

# UTILIZATION OF CO<sub>2</sub> IN SEMI-CONTINUOUS CULTIVATION OF *Spirulina* sp. AND *Chlorella fusca* AND EVALUATION OF BIOMASS COMPOSITION

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**Abstract** - Cultivation conditions and the process considerably influence the composition of microalgae. The objective of this study was to use CO<sub>2</sub> as a carbon source in semi-continuous cultivation of *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 and to evaluate the influence of the renewal rate on the biomass composition and production of these microalgae. *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 were cultivated with 10% (v/v) CO<sub>2</sub>. The blend concentration was 1.6 g L<sup>-1</sup>, and 20 and 40% (v/v) renewal rates were studied. *Spirulina* sp. LEB 18 presented the best kinetic results and the maximum biomass concentration and biopolymer yield when grown with CO<sub>2</sub> as the carbon source. Under the same conditions (10% (v/v) CO<sub>2</sub>), the microalgae *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 exhibited maximum levels of protein, carbohydrates and lipids.

**Keywords:** Bioproducts; Semi-continuous process; Sustainability.

## INTRODUCTION

The rising price of fossil fuels, the environmental impact of gaseous emissions, the improper disposal of polymers of petrochemical origin and the waste of raw materials have led to the demand for renewable resources and technologies that meet the needs of the world market (Antunes and Silva, 2010).

Microalgae are considered to be an efficient biological system for capturing solar energy and for the production of organic compounds. Microalgae can be produced throughout the year, requiring little water compared to terrestrial plants. The biochemical composition of microalgae can be manipulated by changing the growth conditions and environmental stresses (Hu *et al.*, 2008), inducing the production of high concentrations of commercially important biocompounds (Brennan and Owende, 2010).

The mode of operation is directly related to the biotechnological process, because the microorganism needs appropriate conditions to stimulate the synthesis of the desired product (Henrard *et al.*, 2014). The microalgal biomass has high concentrations of lipids, proteins and carbohydrates, which can be used for different applications. When the full potential of the microalgal biomass constituents is exploited, many byproducts can be obtained simultaneously and the market value is greater than the production costs (Wijffels *et al.*, 2010).

Semi-continuous cultures are alternatives for larger scale production of microalgal biomass. Periodic withdrawal of the product and the addition of substrates reduces the stagnant time for the collection of biomass products and photobioreactor cleaning. Moreover, self-sustaining and environmentally friendly microalgae cultivation must utilize alternative sources

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of carbon that originate from industrial effluents (CO<sub>2</sub>) to reduce production costs.

The objective of this study was to use CO<sub>2</sub> as a carbon source in semi-continuous cultivation of *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 and to evaluate the influence of the renewal rate on the biomass composition and production of these microalgae.

## MATERIALS AND METHODS

### Microalgae and Growing Conditions

The microalgae used in this study were *Spirulina* sp. LEB 18 (Morais *et al.*, 2008) and *Chlorella fusca* LEB 111 isolated from ponds near the region of the Presidente Médici Thermoelectric Station, Candiota-RS (Morais and Duarte, 2012). *Spirulina* sp. LEB 18 cultures were grown in Zarrouk medium (Zarrouk, 1966) and modified Zarrouk medium, which was modified by replacing the original source of carbon (16 g L<sup>-1</sup> de NaHCO<sub>3</sub>) by 10% (v/v) CO<sub>2</sub>. The microalgae *Chlorella fusca* LEB 111 were grown in BG-11 medium (Rippka *et al.*, 1979) that was modified by adding 0.4 g L<sup>-1</sup> of NaHCO<sub>3</sub>. *Chlorella fusca* LEB 111 cultures were also studied, in which the carbon sources (Na<sub>2</sub>CO<sub>3</sub> of the BG-11 medium and NaHCO<sub>3</sub>) were replaced by 10% (v/v) CO<sub>2</sub>.

The cultures were carried out in duplicate under controlled conditions in a thermal chamber at 30 °C and 41.6 μmol/m<sup>2</sup> s and under a 12 h light/dark photoperiod, with light provided by 40 W fluorescent lamps. The initial biomass concentration in the *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 cultures was 0.2 g L<sup>-1</sup>. The microalgae were grown in 2 L vertical tubular photobioreactors with a working volume of 1.5 L.

Aeration was carried out by aspirating using a porous stone. The compressed air was mixed with CO<sub>2</sub> in an industrial cylinder (White Martins). The input flow of the mixture into the cultures was 0.3 vvm, which was controlled using a solenoid valve. The 10% (v/v) CO<sub>2</sub> was injected directly into the photobioreactors every 2 h for 10 min during the light period.

### Microalgal Biomass Concentration and pH Monitoring

Samples were collected daily to determine the concentration of biomass, which was calculated by measuring the optical density at 670 nm (Costa *et al.*, 2002) using a spectrophotometer (Q7980RM, Quimis, Brazil) according to a calibration curve that related the optical density with the dry weight of the micro-

algal biomass. The pH was also monitored daily using a digital pH meter (Q400AS, Quimis, Brazil).

### Kinetic Parameters and Biomass Produced

The specific growth rate (μ<sub>x</sub>) and biomass productivity (P<sub>x</sub>) (Equation (1) and (2), respectively) were determined for each growth cycle. The produced biomass (B<sub>p</sub>) was calculated using Equation (3):

$$\mu_x = \frac{1}{X} \frac{dX}{dt} \quad (1)$$

$$P_x = \frac{X - X_0}{t - t_0} \quad (2)$$

$$B_p = \left[ \sum_{i=1}^n (X_i V_i) \right] + (X_f V_f) \quad (3)$$

where X (g L<sup>-1</sup>) is the final cell concentration in the growth cycle; X<sub>0</sub> (g L<sup>-1</sup>) is the initial cell concentration in the growth cycle; t (d) is the final time of the growth cycle; t<sub>0</sub> is the initial time of the growth cycle; V (L) is the volume removed in the growth cycle; X<sub>f</sub> (g L<sup>-1</sup>) and V<sub>f</sub> (L) are the concentration of biomass and the volume at the end of the cultivation, respectively. The mean value of the specific growth rate and biomass productivity results are presented.

### Characterization of the Microalgal Biomass

For each dilution during the semi-continuous cultivation, the microalgal biomass was recovered via centrifugation at 16920 g, and 20 °C for 20 min to separate the biomass from the culture medium. Subsequently, the biomass was washed with distilled water to eliminate salt residues in the culture and centrifuged again. Then, the microalgal biomass was refrigerated at -20 °C. At the end of the semi-continuous cultivation, the biomasses obtained at each dilution were mixed together, and the samples were lyophilized. The lyophilized biomass was subjected to pre-treatment in an ultrasonic probe. This procedure consisted of adding 10 mL of distilled H<sub>2</sub>O to 5 mg of microalgal biomass and sonicating for 10 min in 59 s cycles (59 s on and 50 s off). The extract was homogenized using a magnetic stirrer for later characterization analyses.

### Proximate Composition

The biomasses obtained from the semi-continuous cultivation of *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 were characterized in terms of carbo-

hydrate content using the phenol-sulfuric method (Dubois *et al.*, 1956), protein content using the colorimetric method of Lowry (Lowry *et al.*, 1951), and lipid content using the method reported by Folch *et al.* (1957) and adapted by Colla (2002). The moisture and ash content were determined according to the AOAC (2000) methodology. The analyses were carried out in triplicate, and the results are presented on a dry basis.

### Biopolymer Yield

The biopolymer extraction was carried out for *Spirulina* sp. LEB 18 using differential digestion. The lyophilized biomass was stirred using a magnetic stirrer (753A, Fisatom, Brazil) with sodium hypochlorite (NaOCl) 10% (v/v) and distilled water. Next, it was centrifuged (14,100 g, 20 °C for 20 min). The precipitate was washed and stirred for 10 min with distilled water, and the centrifugation process was repeated. Subsequently, the precipitate was washed with acetone while being stirred, followed by centrifugation (14,100 g, 15 °C for 20 min) and oven drying (35 °C) for approximately 48 h (Morais, 2008).

The biopolymer yield ( $\eta$ ) was calculated according to Equation (4), where  $\eta$  is the yield of biopolymers with respect to the microalgal biomass (% w/w),  $m_b$  is the final weight of the biopolymer (g), and  $m$  is the microalgal biomass (g).

$$\eta = \frac{m_b}{m} \quad (4)$$

The extraction of biopolymers was carried out using the biomass obtained from the culture with Zarrouk medium and also from the assays with 10% (v/v) CO<sub>2</sub>, at both 20% and 40% renewal rates (v/v).

### Statistical Analysis

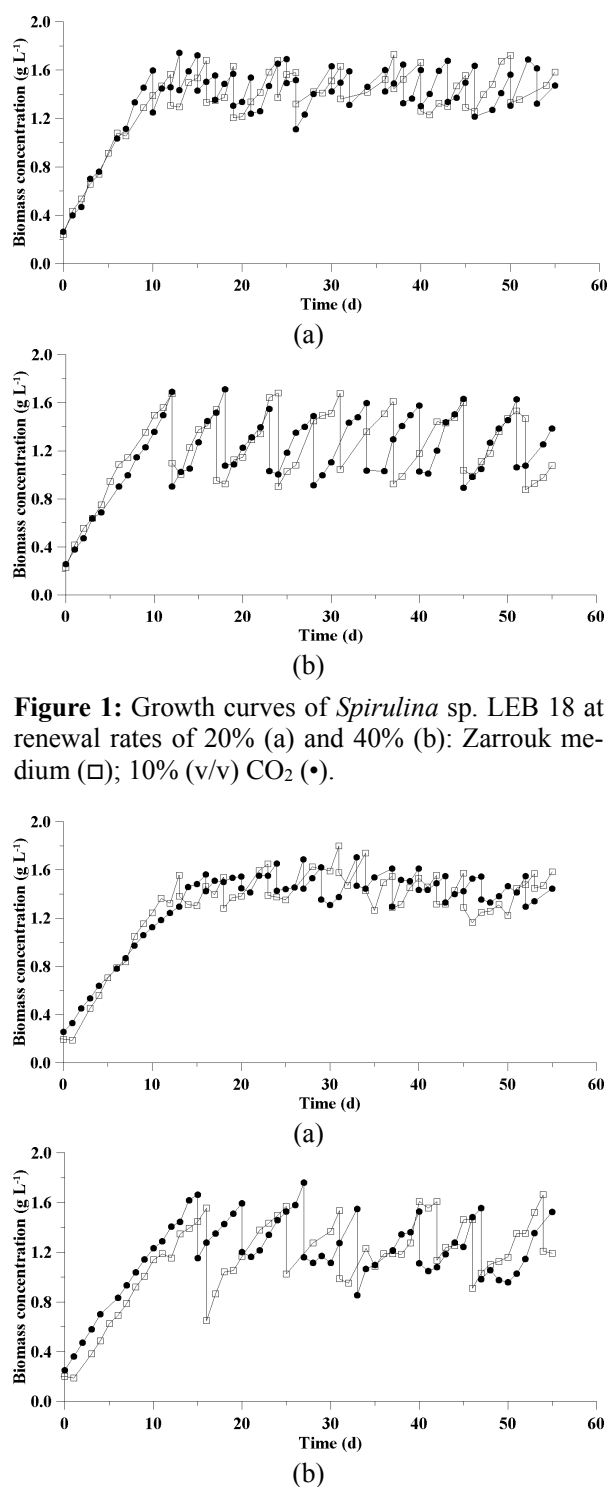
Analysis of variance (ANOVA) was used at a confidence level of 99% ( $p \leq 0.01$ ), followed by Tukey's post-hoc test to compare the means of the growth kinetics of the results and to characterize the biomass of each microalgae.

## RESULTS AND DISCUSSION

### Growth of *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111

The cultures of both types of microalgae grown with CO<sub>2</sub> as the carbon source at a 20% renewal rate

(v/v) exhibited more growth cycles, favoring the production of microalgal biomass, compared to the assays grown with NaHCO<sub>3</sub> (Figures 1 and 2).



**Figure 1:** Growth curves of *Spirulina* sp. LEB 18 at renewal rates of 20% (a) and 40% (b): Zarrouk medium (□); 10% (v/v) CO<sub>2</sub> (•).

**Figure 2:** Growth curves of *Chlorella fusca* LEB 111 at renewal rates of 20% (a) and 40% (b): modified BG-11 medium (□); 10% (v/v) CO<sub>2</sub> (•).

In accordance with the growth curves a linear increase of biomass concentration was observed in each cycle. As a consequence of a system where there is a limitation to the growth, the specific growth rate decreased during a cycle. Among the studied microalgae, *Spirulina* sp. LEB 18 exhibited higher productivity, specific growth rates and biomass production (Table 1).

The renovation rate of the medium in the cultures with 10% (v/v) CO<sub>2</sub> did not influence the kinetic results of *Spirulina* sp. LEB 18. However, it did influence the results obtained for *Chlorella fusca* LEB 111. Thus, the semi-continuous cultivation of *Spirulina* sp. LEB 18 can reduce the cost of nutrients during the process, as there is a lower renovation rate of the medium (20% v/v).

For both microalgae, the cultures with 10% (v/v) CO<sub>2</sub> exhibited lower pH values. The maximum and minimum pH values were found in the assays with a renewal rate of 40% (v/v) (Table 2). However, during the cultivation, the pH values tended to stabilize around 9.0. Zeng *et al.* (2012) observed that high aeration

with CO<sub>2</sub> resulted in a reduction of the culture pH during cultivation. However, the control experiments also exhibited stable pH profiles. According to Barsanti and Gualtieri (2006), the addition of CO<sub>2</sub> to the culture medium is an alternative method that can be used to decrease the pH of the medium and prevent abrupt changes. CO<sub>2</sub> dissolves in the medium, and the carbonic acid that is formed prevents an increase in pH during the growth of the microalgae.

For *Spirulina* sp. LEB 18 cultured in Zarrouk medium, the maximum pH was 11.20, and for *Chlorella fusca* LEB 111 cultured in modified BG-11 medium, the maximum pH was 11.59. According to Cuaresma *et al.* (2006), the increase in pH in photosynthetic cultures occurs due to the biological activity of the cells, which reduces the dissolved inorganic carbon content due to the consumption during cell growth. Thus, there exists a displacement of the carbonate-bicarbonate equilibrium in the buffer system. The pH of the culture medium determines the forms of inorganic carbon (CO<sub>2</sub>, CO<sub>3</sub><sup>2-</sup> or HCO<sub>3</sub><sup>-</sup>) dissolved in the liquid phase.

**Table 1: Kinetic parameters and produced biomass (B<sub>p</sub>, g) obtained from the semi-continuous cultivation of the microalgae.**

TR (% v/v)	N	P <sub>mean</sub> (g L <sup>-1</sup> d <sup>-1</sup> )*	μ <sub>mean</sub> (d <sup>-1</sup> )*	B <sub>p</sub> (g)*
<b><i>Spirulina</i> sp. LEB 18 (Zarrouk Medium)</b>				
20	11	0.081 ± 0.005 <sup>bcC</sup>	0.060 ± 0.002 <sup>dC</sup>	7.49 ± 0.04 <sup>bcB</sup>
40	8	0.092 ± 0.003 <sup>Bb</sup>	0.079 ± 0.003 <sup>bbB</sup>	8.37 ± 0.14 <sup>abcAB</sup>
<b><i>Spirulina</i> sp. LEB 18 (10% (v/v) CO<sub>2</sub>)</b>				
20	18	0.105 ± 0.004 <sup>aA</sup>	0.091 ± 0.005 <sup>aA</sup>	10.46 ± 0.12 <sup>aA</sup>
40	9	0.108 ± 0.004 <sup>aA</sup>	0.092 ± 0.001 <sup>aA</sup>	9.81 ± 0.62 <sup>abA</sup>
<b><i>Chlorella fusca</i> LEB 111 (Modified BG-11 Medium)</b>				
20	10	0.060 ± 0.002 <sup>dC</sup>	0.058 ± 0.001 <sup>dB</sup>	6.92 ± 0.21 <sup>cA</sup>
40	7	0.075 ± 0.010 <sup>cAB</sup>	0.058 ± 0.001 <sup>dB</sup>	7.42 ± 0.72 <sup>cA</sup>
<b><i>Chlorella fusca</i> LEB 111 (10% (v/v) CO<sub>2</sub>)</b>				
20	12	0.062 ± 0.002 <sup>dBc</sup>	0.057 ± 0.002 <sup>dB</sup>	7.45 ± 0.50 <sup>cA</sup>
40	7	0.079 ± 0.009 <sup>bcA</sup>	0.071 ± 0.002 <sup>cA</sup>	8.08 ± 0.56 <sup>bcA</sup>

TR: renewal rate; N: growth cycle; \*: Values are mean ± standard deviation; Lowercase letters in the same column represent comparisons among all tests; capital letters in the same column compare microalgae with one other; equivalent letters in the same column do not differ statistically (p > 0.01) according to Tukey's test.

**Table 2: pH values of semi-continuous cultivation of microalgae.**

Microalga / Assay	TR (% v/v)	pH (Minimum)	pH (Maximum)
<i>Spirulina</i> sp. LEB 18 / Zarrouk Medium	20	9.74	11.20
<i>Spirulina</i> sp. LEB 18 / Zarrouk Medium	40	9.76	10.86
<i>Spirulina</i> sp. LEB 18 / 10% (v/v) CO <sub>2</sub>	20	7.51	10.03
<i>Spirulina</i> sp. LEB 18 / 10% (v/v) CO <sub>2</sub>	40	7.25	10.04
<i>Chlorella fusca</i> LEB 111 / Modified BG-11 Medium	20	9.11	11.59
<i>Chlorella fusca</i> LEB 111 / Modified BG-11 Medium	40	9.13	10.68
<i>Chlorella fusca</i> LEB 111 / 10% (v/v) CO <sub>2</sub>	20	7.68	10.39
<i>Chlorella fusca</i> LEB 111 / 10% (v/v) CO <sub>2</sub>	40	7.35	10.62

TR: Renewal rate.

The CO<sub>2</sub> concentration influenced the *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 microalgae cultures, as the best kinetic responses were found for 10% (v/v) CO<sub>2</sub>. For *Spirulina* sp. LEB 18, the increase in CO<sub>2</sub> in the cultures caused a 39.7% (w/w) increase in the concentration of biomass produced, and the renewal rate was 20% (v/v). Zeng *et al.* (2012) also observed that *Spirulina platensis* microalgae cultured with CO<sub>2</sub> exhibited better microalgal growth compared to controls that did not contain CO<sub>2</sub>.

The kinetic responses obtained for the culture with 10% (v/v) CO<sub>2</sub> can be explained by the uptake of CO<sub>2</sub> gas due to the Calvin cycle. The initial reaction that occurs in the Calvin cycle is CO<sub>2</sub> fixation in ribulose, which is catalyzed by the enzyme ribulose 1.5 biphosphate carboxylase/oxygenase, known as Rubisco. Because Rubisco requires high CO<sub>2</sub> concentrations at its active site to maintain the carboxylase activity and inhibit oxygenase (Schenk *et al.*, 2008), the addition of CO<sub>2</sub> to cultures of microalgae provides better kinetic results.

The semi-continuous process and CO<sub>2</sub> as a carbon source is favorable in terms of sustainability factors. For a semi-continuous cultivation, the harvesting of the product and the periodic renewal of the substrate saves time that would otherwise be spent harvesting the formed biomass, cleaning the photobioreactor and re-starting the process. The use of CO<sub>2</sub> reduces the process costs because carbon is the most-required nutrient in microalgal cultures. CO<sub>2</sub> can be obtained free of charge from the burning of coal, which provides environmental benefits by reducing the emission of this gas into the atmosphere.

### Characterization of the Microalgal Biomass

For each complete Calvin cycle, carbohydrates are produced; however, fatty acids, amino acids and organic acids may also be synthesized during the photosynthetic fixation of CO<sub>2</sub> (Schenk *et al.*, 2008). The microalga *Chlorella fusca* LEB 111 produced the maximum content of carbohydrates when cultivated at 10% (v/v) CO<sub>2</sub> and a renewal rate of 40% (v/v) (Table 3).

The highest protein concentrations were obtained for *Spirulina* sp. LEB 18 in assays using CO<sub>2</sub> as a carbon source. *Chlorella fusca* LEB 111 yielded the maximum protein content in the assay with 10% (v/v) CO<sub>2</sub> and a renewal rate of 40% (v/v), although no statistical difference ( $p > 0.01$ ) was found compared to the higher value of *Spirulina* sp. LEB 18. According to Lourenço *et al.* (2004), if nitrogen is

abundant in cultures, the concentrations of proteins and chlorophyll in the cells tend to increase.

The analysis of the chemical composition of *Spirulina* sp. LEB 18 revealed an inverse relationship between the contents of protein and carbohydrates. Higher levels of protein and lower levels of carbohydrates were obtained for the microalgae cultivated with 10% (v/v) CO<sub>2</sub>. Other studies have shown similar results. Derner (2006) reported that the use of CO<sub>2</sub> increased the protein content, especially in cultures of *Thalassiosira fluviatilis*. Castro Araújo and Garcia (2005) found that the concentration of carbohydrates was lower due to the use of CO<sub>2</sub> in cultures of *Chaetoceros* cf. *weighamii*.

The microalgae *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 exhibited the maximum lipid levels for the assays conducted with 10% (v/v) CO<sub>2</sub> and a 20% (v/v) renewal rate. Lipid accumulation occurs when acetyl-CoA is converted to malonyl-CoA followed by fatty acids after continuous cycles, which is catalyzed by acetyl-CoA carboxylase (ACCase). The accumulation of lipids occurs in the chloroplasts, and ACCase regulates the fatty acid synthesis by microalgae (Lv *et al.*, 2010). In the chloroplasts, depending on the developmental stage of the cell, pyruvate dehydrogenase activity is often low. In contrast, acetyl-CoA synthetase in the chloroplasts has a high affinity for acetate and consumes adenosine triphosphate (ATP) to convert it to acetyl-CoA (Heldt, 2005). Thus, the addition of CO<sub>2</sub> to the *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 cultures induced the rapid conversion of ATP to acetyl-CoA and initiated the biosynthesis of lipids.

The *Spirulina* sp. LEB 18 assays grown with NaHCO<sub>3</sub> as the carbon source exhibited the maximum ash content, as indicated by the high concentration of minerals in the Zarrouk medium (16.8 g L<sup>-1</sup>). For the *Spirulina* sp. LEB 18 assays grown with CO<sub>2</sub> as the carbon source, the ash levels were lower, as NaHCO<sub>3</sub> from the Zarrouk medium was replaced with 10% (v/v) CO<sub>2</sub>. The *Chlorella fusca* LEB 111 assays exhibited lower levels of ash compared to the *Spirulina* sp. LEB 18 assays. In the BG-11 medium, used in the cultivation of *Chlorella fusca* LEB 111, the concentration of nutrients was lower compared to the use of the Zarrouk medium. Furthermore, NaHCO<sub>3</sub> may have affected the ash content of the *Chlorella fusca* LEB 111 assays, which exhibited higher ash levels when grown with the modified BG-11 medium containing 0.4 g L<sup>-1</sup> NaHCO<sub>3</sub>. For the *Chlorella fusca* LEB 111 assays grown with CO<sub>2</sub>, the ash levels were lower due to the replacement of the carbon source of the medium with 10% (v/v) CO<sub>2</sub>.

**Table 3: Determination of protein, carbohydrates, lipids and ash in the microalgal biomass.**

TR (%, v/v)	Carbohydrates (%, w/w)	Protein (%, w/w)	Lipids (%, w/w)	Ash (%, w/w)
<b><i>Spirulina</i> sp. LEB 18 (Zarrouk Medium)</b>				
20	19.1 ± 0.97 <sup>cA</sup>	42.9 ± 4.50 <sup>dB</sup>	9.2 ± 0.65 <sup>bcA</sup>	28.1 ± 1.90 <sup>aA</sup>
40	18.8 ± 1.00 <sup>cA</sup>	47.8 ± 2.25 <sup>cB</sup>	9.3 ± 1.39 <sup>bcA</sup>	22.7 ± 0.97 <sup>bB</sup>
<b><i>Spirulina</i> sp. LEB 18 (10% (v/v) CO<sub>2</sub>)</b>				
20	13.7 ± 0.45 <sup>dB</sup>	60.1 ± 1.18 <sup>aA</sup>	9.8 ± 0.61 <sup>abcA</sup>	14.4 ± 0.94 <sup>cC</sup>
40	14.4 ± 0.35 <sup>dB</sup>	59.7 ± 1.66 <sup>aA</sup>	8.8 ± 1.01 <sup>cA</sup>	13.8 ± 0.92 <sup>cC</sup>
<b><i>Chlorella fusca</i> LEB 111 (Modified BG-11 Medium)</b>				
20	27.4 ± 1.37 <sup>bB</sup>	51.3 ± 1.91 <sup>bcAB</sup>	11.8 ± 1.38 <sup>abcA</sup>	5.3 ± 0.30 <sup>dA</sup>
40	30.1 ± 1.32 <sup>aA</sup>	49.5 ± 0.81 <sup>cB</sup>	12.9 ± 1.62 <sup>abA</sup>	5.7 ± 0.28 <sup>dA</sup>
<b><i>Chlorella fusca</i> LEB 111 (10% (v/v) CO<sub>2</sub>)</b>				
20	29.4 ± 0.59 <sup>abAB</sup>	51.0 ± 2.49 <sup>bcAB</sup>	13.3 ± 1.46 <sup>aA</sup>	3.5 ± 0.05 <sup>dB</sup>
40	30.2 ± 1.10 <sup>aA</sup>	56.1 ± 3.60 <sup>abA</sup>	11.6 ± 1.72 <sup>abcA</sup>	4.0 ± 0.43 <sup>dB</sup>

Results are on a dry basis. TR: Renewal rate; \*: Values are mean ± standard deviation; Lowercase letters in the same column represent a comparison among all of the assays; capital letters in the same column compare each microalga with one another; equal letters in the same column do not differ statistically ( $p > 0.01$ ) according to Tukey's test.

Elucidating the kinetic parameters for the synthesis of biopolymers is important, and the aim is to identify strains with specific growth rates and high yields that utilize low-cost substrates and yield high concentrations of biopolymers in relation to the total dry mass. The production costs of the biopolymers are directly linked to the selection of the type of microorganism and substrate (Chen, 2009; Khanna and Srivastava, 2005).

The maximum value of biopolymer yield was 7.1% (w/w), obtained by the *Spirulina* sp. LEB 18 assay with 10% (v/v) CO<sub>2</sub> and a renewal rate of 40% (v/v), which was significantly different compared to the other assays ( $p < 0.01$ ). At a 20% (v/v) renewal rate, the yield was 3.9% (w/w). CO<sub>2</sub> stimulated the synthesis of biopolymers by the microalga, as this nutrient was made available throughout the cultivation.

For the cultivation of *Spirulina* sp. LEB 18 with NaHCO<sub>3</sub> (16.8 g L<sup>-1</sup>) as the carbon source, the biopolymer yield was 3.7% (w/w) for both renewal rates. In previous studies carried out by our team, we found that the optimal concentration of NaHCO<sub>3</sub> for *Spirulina* in biopolymer synthesis was 8.4 g L<sup>-1</sup>. Khanna and Srivastava (2005) suggested using an excess of carbon to produce polyhydroxybutyrate (PHB). However, concentrations above 8.4 g L<sup>-1</sup> for *Spirulina* produce high concentrations of nicotinamide adenine dinucleotide phosphate (NADPH), inhibiting the enzyme citrate synthase, which is responsible for the entry of acetyl-CoA into the carboxylic acids cycle, where it is available to 3-β-cetatiolase. 3-β-Cetatiolase binds two molecules of acetyl-CoA to form acetoacetyl-CoA, which is reduced to (R)-3-hydroxybutyryl-CoA. Thus, PHB is synthesized by the polymerization of (R)-3-hydroxybutyryl-CoA by the

PHA synthase enzyme (Khanna and Srivastava, 2005).

The assays using NaHCO<sub>3</sub> as the carbon source yielded the required conditions for the cellular multiplication of *Spirulina* sp. LEB 18. According to Lima *et al.* (2001), under balanced growth conditions when all nutrients necessary for cell multiplication are available, high levels of free coenzyme A (CoA) are expected. Thus, it is possible to supply the high demand for acetyl groups during the Krebs cycle for the formation of carbon skeletons and generation of energy. Free CoA inhibits the β-ketothiolase enzyme, thus preventing the synthesis of PHB. Therefore, the yields of biopolymers presented in this study, in the assays where NaHCO<sub>3</sub> was used as the carbon source, were low compared to the assays that used CO<sub>2</sub>.

Further studies of biopolymer production in semi-continuous cultivations of *Spirulina* sp. LEB 18 microalgae can be conducted to determine the cultivation limitations of other nutrients, such as nitrogen and phosphorus. Miyake *et al.* (1996) studied the cultivation of *Synechococcus* sp. MA19 microalga using CO<sub>2</sub> but without nitrogen and reported 30% (v/v) PHB. Nishioka *et al.* (2001) achieved 66% PHB with the same strain of microalga grown with CO<sub>2</sub> but using a medium that was phosphate-free. The limitation of phosphate changes the balance of ATP and NADPH production during photosynthesis, which is potentially responsible for the metabolic changes in the synthesis of PHB (Marzan and Shimizu, 2011). Under nitrogen limitation, cells no longer produce protein, and there is an accumulation of ATP. Excess ATP leads to decreased oxidative phosphorylation and the accumulation of reduced coenzymes (NADH), which leads to the formation of PHB (Carlson *et al.*, 2005).

## CONCLUSION

*Spirulina* sp. LEB 18 showed the best kinetic results and maximum biomass production and yield of biopolymer when cultivated with CO<sub>2</sub> as the carbon source. In cultivations with CO<sub>2</sub> it was found that the renewal rate did not influence the values of kinetic parameters obtained for *Spirulina* sp. LEB 18. In addition, in assays with CO<sub>2</sub> the microalgae *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 presented maximum levels of protein, carbohydrates and lipids.

In the semi-continuous cultivation of these microalgae, using CO<sub>2</sub> as the carbon source and a medium renewal rate of 20% (v/v) is favorable. Lower renewal rates result in lower nutrient costs for every dilution carried out. Furthermore, the use of CO<sub>2</sub> reduces the cost of the carbon source, which is the main nutrient in the culture, and also reduces the negative environmental effects cause by the emission of this gas into the atmosphere.

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## NOMENCLATURE

B <sub>p</sub>	Produced biomass (g)
m	Microalgal biomass (g)
m <sub>b</sub>	Final weight of the biopolymer (g)
N	Growth cycle
P <sub>x</sub>	Biomass productivity (g L <sup>-1</sup> d <sup>-1</sup> )
P <sub>mean</sub>	Mean value of the biomass productivity (g L <sup>-1</sup> d <sup>-1</sup> )
t	Final time of the growth cycle (d)
t <sub>0</sub>	Initial time of the growth cycle (d)
TR	Renewal rate (%)
V	Volume removed in the growth cycle (L)
V <sub>f</sub>	Volume at the end of the cultivation (L)
X	Final cell concentration in the growth cycle (g L <sup>-1</sup> )
X <sub>0</sub>	Initial cell concentration in the growth cycle (g L <sup>-1</sup> )
X <sub>f</sub>	Concentration of biomass at the end of the cultivation (g L <sup>-1</sup> )
η	Biopolymer yield (%)
μ <sub>x</sub>	Specific growth rate (d <sup>-1</sup> )
μ <sub>mean</sub>	Mean value of the specific growth rate (d <sup>-1</sup> )

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