%, respectively). Among the protein compounds present in *Spirulina* phycocyanin, a blue biocorante applied in the food industry and cosmetics, stands out with a recognized antioxidant capacity [20].

Spirulina sp LEB 18, cultivated with nitrogen source reduction and by replacing the carbon source of Zarrouk ($NaHCO_3$) with 10% (v/v) CO_2, increased the lipid concentration (32.7%) by nearly 6 times when compared with growing the same species on a commercial scale (5.7%). The cultivation time until the stationary phase and the reduction of the nitrogen component may have contributed to the increase of lipid concentration. In the exponential growth phase, protein content is generally higher because of cell proliferation and nucleic acids. The increased production of lipids and carbohydrates, however, occurs in the stationary phase as an energy accumulation form [21]. The nitrogen source reduction in microalgal cultivation is capable of shifting the metabolism of cells for the production of lipids or carbohydrates [22]. In the case of *Spirulina* sp LEB 18 cultivation in a tubular bioreactor, this difference was favorable for lipid production and decreased carbohydrate production by approximately 2 times (13.4% and 21.7% tubular and cultivation on a commercial scale, respectively). Microalgae carbohydrates and lipids have been studied for energy production and food applications. Thus, the obtained carbohydrates can be applied in the production of bioethanol, and lipids can be applied for biodiesel production, obtaining of essential fatty acids and as pigments such as astaxanthin or biopolymers.

Biodiesel consists of fatty acid methyl esters (FAMEs) produced by transesterification of lipids that directly influence the quality of the produced biofuel. According to the FAME composition in biodiesel, there may be changes in the viscosity characteristics, density and cetane number [23]. Through gas chromatographic analysis, 82.7% and 87.6% of FAMEs were identified in *Spirulina* sp LEB 18 biomass grown on a laboratory scale (horizontal PTBR) and a commercial scale (raceway bioreactor), respectively (Table 2).

Table 2 Fatty acids composition (%, $w.w^{-1}$) for *Spirulina* sp LEB 18 cultured in tubular PTBR with CO_2 (laboratory scale) and commercially in raceway ponds (commercial scale).

	Fatty acids	Trivial names	Laboratory scale	Commercial scale
Saturated	13:0	Tridecylic	0.50± 0.01	0.25± 0.01
	14:0	Myristic	0.56± 0.02	0.39± 0.01
	15:0	Pentadecanoic	0.33± 0.01	0.17± 0.01
	16:0	Palmitic	44.87± 7.20	47.20± 5.11
	17:0	Margaric	0.44± 0.02	0.19± 0.02
	18:0	Stearic	0.91± 0.02	1.40± 0.03
	22:0	Beenic	0.03± 0.01	nd
	Sum		47.64	49.60
Unsaturated	14:1ω5	Miristolyc	0.06± 0.01	0.14± 0.01
	15:1	Pentadecanoic	0.87± 0.03	1.42± 0.08
	16:1 ω 7	Palmitoleic	2.40± 0.23	4.25± 0.80
	16:2 ω4	Hexadecadienoic	1.39± 0.11	1.24± 0.01
	17:1 ω5	Heptadecanoic	0.18± 0.01	0.50± 0.12
	16:3 ω4	Hexadecatrienoic	0.15± 0.02	0.05± 0.01
	18:1 trans	Elaidic	0.47± 0.02	0.15± 0.01
	18:1 ω9	Oleic	3.44± 0.03	3.05± 0.01
	18:1 ω7	Vaccenic	1.10± 0.01	0.96± 0.01
	20:1 ω9	Gondoic	0.08± 0.01	nd
	18:2 trans	Linoelaidic	0.14± 0.02	0.10± 0.01
	18:2 ω6	Linoleic	11.15± 2.41	12.23± 2.07
	18:3 ω6	γ-Linolenic	12.66± 4.20	13.09± 3.59
	18:3 ω3	α-Linolenic	0.14± 0.01	nd
	18:4 ω3	Stearidonic	0.09± 0.01	nd
	20:2 ω6	Eicosadienoic	0.48± 0.01	0.31± 0.01
	20:3 ω6	di-homo-γ-linolenic	0.24± 0.01	0.47± 0.01
	Sum		35.04	37.96
Total			82.68	87.56

Means ± standard deviations

The main fatty acids found in both samples were palmitic, palmitoleic (C16), oleic, linoleic and linolenic (C18) acids. These results are in agreement with the literature that reports that the fatty acid composition of microalgae consists mainly of C16 and C18 fatty acids, approaching the vegetable oils that are generally used for biodiesel production and are g suitable for the production of this biofuel [24,25].

Oleic acid (18:1), considered as a biodiesel quality indicator, has been identified in both conditions of *Spirulina* sp LEB 18 cultivation, with 3.4% and 3.0%, on a commercial and laboratory scale, respectively. Addition of the methyl oleate is suggested to improve biodiesel properties such as oxidative stability and melting temperature [25]. Palmitic acid was the dominant FAME among the total FAME that were identified (44.9%, growing tubular bioreactor and 47.2%, commercial cultivation). The content was similar to that of palm oil, which was between 39.2% and 45.8% of 16:0 [26]. When comparing the composition of microalgae grown on a laboratory scale with the commercial scale, it can be said that the reduction in the nitrogen source and CO_2 injection did not alter the FAME composition in the biomass.

In cultures performed in a horizontal FBRT using 10% (v / v) CO_2 and reduction of the nitrogen source on a laboratory scale, the direct esterification of microalgal lipids formed saturated FAMEs (47.6%) and unsaturated FAMEs (35.0%). The highest percentage of saturated FAMEs is an advantage because the higher the concentration of these compounds in biodiesel, the greater the oxidation resistance [27]. The presence of large amounts of unsaturated FAMEs reduces the stability of the biodiesel, causing it to rapidly oxidize (Sarin et al, 2007). According to Sarin et al. [28], palm oil has 43.4% saturated and 41% unsaturated fatty acids, thereby *Spirulina* sp LEB 18 biodiesel would be more stable compared to palm oil.

The yield obtained for esterification of the lipid extract of the biomass (47.4 ± 1.40%) was approximately 2 times higher than the *in situ* yield (19.8 ± 1.70%). This result highlights the importance of studying the extraction of microalgal lipids primarily for the reduction of costs and reaction time and the increased efficiency of the applied methodologies, enabling even more conventional transesterification. Conventional transesterification is the chemical conversion of triacylglycerides (oil) in FAMEs through a solvent and a catalyst which comprises two steps: the extraction of oil feedstock and esterification of the obtained oil. When the process occurs *in situ*, extraction and esterification steps occur together in a single step. Use of this method to obtain biodiesel from microalgal biomass reduces extraction costs in addition to the reaction time [29].

CONCLUSION

Spirulina sp LEB 18 cultured in tubular air-lift photobioreactor biofixed 160 $mg.L.d^{-1}$ CO_2 reaching 1.07 $g.L^{-1}$ of maximum cell concentration. The biomass obtained presents potential to biodiesel production with 32.7% of lipids, an interesting fatty acid composition and yield of 47.9% in conventional transesterification. The other evaluated compounds (proteins and carbohydrates) can be used to obtain high value-added products such as pigments and biofuels. The biodiesel production from microalgal biomass cultured with CO_2 combined with other compounds production supports the application of a photobiorefinary concept from this raw material. Along with this, CO_2 using make the process more feasible reducing costs with the carbon source.

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