

Article

Cultivation of *Chlorella* sp. in a Closed System Using Mining Wastewater and Simulated Flue Gas: Biomass Production and CO₂ Fixation Potential

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Abstract: *Chlorella* sp. was cultivated in a closed system using PET bottles (5 L) and with the continuous injection of air and commercial gas (98% CO₂) and in simulated conditions (15% CO₂, 73% N₂, and 12% O₂). The culture medium was prepared using well water and mining wastewater, the cultivation period occurred in a 10-day cycle, and the cell growth curves were evaluated through cell counting using a Neubauer chamber. The cultivation was carried out under the following conditions: temperature at 22 °C to 25 °C; aeration rate with commercial and simulated CO₂ gas at 0.01 vvm; and synthetic air containing 0.042% CO₂. The dry biomass productivity was 0.81 g·L⁻¹·day⁻¹ and the maximum CO₂ fixation rate was 0.90 g·L⁻¹·day⁻¹ when the microalgae were cultivated with a continuous flow of simulated waste gas and a culture medium composed of wastewater. The percentages of macromolecules obtained in the biomass cultivated in wastewater reached 20.95%, 26.48%, and 9.3% for lipids, proteins, and carbohydrates, respectively.

Keywords: CO₂ biofixation; *Chlorella* sp.; wastewater cultivation; growth curves



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1. Introduction

Population growth, rapid industrial development, and urbanization have increased the demand for energy production, especially the use of fossil fuels, causing the large-scale release of CO₂ into the atmosphere. It is estimated that fossil fuel plants emit almost 33.4 Gt of CO₂ each year, representing approximately 40% of the total CO₂ emitted on the planet, significantly increasing the effects of global warming [1–4].

In attempts to mitigate these effects, the use of renewable energy, such as wind, solar, and biomass, has been gaining ground on the world stage to replace the use of energy derived from fossil fuels [5]. Carbon capture and utilization technologies have emerged as promising and increasingly recognized approaches, attracting attention for their potential to solve the global issue of climate change [6]. Industries that require combustion mechanisms to function have sought to find viable techniques to achieve a carbon neutral economy. Studies have indicated microalgae as a potential platform for large-scale biochemical production, CO₂ capture, and environmental remediation [7].

The use of CO₂-fixing microalgae plays an important role in mitigating residual carbon dioxide, minimizing the environmental impacts caused by the greenhouse effect. They can effectively capture CO₂ from the atmosphere and other sources, such as industrial environments with high concentrations of emitted CO₂ flue gas, exhibiting a CO₂ utilization efficiency that exceeds that of typical terrestrial plants by 10–50% [6]. Studies in the literature have indicated that every 1.8 kg of CO₂ fixed corresponds to 1 kg of biomass produced [3,8]. Methods based on the cultivation of microalgae have an efficiency of 80% to 99% in CO₂ biosequestration [2]. Furthermore, microalgae act as environmental bioremediators, removing excess nutrients from water bodies, reducing the possibility of contamination caused by industrial waste.

Therefore, microalgae offer economic and ecological advantages including photosynthetic efficiency, high growth rates, and cooperation with CO₂ sequestration and wastewater treatment to produce biomass that can be transformed into high-value-added products [2,9].

Microalga biomass has been explored for multifaceted applications in various domains, including biofuels, bioplastics, natural pigments, food, feed, cosmetic ingredients, nutraceuticals and pharmaceuticals, and clinical medicine [6]. For example, microalgae are an important source of bioproducts, such as proteins and polyunsaturated fatty acids like omega-3 [7]. Some microalgae contain more than 50% protein in their cells on a dry weight basis. It is observed that microalgae are an alternative source of omega-3 essential fatty acids for human consumption. Upon administering 2.4% algae-mixed feed to chickens, the effectiveness of the assimilation of DHA (fatty acids found in omega-3) from microalgae to eggs was increased to 42.6% [7,8]. It is estimated that the global market value of the microalga industry could reach USD 1.4 billion by 2030 [6].

The present study aimed to evaluate the potential for CO₂ biofixation in *Chlorella* sp. using wastewater from mining operations as a culture medium and injecting a simulated mixture of exhaust gas derived from refractory industries containing 73% N₂, 15% CO₂, and 12% O₂. In addition, the macromolecular biochemical composition of the obtained biomass and the rate of CO₂ fixation in the cultivated biomass were analyzed. The experimental tests were performed in two stages: the first stage evaluated the behavior of *Chlorella* sp.'s growth curve and the variation in pH during the insertion of commercial carbon dioxide at a concentration of 98% and simulated gas.

2. Materials and Methods

2.1. *Chlorella* sp. Cultivation and Experimental Design

For the development of this work, a *Chlorella* sp. strain provided by the Sustainable Mariculture Laboratories (LAMARSU) and the Live Food Production Laboratory (LAPAVI) of the Federal Rural University of Pernambuco UFRPE was used (Figure 1).

For the experimental design, samples for each treatment were randomly distributed. The culture in the PET bottles contained approximately 4 L of culture medium, it was kept under artificial lighting from fluorescent lamps with a light intensity ranging from 7000 to 10,000 lux (digital lux meter: MT 30 Ruining/Walfront, Wuhan, China), and the temperature was maintained between 22 and 24 °C.

The experimental test was performed in two stages: the first stage evaluated the behavior of *Chlorella* sp.'s growth curve and the injection of commercial carbon dioxide at a concentration of 98% and simulated gas. The pH variation was measured 5 times a day every 2 h.

The second stage evaluated microalgal growth using mining wastewater and simulated gas insertion, the quantification of the CO₂ fixed in the biomass, and the macromolecular biochemical composition of the biomass obtained.

The simulated gas was purchased from GASMED (Comércio e Serviço de Gases LTDA, Curitiba, Brazil); it consisted of approximately 9.3 m³ of a simulated mixture of gases emanating from the refractory industry with 15% CO₂, 73% N₂, and 12% O₂, and was stored in a 9.3 m³ WM valve T cylinder of the CGA580 type at a pressure of 185 kgf/cm². Although the oxygen composition was higher than that observed under real conditions (see Table 1), this study aimed to evaluate the CO₂ content; the effects that N₂ and O₂ could have on the cultivation of *Chlorella* sp. were not reported.

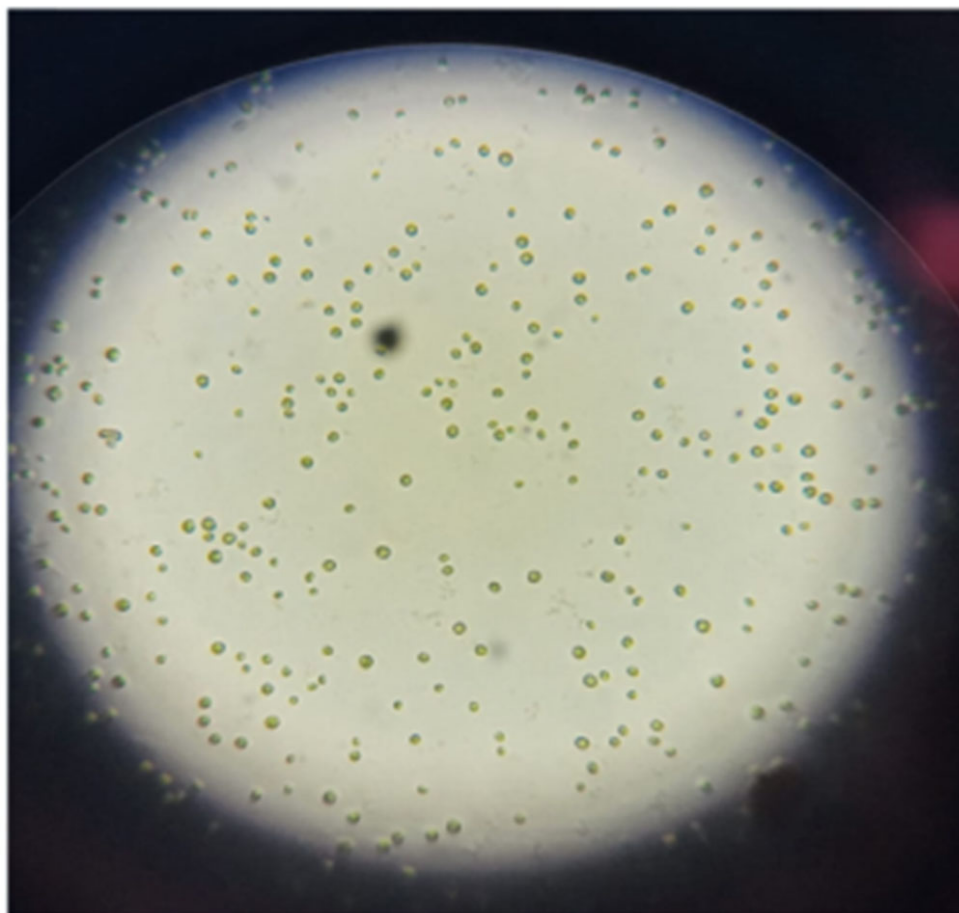


Figure 1. *Chlorella* sp. strain inoculum; image of the microalgae observed under an optical microscope with 100× magnification.

Table 1. Gas composition at two gas capture points in the gas industry (RHI Magnesite) in a calcination oven.

Gas	Calcination Oven Output	Slender Filter Chimney
O ₂ (%)	10.0	15.0
CO ₂ (%)	15.8	9.3
CO (ppm)	2550	1500
NO _x (ppm)	65	38
SO ₂ (ppm)	<20	<20
SO ₃ (ppm)	<3.6	<2.1
H ₂ O (%)	4.8	2.9
N ₂ (%)	73.4	75.7
Particulate Material (mg/Nm ³)	60	35
Temperature (°C)	349	-

The medium used was BG11 (Table 2) prepared with well water and wastewater.

Table 2. Chemical composition of BG11 medium.

Solution	Concentration	Used Amount
Solution 1 (sodium nitrate)	300 g/L	5 mL/L
Solution 2 (potassium phosphate)	80 g/L	5 mL/L
Solution 3 (magnesium sulfate)	15 g/L	5 mL/L
Modified Solution 4 (calcium chloride)	7.2 g/L	5 mL/L
Solution 5 (citric acid)	1.2 g/L	5 mL/L
Modified Solution 6 (ammonium ferric sulfate)	1.2 g/L	5 mL/L
Solution 7 (EDTA)	0.2 g/L	5 mL/L
Solution 8 (sodium bicarbonate)	40 g/L	5 mL/L
Solution 9 (trace metals)	4.849 g/L	1 mL/L
Boric acid	2.8 g/L	
Manganese chloride	1.8 g/L	
Zinc sulfate	0.22 g/L	
Sodium molybdate	0.021 g/L	
Copper sulfate	0.008 g/L	

2.2. Cultivation of *Chlorella* sp. with 98% CO₂ (v/v) Injection and Simulated Gas at Concentrations of 15% CO₂, 73% N₂, and 12% O₂

The experiments were carried out with the addition of 98% and 15% CO₂ and with an aeration rate varying between 0.03 vvm (120 mL/min) and 0.01 vvm (50 mL/min), respectively. At 98% CO₂, the injection time was varied between 0.5 h/day and 1 h/day, and at 15% CO₂, between 6 h/day and 24 h/day.

Microalgal growth curves were obtained through cell counting (Cells/mL) by observing the number of cells/mL in a Neubauer chamber using an optical microscope. To control acidity, the pH in the cultivation medium was analyzed as CO₂ was injected. Artificial light was provided using fluorescent lamps for 24 h. The microalgae were cultivated over a 10-day cycle, and at the end of the cycle, the biomass was flocculated using 2 mg/L of CaCl₂. After flocculation, the biomass was decanted, and then the excess supernatant was removed using the siphoning technique. The collected biomass was deposited in falcon tubes, frozen in liquid nitrogen and lyophilized.

The steps performed in the dry mass analysis were based on the methodology of Cruz et al. (2018) [10]. Initially, the membranes (Whatman GF/Ctm; diameter, 47 mm; pore size, 0.45 µm) were washed with deionized water to remove particulate matter. Then, they were calcined at 400 °C for 4 h to remove organic matter. After calcination and cooling, 20 mL of the microalga sample was filtered using a vacuum pump. After filtration, the samples were placed in an oven at 60 °C for 24 h.

The production was calculated according to the equation (Equation (1)). The analyses to obtain the dry mass were carried out in triplicate.

For the experimental design, samples of each treatment were randomly distributed. The cultivation took place in 5 L PET bottles under artificial lighting with fluorescent lamps with a light intensity ranging from 7000 to 10,000 lux (digital lux meter: MT 30 Ruining/Walfront, Wuhan, China) and temperature maintained between 22 and 24 °C. The variation in lux occurred due to the distribution of the PET bottles on the bench.

$$P(\text{g/Ld}) = (m_f(\text{g}) - m_i(\text{g})) / V_f(\text{mL}) \times 1000 \quad (1)$$

where P is the biomass produced in g/L·d, m_i is the initial mass of the calcined membrane without the microalgae, m_f is the final mass of the membrane with the dry microalga sample, and V_f is the volume of the filtered microalga culture.

The CO₂ fixation rate in the sample was obtained following the protocol described by Farroq et al. (2023) [9] and Kassim et al. [11] and calculated using Equation (2) as follows:

$$T_f(\text{CO}_2) = C(\text{ultimate}) \times P \times (\text{MCO}_2/\text{MC}) \quad (2)$$

where T_f is the fixation rate, C is the carbon content (obtained through Ultimate Analysis—CHN), P is the production (g/L·d), MCO_2 is the molar mass of carbon dioxide (g/mol), and MC is the molar mass of carbon (g/mol).

The biomass collected in the flocculation process was freeze-dried at $-40\text{ }^\circ\text{C}$ and 0.133 mbar, using a 4.5 L capacity Labconco freeze-dryer.

To obtain the protein content (%), the Kjeldahl method, recommended by the Association of Official Analytical Chemists, was used, in which the organic matter is removed by digesting the sample at $400\text{ }^\circ\text{C}$ with concentrated sulfuric acid, in the presence of copper sulfate as a catalyst, which accelerates the oxidation of organic matter. In the analysis of the final protein calculation, a conversion factor of 6.25 N was used. The nitrogen present in the resulting acid solution was determined by steam distillation, followed by titration with dilute acid [12]. The lipid content (%) was obtained by the Bligh–Dyer method, and the carbohydrate content (%) was obtained as the difference, $100 - (\% \text{ ash} + \% \text{ lipids} + \% \text{ proteins})$. To determine the ash content, the samples were subjected to thermogravimetric analysis (TGA/DTGA) using a TG 209 F3 Tarsus NETZSCH instrument (Selb, Germany). The test was performed under a gas flow current of 20 mL/min. Approximately 10 mg of the sample was used, conditioned in an Al₂O₃ ceramic crucible. The temperature of the thermogravimetric analyzer was increased by $5\text{ }^\circ\text{C}/\text{min}$ until it reached $105\text{ }^\circ\text{C}$, at which it was held for 12 min; then increased by $10\text{ }^\circ\text{C}/\text{min}$ until it reached $250\text{ }^\circ\text{C}$, at which it was held for 30 min; and finally increased by $20\text{ }^\circ\text{C}/\text{min}$ until it reached $575\text{ }^\circ\text{C}$, at which it was held for 180 min (adapted from [1]).

The growth of the *Chlorella* sp. was analyzed by injecting 98% CO₂ and 15% CO₂ (v/v) separately into well water and later into mining wastewater.

The chemical composition of the wastewater obtained from the ore basins in Brumado—BA was determined. The analysis was performed by ALS environmental LTD, and using the data from the analytical bulletin, some trace metals were identified, such as Cu ($<0.00050\text{ mg/L}$), Mg (0.0117 mg/L), Zn ($<0.0050\text{ mg/L}$), and B (0.516 mg/L) (a complete analysis can be found in Table S1 of the Supplementary Material). These trace metals were also found in the BG11 culture medium. However, it was observed that the concentrations of these metals were very low when compared to the concentrations of trace metals in BG11.

Table 3 briefly summarizes the conditions applied in the cultivation of *Chlorella* sp. in the present study.

Table 3. *Chlorella* sp. samples cultivated under different CO₂ concentrations, aeration rates, and aeration times.

Sample	Medium	Aeration Rate (vvm)	CO ₂ Concentration (%)	Aeration Time (CO ₂ /h)
B1 Control	Well water	Air	0.042	24
B2	Well water	0.03	98	0.5
B3	Well water	0.03	98	1
B4	Well water	0.01	15	6
B5	Well water	0.01	15	24
B6	Wastewater	Air	0.048	24
B7	Wastewater	0.01	15	24

3. Results and Discussion

3.1. Growth Curve of *Chlorella* sp. and pH Control Parameters When Adding 98% CO₂

Figure 2 shows the growth curves of *Chlorella* sp. when injecting 98% CO₂. The addition of 98% CO₂ over a 10-day cycle at a flow rate of 120 mL/min is equivalent to 36 L of carbon dioxide being added for 0.5 h/day and 72 L when the injection time corresponds to 1 h/day. However, even when adding twice as much 98% CO₂, it was observed from the growth curves that samples B2 and B3 had very similar cell growth profiles. Changing the time at which 98% CO₂ was added did not lead to significant changes in the growth curve of *Chlorella* sp. At the end of cultivation, samples B2 and B3 had 7.28×10^7 cells/mL and 7.022×10^7 cells/mL, respectively. The photosynthetic production of microalgae demands light and CO₂. When the cell mass concentration is high, some limitations regarding light and CO₂ penetration can occur, decelerating the production. This limits the total cell concentration.

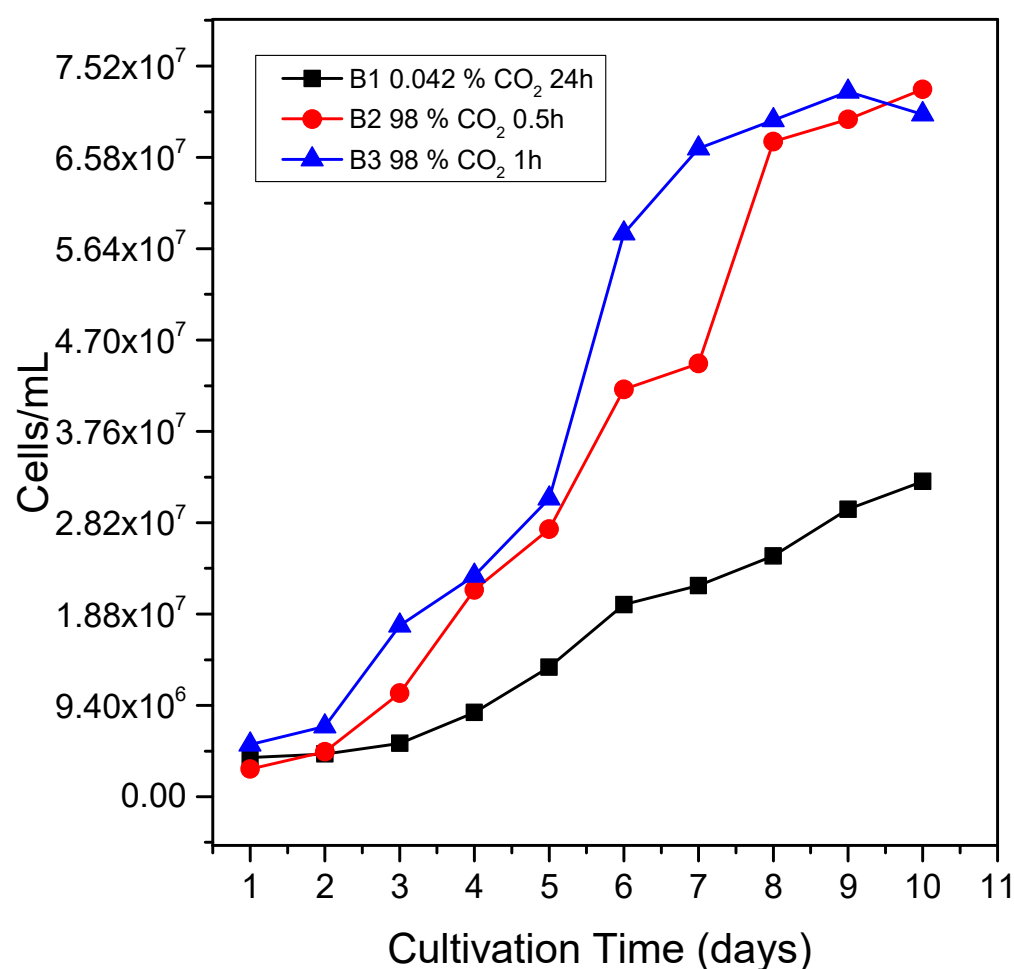


Figure 2. *Chlorella* sp. growth curve (abs) when adding 98% CO₂.

Studies have shown that a high concentration of CO₂ stimulates microalgae to grow faster compared to atmospheric air, as higher CO₂ concentrations increase the photosynthetic efficiency of microalgae, allowing them to reproduce in less time, resulting in a greater amount of biomass [13,14]. However, high concentrations of CO₂ can affect the pH of the culture, influencing the growth of microalgae and the composition of the medium. Excess CO₂ can cause a reduction in pH to cultivation threshold values, inhibiting cell growth, because it reduces the affinity of the microalgae for atmospheric CO₂ [15]. The literature shows that the ideal pH ranges for cultivation vary between 7 and 9 [4]. However,

Kassim and Meng (2017) [11], found that *Chlorella* sp. showed a good cell growth rate at pH values of 6.3 to 8.2, which explains the good cell growth of the microalgae cultivated in this study.

pH variation could be observed after the addition of 98% CO₂ (Figure 3, B2' and B3'). Too-low pH values can cause the death of the crop. It was observed that the pH values in samples B2 and B3 at the beginning of the cultivation varied between 8.5 and 10; after the addition of 98% CO₂ at the determined time intervals, there was a significant drop in pH to values in the range 5.2 to 6.3.

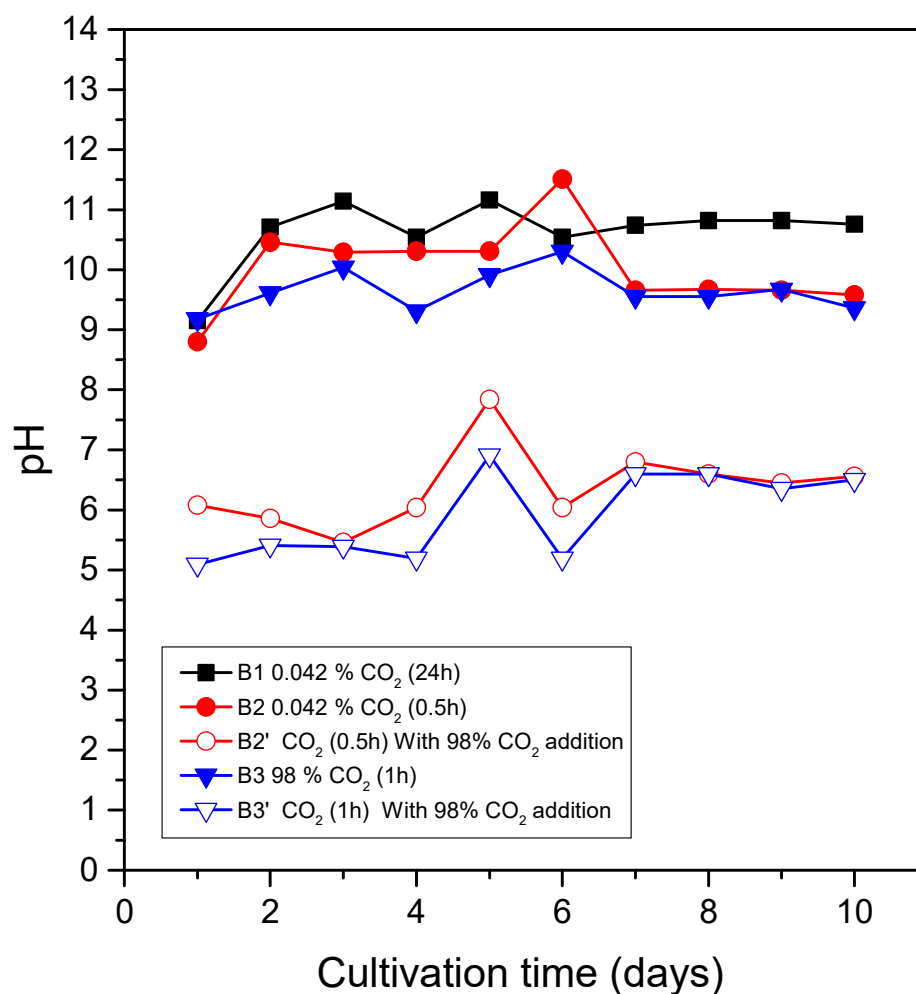


Figure 3. pH variation in relation to CO₂ injection time.

It was found that the high concentration of 98% CO₂ acidified the cultivation medium in a very short period of time, making continued gas insertion unfeasible, given that pH values greater than 5 are ideal for the growth of *Chlorella* sp. Furthermore, it has been reported in the literature that excess acidity or a long exposure time can lead to the death of most species [16].

3.2. Growth Curve of *Chlorella* sp. and pH Control Parameters When Adding Simulated CO₂ at Concentrations of 15% CO₂, 73% N₂, and 12% O₂

The microalgal growth profiles of samples B4 and B5 are shown in Figure 4. The microalgae were subjected to simulated mixture addition conditions, varying the injection period from 6 h/day to 24 h/day, with an aeration rate of 0.01 vvm over a 10-day cycle. It was observed at the end of the cultivation that the concentration of cells per milliliter for treatment B5 is higher than that for treatment B4 because the amount of simulated

mixture applied to sample B5 was higher, favoring greater cell development as already evidenced in the literature [8]. This reveals the potential to use waste gas in the cultivation of *Chlorella* sp. on a laboratory scale and the possibility of staged processes.

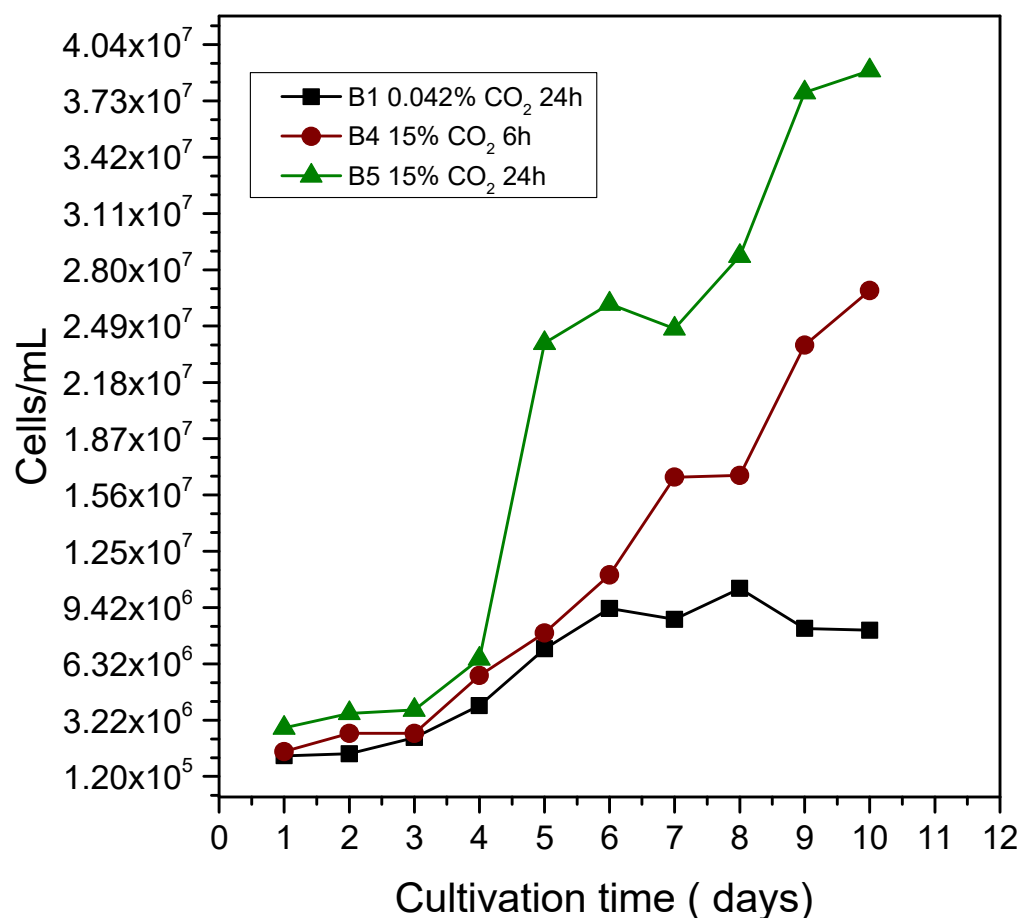


Figure 4. *Chlorella* sp. growth curve (abs) when adding simulated gas.

Regarding the pH monitoring of samples B4 and B5, it was observed that, during cultivation with the addition of simulated gas, the pH underwent few changes, remaining between 7.2 and 8.2, representing favorable conditions for microalgal development. A characteristic of microalgae is their ability to increase the pH of water, as they remove carbon dioxide in water and, through photosynthesis and metabolism, use carbon as a source to produce biomass and generate oxygen as a byproduct, achieving pH stability in the cultivation of *Chlorella* sp. when simulated waste gas is added [16].

3.3. Use of Wastewater in the Cultivation of *Chlorella* sp.

Figure 5 shows the microalgal growth profiles in samples B6 and B7. The cultivation conditions were analyzed for the following samples: B6 (0.042% CO₂ and cultivated in mining basin water) and B7 (15% CO₂ and cultivated in mining tailings basin water). The aeration rate applied was 0.01 vvm (40 mL/min) for 24 h over a 10-day culture cycle.

It can be observed that treatments B1, B6, and B7 led to rapid growth in the first two days, indicating that the samples were adapted to the culture medium, which induced an increase in cell reproduction. It is noted that the curve profile in sample B7 shows higher microalgal growth than that of sample B6, as the addition of the simulated mixture promoted greater cellular photosynthetic development and enhanced cellular reproducibility [11]. In the present study, it was found that wastewater from the mining basin is an important resource that can be reused in the production of microalgae of the

Chlorella sp. strain, as it contains trace elements such as copper (Cu), manganese (Mn), zinc (Zn), and boron (B) that are essential in microalgae's nutrition.

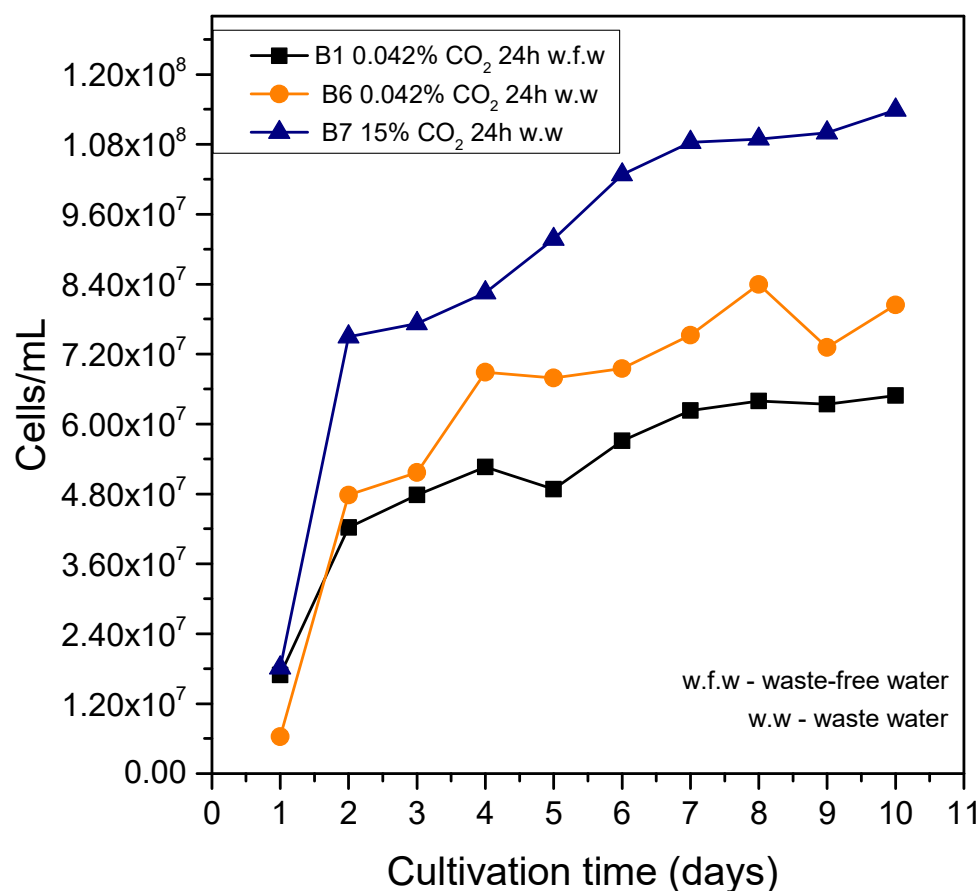


Figure 5. Microalgal growth curves for cultivation in mining basin water.

Regarding the pH monitoring in samples B6 and B7, the same stability dynamics were observed when using the simulated gas mixture; the pH remained between 7.2 and 7.8, establishing acceptable conditions for the cultivation of *Chlorella* sp. even in water containing mineral waste.

3.4. Production and CO₂ Fixation Rate

Treatments B6 (0.042% CO₂) and B7 (15% CO₂) were subjected to elemental analysis of CHN, determining the inorganic carbon dioxide content in % and dry mass productivity in g/L·d.

Table 4 shows the results obtained in the cultivation of *Chlorella* sp. with the injection of simulated gas as well as the CO₂ fixation rate based on the calculations defined in [9].

Table 4. Dry mass production and CO₂ fixation rate in relation to the standard sample with simulated gas.

Treatment	%C	Production (g/L·d)	Fixation Rate (g/L·d)	%CO ₂ Fixed in Biomass (Simplified Method) [17]	%CO ₂ Fixed in Biomass (Advanced Method) [17]
Treatment B6	25.04	0.26	0.23	---	---
Treatment B7	30.64	0.81	0.91	27	17.4

The production and CO₂ fixation rate observed in sample B7 reached values in the order of 0.81 g/L·d and 0.91 g/L·d, respectively. In the literature, it is predicted that

the production of 1 kg of biomass requires 1.8 kg of CO₂ [3]; through this estimate, it was possible to determine the fixed amount of CO₂ in sample B7, obtaining a value of 5.8 g, which is equivalent to 27% of CO₂ fixed in the sample biomass. However, Adamczyk et al. (2017) [17] found that this estimate is based on a simplified method showing little precision regarding the biofixation of CO₂ in biomass. In the present study, a more accurate method for CO₂ fixation based on the analysis of the elemental carbon of the sample (CHN) was used. Based on the elemental carbon value, the CO₂ fixation rate in the biomass was determined to be 0.91 g/L·d. By performing a mass balance, as well as controlling the amount of CO₂ injected into the 72 L culture medium, it was possible to evaluate the amount of CO₂ fixed by the microalgae, obtaining a result of 3.64 g of CO₂ fixed in the biomass, which is equivalent to 17.4% of the value calculated by the ratio $(\text{CO}_2 \text{ input} / \text{CO}_2 \text{ accumulation}) \times 100$, where the input value was 21.38 g of CO₂ and the accumulated value was 3.6 g of CO₂.

3.5. Biochemical Composition (Carbohydrates, Proteins, and Lipids)

Figure 6 shows the lipid, protein, and carbohydrate contents of samples B5 (15% CO₂ 24 h well water) and B7 (15% CO₂ 24 h wastewater containing ores).

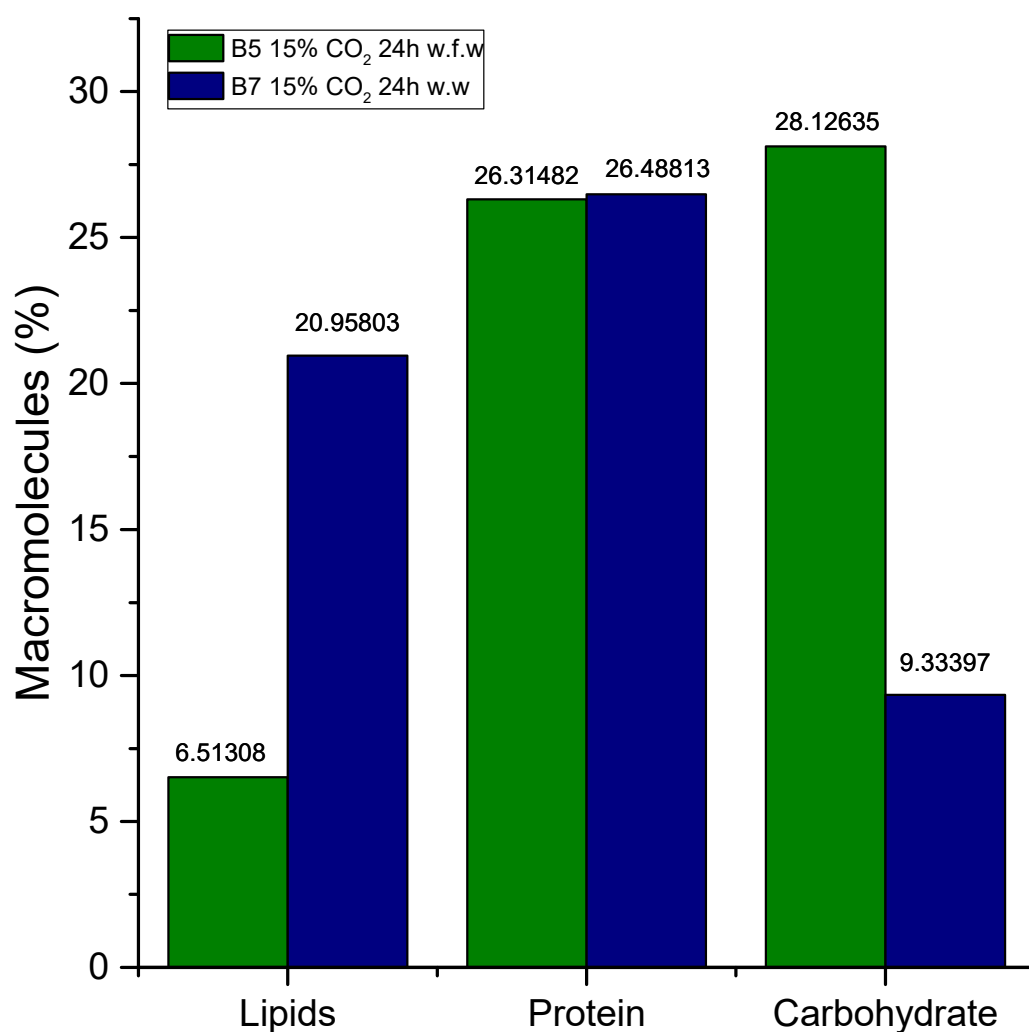


Figure 6. Composition of macromolecules, proteins, lipids, and carbohydrates. (w.f.w—waste-free water; w.w—wastewater).

The lipid yield of *Chlorella* sp. cultivated in the two sampling conditions was verified. It is noted that the percentage of lipids under the cultivation carried out in wastewater presented values much higher than those observed in well water. It is observed that microalgae require some heavy metals as micronutrients to photosynthesize and produce biomass [18]. Vital micronutrients include copper, iron, zinc, and manganese [19]. Other heavy metal micronutrients such as magnesium, nickel, lead, cobalt, and chromium are also required in small amounts for growth; the bioavailability of these resources plays a large role in microalga growth and metabolite production [20]. It is possible that the lipid value presented under the cultivation carried out in wastewater was due to the presence of some heavy metals present in the wastewater. Furthermore, it is observed in the literature that the lipid content is directly influenced by the CO₂ concentration [4]. Regarding the protein values that were determined in the present study, it is observed that, in the two sampling conditions, the values were relatively close; a higher percentage of protein was expected in the samples that were cultivated with the addition of 15% CO₂. In the literature, it is possible to find samples with up to 43 to 63% protein content present in the biomass [13]. Regarding the carbohydrate content, it was observed that there was a marked difference in the values obtained in the cultivation carried out with well water (28.12%) and wastewater (9.34%). In the study by Kassim and Meng (2017) [11], it was observed that higher concentrations of CO₂ reduced the percentage of carbohydrates. However, it was observed in this study that the carbohydrate concentrations increased under cultivation in well water and decreased in wastewater at the same CO₂ concentrations. The presence of macronutrients such as nitrogen, phosphorus, sulfur, and potassium, among others, is essential for the ideal growth of microalgae [11]. However, conditions of nutrient deficiency or limitation are viable and commonly used to alter the composition of metabolites [17]. In this study, it was possible to predict that the non-limitation of macronutrients such as phosphorus, sulfur, potassium, and nitrogen caused low yields of lipids, proteins, and carbohydrates in the cultivated biomass of *Chlorella* sp. Other factors that influence the composition of macromolecules obtained from the biomass of cultivated samples are irradiance and temperature [17]. However, in the present study, it was not possible to predict how much temperature or irradiance influenced the production of macromolecules because this study was limited to analyzing the growth curves of *Chlorella* sp. given the mixture of simulated gas containing 15% CO₂, considering that this was a problem presented by a company that generates residual carbon dioxide.

4. Conclusions

This study showed that the microalga *Chlorella* sp. is a species capable of adapting to a cultivation medium prepared with wastewater from a mining basin, which makes its cultivation viable in places where water is scarce, allowing local industry to save potable water as there is no need to use it as a cultivation medium. It was observed that the *Chlorella* sp. treated in this study showed good CO₂ biofixation capacity under conditions of simulated gas insertion, obtaining biomass with protein and carbohydrate levels favorable for the generation of value-added products. Aiming at the possibility of implementing a microalga cultivation plant, the results from this study can open avenues for the reuse of mining wastewater, the capture of residual CO₂ derived from refractory industries, and the acquisition of high-value-added products from biomass production.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/suschem6020011/s1>, Table S1: Analytical bulletin with analytical data of mining read basin.

Author Contributions: Conceptualization, T.J.T.C., R.M.B., F.R.d.A.C., D.I.A.T. and S.B.C.P.; methodology, T.J.T.C.; software, T.J.T.C.; validation, T.J.T.C., G.Q.C. and S.B.C.P.; formal analysis, T.J.T.C.; investigation, T.J.T.C.; resources, S.B.C.P.; data curation, T.J.T.C.; writing—original draft preparation, T.J.T.C.; writing—review and editing, T.J.T.C., S.B.C.P., R.M.B., F.R.d.A.C., D.I.A.T. and G.Q.C.; visualization, T.J.T.C.; supervision, S.B.C.P., R.M.B. and F.R.d.A.C.; project administration, S.B.C.P.; funding acquisition, S.B.C.P. All authors have read and agreed to the published version of the manuscript.

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