Luminosity and Chemical Stress Improve the Production of Biomass and Biomolecules from *Chlorella vulgaris* Cultivated in Produced Water

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Abstract

Produced water (PW) is an effluent from the petrochemical industry that has a significant environmental impact due to its high salinity and presence of chemical compounds and heavy metal. Microalgae have been considered promising to phytoremediation of this effluent and producing biomass with high added value. Therefore, this study aimed to stimulate the production of biomass and biomolecules from *Chlorella vulgaris* by supplementation with PW and evaluate the synergistic effect of sources of physical and chemical stress. *C. vulgaris* was cultivated in different PW concentrations of PW (not autoclaved) and BG11 medium under a 24 h photoperiod, with inoculum pre-adapted the same photoperiod. The culture containing 70% of BG11 and 30% PW (PW 30%) was the most viable, with a biomass production of 1.35 g/L and a higher concentration of carbohydrates (37.46%) and ash (18.21%) than the control culture (100% BG11). Furthermore, PW 30% also resulted in considerable amounts of lipids (9.92%), proteins (21.94%), chlorophyll-*a* (6.64 µg/mL), chlorophyll-*b* (10.57 µg/ mL), and carotenoids (21.38 µg/mL). The major fatty acids were C18:3n6 (21.50%), C20:0 (19.96%), C16:0 (17.12%), and C18:0 (12.15%). The PW 30% treatment showed removal efficiencies for iron (61.80%), chlorides (79.64%), phosphates (97.18%), and petroleum hydrocarbons including total petroleum hydrocarbons (TPH; 45.39%) and resolved petroleum hydrocarbons (RPH; 57.57%). The analyzed fuel properties presented an ideal profile for the production of biodiesel and bioethanol, which can be obtained from carbohydrates. Simultaneously, treatment PW 30% resulted in the production of biomolecule-rich in biomass in addition to the bioremediation effect.

Keywords Microalgae · Cyanobacteria · Phycoremediation · Bioproducts · Biofuel

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Introduction

The effluent resulting from oil and gas production, also known as produced water (PW), is wastewater typically composed of hydrocarbons, heavy metals, and inorganic salts. The PW composition and volume depend on the location of the well, frequency of water injection, and constituents extracted from the oil [1]. In 1990, the global production of PW was 190 million barrels per day. Thirty years later, the production increased to approximately 320 million barrels per day, and it is estimated that this volume will grow to 600 million barrels per day over the next ten years [2, 3].

The large volume of PW, scarcity of pure water, and greater energy demand have increased interest in the use of microalgae for biomass production as energy sources for the treatment and reuse of effluents. The cultivation of microalgae stands out because of the non-necessity of

using fresh water; microalgae can utilize wastewater due to their bioremediation capacity and assimilating of organic and inorganic pollutants and heavy metals [4].

Some of these pollutants, mainly heavy metals, are considered toxic and are increasingly found in water and soil owing to economic growth and accelerated industrialization. Therefore, various removal treatments are used, including chemical, electrochemical, redox, membrane separation, adsorption, ion exchange, and biological treatment [4, 5]. Biological treatment with microalgae uses organic matter from these wastewaters as a source of nutrients and carbon, reduces toxicity, removes pollutants, and provides biomass rich in fatty acids, proteins, carbohydrates, vitamins, minerals and pigments, making them a source of bioproducts used in the aquaculture, pharmaceutical, food, and biofuel industries [4, 6].

Although PW contains several toxic components that inhibit microalgae growth, it also contains nutrients necessary for cultivation, such as iron (Fe), potassium (K), and manganese (Mn) [6]. Research has been conducted to make the production of microalgae in PW feasible. Ammar et al. [1] cultivated *Nannochloropsis oculata* and *Isocrysis galbana* in PW for 15 days at three percentages (10, 25, and 50%). Silva et al. [7] produced 1.69 g/L of *Chlorella vulgaris* biomass for 26 days in 1.5 L of BG11 medium, when cultivated in medium supplemented with 50 mL of non-autoclaved PW per day.

The *Chlorella* genus is widely used for wastewater phycoremediation. *Chlorella* stands out for its tolerance of wastewater from industrial, agricultural, and municipal environments, making it useful in the treatment of effluents and ideal for biomass production [7]. Its potential is based on its plasticity, which can be modified according to cultivation conditions, allowing for modifications to its growth and biochemical composition. Some strategies are used to increase biomass production and the yields of some compounds, such as modulating chemical or physical stressors. Chemical stress is induced by modifying the composition of the culture medium, and physical stress is induced by changing the luminosity, temperature, salinity, and pH [8].

Light conditions are the main factors that affect phytoplankton physiology and directly affect microalgae photosynthesis. The quantity and quality of light are primordial and limiting factors for the energy available for photosynthesis and all metabolic activities [9]. According to Khoeyi et al. [9], increased light duration at different intensities (37.5; 62.5 and 100 μmol photons/m^{2}s) was associated with increased specific growth rates of *Chlorella vulgaris* (0.81, 1.00, and 1.12 / d) for the highest light cycle (16:8 h light/dark). Moreover, other authors, such as Yusof et al. [10], have stated that with increased light exposure, there is an increase in the microalgal reproduction rate up to the

saturation point. Moreover, each organism has different light requirements and life cycles.

Strategies to maximize microalgae growth grown in wastewater depend on changes in cultures and knowledge of microalgal physiology [11]. Therefore, modifications of physical and chemical factors are viable options to influence cell metabolism, increase growth, and stimulate the synthesis of biomolecules of interest based on their biochemical composition [12]. This study aimed to stimulate the production of biomass and biomolecules from *Chlorella vulgaris* by supplementation with PW and evaluate the synergistic effect of maximum luminosity and PW chemical composition on microalgal growth.

Material and Methods

Collection, the Chemical Composition of the Culture Medium with Produced Water, and Nutrient Removal Efficiency

The PW was collected from the Santo Amaro das Brotas district (latitude 10º46′44′′ south; 37º3′30′′ west), located 41 km from Aracaju, Sergipe (SE), Brazil. The samples were kindly supplied by a local oil exploration company and transported to the Federal University of Bahia (UFBA) in sterile polypropylene vessels under refrigeration. All PW samples were stored at -20 °C until use.

The physicochemical parameters were evaluated by previously reported consolidated methods using the standard method for the examination of water and wastewater [13] for the following analyses: chloride (Cl$^-$), phosphate (PO$_4^{3-}$), lead (Pb), arsenic (As), cadmium (Cd), nickel (Ni), manganese (Mn), vanadium (V), iron (Fe), zinc (Zn), and chromium (Cr). Determination of total petroleum hydrocarbons (TPH) and resolved petroleum hydrocarbons (RPH) by EPA 8015D (SW-846) followed the US standard method [14]. The nutrient removal efficiency (RE) from the treatment was evaluated using Eq. 1, previously reported by Cardoso et al. [15].

$$RE(\%) = \frac{(C_i - C_f)}{C_f}100 \qquad (1)$$

where, Ci and Cf are the initial and final concentrations, respectively.

Growing Conditions and Treatments

The *C. vulgaris* strain was obtained from the Iracema Nascimento Microalgae Bank of the Bioprospecting and Biotechnology Laboratory of the UFBA. To obtain the inoculum for used in the experiments, the strain was autoclaved

and cultivated in a synthetic medium, BG11 (NaHCO$_3$; K$_2$PO$_4$.3H$_2$O; NaNO$_3$; Na$_2$CO$_3$; MgSO$_4$.7H$_2$O; CaCl$_2$.2H$_2$O; (NH$^+$)$_5$Fe(C$_6$H$_2$O$_7$)$_2$; C$_6$H$_8$O$_7$; and EDTA) and trace elements [16] under constant temperature (28 °C), aeration, and continuous photoperiod (24 h) with lighting provided by five fluorescent lamps at 41.60 μmol photons/m^{2}s each. After 18 d of cultivation, the inoculum was centrifuged (Eppendorf 5702 R) at 3450×g for 4 min.

Following this, the experiments were carried out in Erlenmeyer-type photobioreactors (1 L) with a total volume of 800 mL (BG11 medium + PW according to each treatment) and an initial concentration of approximately 0.2 g /L for 18 d. The treatments consisted of a control culture (100% BG11) and treatments containing 30% PW + 70% BG11 (PW 30%), 40% PW + 60% BG11 (PW 40%), and 50% PW + 50% BG11 (PW 50%), all performed in triplicate under conditions of constant temperature (28 °C), aeration, and continuous photoperiod (24 h) with lighting provided by five fluorescent lamps at 41.60 μmol photons/m^{2}s each. PW proportions were determined based on the study by Ammar et al. [1] and in preliminary tests that pointed to a maximum concentration of 50% of PW.

After 18 d of cultivation, the biomass was recovered by centrifugation (Eppendorf 5702 R) at 3450×g for 4 min, washed with distilled water, and centrifuged again (3450×g for 4 min) to remove salts and interferents. After washing, the biomass was frozen at -80 °C and freeze-dried at -43 °C (L101, Liobras) for further analysis.

Growth and pH

The biomass concentration (X, g/L) was determined by optical density and evaluated daily in all cultures. A standard curve was constructed according to the method described by Costa et al. [17], using a spectrometer (Perkin–Elmer Lambda 35 UV–Vis). All readings were recorded at a wavelength of 680 nm. The productivity (P, (g/L)/d) was determined using Eq. 2, and the growth rate (μ, 1/d) was determined by linear regression of the biomass production curve in the log phase [18]. pH was measured daily using a digital pH meter (Gehaka PG2000).

$$P = \frac{(X_t - X_0)}{(t - t_0)} \qquad (2)$$

where, X_t e X_0 are the biomass concentrations (g/L) at times t (d) and t_0 (d) initial.

Biomass Biochemical Composition

Protein concentration was calculated from the total nitrogen concentration determined by Kjeldahl method [19] with a microalgae conversion factor of 5.22 [20]. Total

lipids were extracted and quantified using the method by Folch et al. [21] with chloroform–methanol solution (2:1). Thermogravimetric analysis (TGA) was used to determine ash and moisture contents at temperatures between 25 and 900 °C, as proposed by Jesus et al. [18]. Carbohydrates were determined by the difference in proteins, lipids, ash, and moisture contents [20].

Fatty Acid (FA) Composition

Fatty acid composition was determined by transmethylation of fat with boron trifluoride in hexane, followed by gas chromatography. The FA methyl esters were separated with a column (DB-FFAP; 30 m × 0.25 mm × 0.25 μm) in a gas chromatograph equipped with a flame ionization detector (CG-FID Clarus 680; Perkin–Elmer). Fatty acid methyl esters were identified by comparing the retention times obtained from the standard chromatogram (C4–C24, 189–19-AMP, Sigma-Aldrich) according to the method proposed by Souza et al. [22].

Evaluation of Biodiesel Properties

The most important biofuel properties were evaluated. The iodine value (IV), saponification value (SV), cetane number (CN), long-chain saturation factor (LCSF), degree of unsaturation (DU), and cold filter plugging point (CFPP) were determined from empirical equations based on the FA profile [23]. SV and IV calculations were based on the SV and IV values of soybean, palm, and peanut vegetable oils.

$$IV = \sum \left(\frac{254DN}{M} \right) \qquad (3)$$

$$SV = \sum \left(\frac{560N}{M} \right) \qquad (4)$$

$$CN = 46.3 + \frac{5.458}{SV} - (0.225 IV) \qquad (5)$$

$$LCSF = (0.1 C16) + (0.5 C18) + (1 C20) + (1.5 C22) + (2 C24) \qquad (6)$$

$$DU = MUFA + (2 PUFA) \qquad (7)$$

$$CFPP = (3.1417 LCSF) - 16.477 \qquad (8)$$

where, D = number of double bonds, M = molecular weight of the fatty acid, and N = percentage of each fatty acid present in the oil.

Measurement of Chlorophyll a, Chlorophyll b, and Total Carotenoids

The analysis of chlorophyll *a* and *b* and carotenoid contents was performed as described by Andrade et al. [20]. Briefly, 0.2 g of lyophilized sample was added to 25 mL of 80:20 acetone:water, vortexed (Phoenix AP 56), and centrifuged (Eppendorf 5702R) at 3450 g for 5 min. The supernatant was centrifuged again, and the absorbance was measured using a spectrophotometer (Perkin – Elmer Lambda 35 UV–Vis) at wavelengths 663, 647, and 470 nm. Yields were calculated following the equations proposed by Lichtenthaler and Buschmann [24] and expressed in μg/ mL.

$$C_a = 12.25 A_{663} - 2.79 A_{647} \tag{9}$$

$$C_b = 21.50 A_{647} - 5.10 A_{663} \tag{10}$$

$$C_{CT} = \frac{[1000 A_{470} - (1.82 C_a - 85.02 C_b)]}{198} \tag{11}$$

where, Ca = Chlorophyll *a*; Cb = Chlorophyll *b*; C_{CT} = Total Carotenoids.

Theoretical Conversion of Carbohydrates from Microalgae Biomass to Ethanol

The theoretical conversion of carbohydrate, obtained from *C. vulgaris* biomass, for the production of ethanol and carbon dioxide, originating from the alcoholic mixture, can be represented in stoichiometric doses. The Gay-Lussac prescription represents the transformation of carbohydrate into ethanol and carbon dioxide (Eq. 12) [25].

$$C_6H_{12}O_6 + 2Pi + 2ADP => 2C_2H_5OH + 2CO_2 + 2ATP + 2H_2O \tag{12}$$
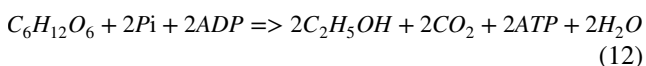
Theoretical calculations were performed to evaluate the conversion of carbohydrates produced in the biomass of *C. vulgaris* into bioethanol. The calculation considered 100 g of biomass, theoretical stoichiometric conversion of glucose into ethanol (kg) of 0.511 with 70% efficiency (EE), and an equivalence of ethanol gallons to GGE of 1.5:1.0 [26].

$$\textit{Theoretical Ethanol (L)} = \text{kg} \times \text{EE} \times \frac{\text{carbohydrates (\%)}}{\rho \text{ ethanol}} \tag{13}$$

Thermogravimetric Analysis (TGA)

Thermogravimetric analyses (TGA) (PerkinElmer Model Pyris 1 TGA) was performed to determine the biomass pyrolysis behavior. A total of 5 mg of biomass was subjected to different temperatures ranging from 25 to 900 °C at a heating rate of 10 °C/min and a nitrogen flow rate of 20 mL/min [18]. The initial decomposition temperature and the final and maximum decomposition temperatures were denoted as (T_{onset}) and (T_{decomp}), respectively.

Attenuated Total reflectance Fourier Transform Infrared Spectroscopy (FTIR-ATR)

The samples were analyzed by Fourier transform infrared spectroscopy using an attenuated total reflectance device (Spectrum two spectrometer, Perkin-Elmer) with a wavenumber ranging from 4000 – 600/cm, accumulating 64 scans [27].

Statistical Analysis

The results were analyzed using two statistical tests to compare and demonstrate the efficiency of the PW-supplemented treatments. An analysis of variance (ANOVA) and Tukey's test at a 95% confidence level were performed using the Statistica software (version 10.0) for comparison of the means. The Shapiro–Wilk test was used to test the normality of the data. The experimental results are presented as the mean ± standard deviation of triplicates experiments. Statistical treatment was applied to all quantitative analyzes performed in this study, with the exception of FTIR, TGA, and evaluation of the theoretical properties of biodiesel, as they are qualitative analyses.

Results and Discussion

Growth and pH

The growth curve of *C. vulgaris* at different PW concentrations (30%, 40%, and 50%) are shown in Fig. 1a. The PW 40% and PW 50% treatments did not show microalgal growth throughout cultivation. This behavior may be related to the disruption of the cell membrane caused by cell-PW contact, as PW is rich in contaminants such as metals, organic compounds, and nutrients, causing interactions between the toxic components and intracellular organelles of the microalgae and hindering their normal biological functions, including growth [1, 28]. Ammar et al. [1] also observed growth inhibition of *Isocrysis galbana* with increasing PW concentration (10%-50%).

In addition to the control, the only treatment that showed cell growth was PW 30%, which may be related to the ability of microalgae to degrade toxic constituents into less toxic products [28]. An adaptation period lasting two days (0 to 2 nd day) was observed in PW 30% and the control groups, followed by exponential growth until the 18th day of cultivation
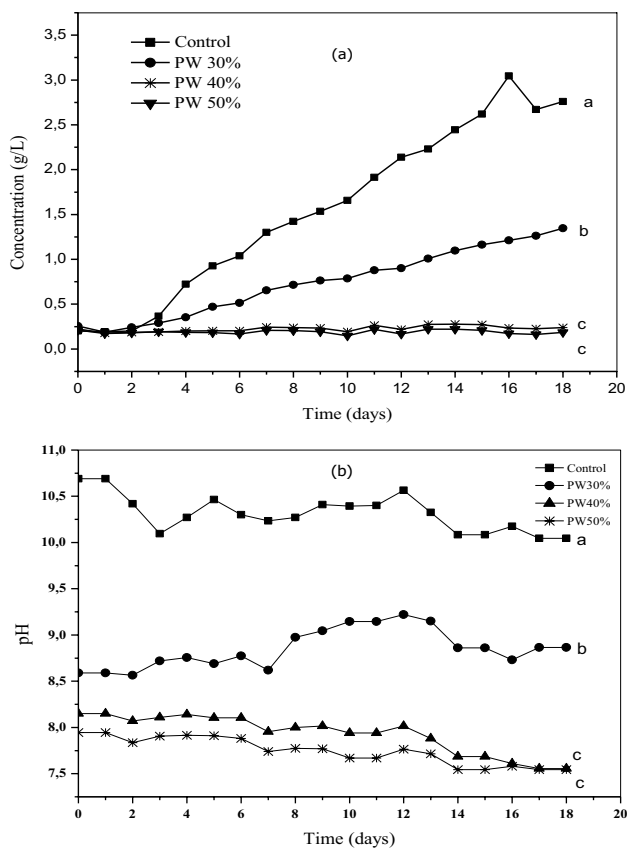
Fig. 1 Production of *Chlorella vulgaris* in BG11 medium with different (**a**) produced water (PW) proportions and (**b**) pH level. Equal letters indicate no statistical difference between treatments ($p < 0.05$) ANOVA and Tukey's test at a 95% confidence level

(Fig. 1a). According Cardoso et al. [15], the growth after two days of cultivation indicates the ability of microalgae to adapt to the imposed environmental conditions. In the present study, *C. vulgaris* showed good adaptation to PW 30% supplementation, similar to the results found by Mehta et al. [29] in cultivating *Chlorella pyrenoidosa* at different salt concentrations, with a comparable adaptation phase of two days. The similar exponential growth of the PW 30% treatment and the control demonstrates the existence of a positive synergism between the luminosity and the chemical

composition of the culture, which allowed the cell growth and exponential development. The PW 40% and PW 50% treatments remained in the lag phase (latency) throughout the 18 days of culture, maintaining the same cell concentration as the initial inoculum concentration (~ 0.2 g/L).

The initial pH values (day 0) decreased with increasing PW concentration in the treatments. Figure 1b shows a variation in the pH from 10.31 (control) to 7.75 (PW50%). In the PW 40% and PW 50% treatments, the pH reduction may be associated with the chemical composition of PW, limiting growth. pH is affected by the availability of inorganic nutrients, carbonaceous species in the medium, and the presence of metals. According to Gómez-Luna et al. [30], pH reduction is usually lethal for microalgae because its tolerance limits are determined by the chemical composition of the culture medium and cell metabolic effects.

The PW 30% and the control treatments showed an increase and a decrease in pH over the cultivation period (Fig. 1b). The pH increase is due to the consumption of CO_2 during photosynthesis and the release of hydroxide (OH^-) by reducing the oxygen molecules produced [31]. Otondo et al. [4] observed higher growth rates during the cultivation of *Nannochloropsis salina* in domestic wastewater at pH 8.0 and 9.0. Here, the control and PW 30% treatments (pH > 8.0) showed better growth and biomass production. pH 8.0 is indicated for most freshwater species, with a pH of 10.0 being ideal for *C. vulgaris* [30]. Therefore, a pH range between 8.0 and 10.0 is ideal for culturing *Chlorella* supplemented with PW 30%.

The PW 30% culture showed a maximum production of 1.35 g/L on the 18th day (Table 1), compared to the control culture with a maximum production of 3.05 g/L on the 16th day. The lower values found in the PW 30% treatment occurred because of the stress caused to the microalgae by PW components, such as petroleum hydrocarbons, excess nutrients, and metals [1]. Even with these characteristics, the dilution of PW allowed the growth of *C. vulgaris* because of the ability of microalgae to degrade petroleum hydrocarbons into their less toxic forms, converting acetyl-CoA into H_2O and CO_2, enabling the production of biomass [32]. Church et al. [31] also observed a reduction in biomass production in the cultivation of *Chlorella vulgaris* in saline wastewater,

Table 1 Parameters of *Chlorella vulgaris* grown in BG-11 medium supplemented with different concentrations of PW or the control

Treatments	Final production (g/L)	Maximum production (g/L)	Mean productivity (g/Ld)	µ (1/d)
Control	2.76 ± 0.17^a	3.05 ± 0.37^a	0.13 ± 0.01^a	0.34 ± 0.01^a
PW 30%	1.35 ± 0.16^b	1.35 ± 0.16^b	0.04 ± 0.01^b	0.20 ± 0.02^b
PW 40%	0.24 ± 0.01^c	0.28 ± 0.002^c	0	-
PW 50%	0.19 ± 0.002^c	0.22 ± 0.003^c	0	-

Equal letters in the same column indicate that there was no statistical difference between treatments ($p < 0.05$) ANOVA and Tukey's test at a 95% confidence level

from 1.30 g/L in the standard culture to 0.74 g/L in saline wastewater.

The specific growth rate (μ) of the control culture was higher than that of the PW 30% treatment (Table 1). This result may be related to the phosphorus (P) limitation of PW because P acts as a chemical energy carrier. The presence of P increases the growth of some microalgae species, including *C. vulgaris* [33]. In addition, low μ rates were found with increasing salt concentration in the medium [31]. According to Mehta et al. [29] and Matos et al. [34] moderate concentrations of NaCl (up to 1%) can help with nutrient absorption and intracellular regulation of photosynthesis. However, a 2–6% NaCl medium can cause cell osmotic stress, interfering with electron transport and photophosphorylation. Therefore, highly saline media tend to reduce algal growth, possibly by accumulating reactive oxygen species. Ammar et al. [1] reported similar behavior in cultivating *Nanochloropsis oculata* with 25% PW (0.1796/d). Thus, a higher growth rate occurs in treatments with higher biomass concentrations [31].

Chemical Composition and Treatment Removal Efficiency

The chemical composition and treatment efficiency of cultivating *C. vulgaris* in BG11 culture medium supplemented with PW 30% are shown in Table 2. The culture medium contained metals important for the metabolism and growth of microalgae. Iron (Fe), for example, participates in electron transport and cellular respiration, acting as an enzymatic cofactor that increases the degradation power of the medium [35].

The Fe removal rate of the PW 30% treatment reached 61.80%. Yaghmaeian and Jaafari [35] found an Fe removal of 86.7% in 2.6 g/L of *C. Coloniales* biomass grown in BG11 medium supplemented with some metals (Cr, Cd, Co, Fe, and As). The lower removal efficiency found in the present study may be related to the lower number of cells used in the inoculum in the PW 30% treatment (~ 0.2 g/L). A lower inoculum concentration represents fewer active biosorption sites. The cell wall contains polysaccharides, proteins, and lipids with functional groups composed of electronegative atoms. The high electron densities of these groups are responsible for the binding of cations to the cell wall surface [35]. Znad et al. [33] reported results similar to those found in the present study cultivating *Chlorella vulgaris* in petroleum effluents, with 57% Fe removal.

Even with low removal efficiency (16.45%), manganese (Mn) is another essential element in microalgae metabolism. Like iron (Fe), Mn also acts as a cofactor in enzyme systems, mainly to maintain a regular photosynthetic rate [33]. The components with the highest removal efficiency were phosphates (97.18%) and chlorides (79.64%). The Cl^- removal efficiency may be related to the ability of microalgae to remove chlorine ions and produce lipids and polyols as an osmoregulatory response to extracellular solute concentration, minimizing osmotic stress and dehydration. Chloride ion are essential cofactor for photosynthesis and are vital for plant growth [36]. Brar et al. [36] reported similar findings to the present study regarding chloride

Table 2 The efficiency and chemical composition of the culture medium before and after treatment with BG11 supplemented with PW 30%

Component	Unit	LQ	Before treatment	After treatment	Removal efficiency (%)
Chloride (Cl^-)	mg/L	1000	24,542.79 ± 245.43[b]	4996.10 ± 49.96[a]	79.64
Phosphate (PO_4)	mg/L	0.30	10.63 ± 1.17[a]	< 0.30 ± 0.01[b]	97.18
Nitrate (NO_3)	mg/L	2.50	< 2.50	< 2.50	-
Lead (Pb)	mg/L	0.01	< 0.01	< 0.01	-
Arsenic (As)	mg/L	0.001	< 0.001	< 0.001	-
Cadmium (Cd)	mg/L	0.001	0.00148 ± 0.000059[a]	0.0017 ± 0.000068[a]	-
Nickel (Ni)	mg/L	0.01	< 0.01	< 0.01	-
Manganese (Mn)	mg/L	0.01	1.10 ± 0.03[a]	0.92 ± 0.03[b]	16.45
Vanadium (V)	mg/L	0.10	< 0.01	< 0.01	-
Iron (Fe)	mg/L	0.10	1.00 ± 0.07[a]	0.38 ± 0.03[b]	61.80
Chromium (Cr)	mg/L	0.01	< 0.01	< 0.01	-
Salinity	g/L	5.00	76.80 ± 0.18[a]	60.40 ± 0.27[a]	21.35
Total Petroleum Hydrocarbons (TPH)	µg/L	300	911.80 ± 45.60[a]	498.40 ± 24.90[b]	45.39
Resolved Petroleum Hydrocarbon (RPH)	µg/L	300	706.90 ± 35.30[a]	< 300 ± 0.38[b]	57.57

Equal letters on the same line indicate no statistical difference between treatments ($p < 0.05$) ANOVA and Tukey's test at a 95% confidence level. LQ is the limit of quantification

removal (61.1%) from wastewater from the textile industry using *C. pyrenoidosa*.

The high efficiency of PO_4^- removal in the treatment is directly associated with the absorption of phosphorus (P) for microalgae growth, as microalgae accumulate phosphorus in their cells as polyphosphates. Microalgae use phosphorus to synthesize cellular constituents, such as phospholipids and nucleic acid, and for reactions related to cell division [36]. Brar et al. [36] reported a 70.8% phosphate removal from wastewater from the textile industry and 99% phosphorus removal in the cultivation of *Chlorella vulgaris* in synthetic wastewater.

Total petroleum hydrocarbons represent the sum of the fractions of RPH and the unresolved complex mixture (UCM). The TPH and RPH removals were 45.39 and 57.57%, respectively. These results indicate the synergistic action of microalgae and bacteria in the treatment, considering that the medium was not autoclaved. The removal of petroleum hydrocarbons depends on the microalgae species used, cultivation conditions, cell wall size, and extracellular and intracellular physiological processes. These processes are characterized by the adsorption of hydrocarbons on the surface, absorption of hydrocarbons into the cells, and transformation of hydrocarbons by enzymes into less toxic molecules [37]. Petroleum hydrocarbons are resistant to degradation by microorganisms mainly because of their high molecular mass. However, the synergistic action of microalgae and bacteria resulted in good removal efficiency. This process transforms hydrocarbons into cis-dihydrodiols by dioxygenases, leading to pyruvic, acetic, fumaric, succinic, aldehydes, and acids production [37].

Abid et al. [38] reported a TPH removal efficiency higher than that of the present study (99.18%) in *Spongiochloris* sp. cultivation in PW for 120 days. The high removal efficiency found by the authors is related to the time of cultivation and the adaptation of the symbiotic culture with aerobic bacteria in the bioreactor because, in the 50-day culture, the removal of TPH was only 22%. Silva et al. [7] presented similar results to those found in this study in cultivating *Chlorella vulgaris* with 10% of PW for day, removing 48.59% of TPH.

Biochemical Composition of Biomass

The biochemical compositions of the biomass obtained from the PW 30% and control treatments are shown in Fig. 2. The lipids concentration in the PW 30% treatment (9.92%) was lower than that in control (17.25%). This result may be related to the high salinity of PW and the stress caused by the metallic cations, forcing the degradation of macromolecules to maintain metabolism [31]. In addition, the high concentration of organic matter present in PW causes additional stress on microalgae, which can inhibit lipid synthesis [23].
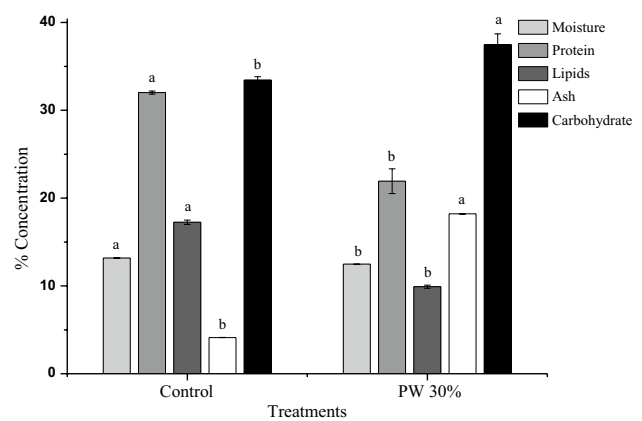

Fig. 2 Biochemical composition of *Chlorella vulgaris* biomass grown in BG11 medium supplemented with PW 30% or the control. Equal letters in the same biochemical composition parameter indicate no statistical difference between treatments ($p < 0.05$) ANOVA and Tukey's test at a 95% confidence level

In this study, the salinity of PW and the salts present in the synthetic medium BG11 may have been higher than that tolerated by *C. vulgaris*, causing a reduction in lipids. Jawaharraj et al. [39] also found a low concentration of lipids in *Oscillatoria* sp. in wastewater from the agro-industry (9.6%). Gao et al. [40] reported a high lipid content (29%) during *Chlorella* sp. Cultivation in untreated wastewater. The variation in lipid levels found in these studies is due to the diversity of chemicals present in wastewater [8].

The protein content in the PW 30% treatment (21.94%) was significantly reduced ($p < 0.05$) compared with that in the control treatment (32.03%). The poor protein content of PW is related to saline stress. Cells subjected to high salinities redirect the available energy to osmoregulation rather than protein synthesis [34]. In addition, stress caused by N limitation can decrease protein synthesis and increase the metabolism of biomolecules, resulting in higher carbohydrates and ash leves [15]. Matos et al. [34] reported results similar to those of the present study in *Chlorella vulgaris* grown in saline medium, with 20% protein content in the treatment group and 37.2% in the control.

The moisture content in the PW 30% and control groups was 12.48 and 13.18%, respectively. Matos et al. [34] reported 18.8% moisture from cultivating *Chlorella vulgaris* in a saline medium, and these values were considered typical. However, moisture affects the quality of bio-oil production because of the reduction in calorific value during pyrolysis [41]. The ash content was higher in the PW 30% treatment (18.21%) than that in the control (4.12%). It is possible that the excess salts found in PW, such as calcium carbonate, sodium, and chlorides, and the accumulation of alkali metals in microalgal cells hinder the degradation of biomass by pyrolysis [15]. High ash values decrease the

bio-oil yield owing to the catalysis of the secondary cracking of the pyrolysis steam [41]. In the cultivation of *Chlorella* in sewage wastewater, Xu et al. [42] found an ash content (16.06%) similar to that observed in the present study.

The carbohydrate content in the PW 30% and control was 37.55% and 33.42%, respectively. The high levels may be related to the continuous photoperiod (24 h) used, which provides the formation of starch and sugars by the reduction of pentose phosphate [43]. Another factor for the higher carbohydrates concentration in PW cultivation is the stress caused by the salinity of the medium, which induces the microalgae to store energy for cellular protection and as a structural component [26]. Mata et al. [6] also found high carbohydrates levels (42.29%) in the cultivation of *Spirulina* sp. LEB18 in saline wastewater. The abundance of carbohydrates in this study is an important biotechnological source of bioethanol because microalgae produce lignin-free fermented carbohydrates [20].

Fatty Acids (FA) Composition

The fatty acids concentrations in the PW and control treatments groups are listed in Table 3. The PW 30% treatment resulted in 54.14% saturated fatty acids (SFA), 10.34% monounsaturated fatty acids (MUFA), and 21.50% of polyunsaturated fatty acids (PUFA). The control showed 66.41%, 7.26%, and 13.09% SFA, MUFA, and PUFA, respectively. The high SFA content was due to the continuous photoperiod. Continuous illumination stimulates ATP and NADPH production, which helps distribute excess light energy and prevents cell damage [9]. In addition, large amounts of salts stimulate desaturase synthesis, which helps in SFA production [15, 23]. Church et al. [31] also observed a difference in SFA levels, from 16.99% to 21.53%, when *C. vulgaris* was cultivated in a medium with high salinity. Zhang et al. [44] reported similar SFA values found to those found in this study (56.76%) by cultivating *Chlorella sorokiniana* SDEC-18 in wastewater from kitchen effluents at various dilutions. The degree of saturation is favorable for obtaining high-quality biodiesel. Higher amounts of SFA increase ignition quality, and a lower percentage of PUFA prevents auto-oxidation [15].

The major FAs from the PW 30% treatment were C18:3n6, C20:0, C16:0, and C18:0. Large concentrations of SFA from the C16 and C18 families occur due to the action of acetyl-CoA carboxylase. This enzyme converts the carbon present in the medium by elongation and desaturation into malonyl-CoA, mainly forming C16 and C18 carbon chains [15, 23]. Gao et al. [40] reported the predominance of C16:0 (26,4–31,5%), C18:0 (18,2–27,2%), and C18:1 (19,1–25,4%) in the cultivation of *C. vulgaris* in different seafood processing wastewater.

Table 3 Composition of fatty acids from the *Chlorella vulgaris* biomass in BG11 medium supplemented with PW 30% or the control

Fatty acid	Tratament		
	Common name	Control	PW 30%
C4:0	Butyric acid	1.29 ± 0.88	2.02 ± 1.68
C10:0	Capric acid	-	0.05 ± 0.01
C11:0	Hendecanoic acid	0.24 ± 0.05	0.32 ± 0.00
C12:0	Lauric acid	-	0.09 ± 0.02
C13:0	Tridecylic acid	3.16 ± 0.04	1.83 ± 0.10
C14:0	Myristic acid	0.17 ± 0.06	0.35 ± 0.01
C14:1ω5	Myristoleic acid	0.85 ± 0.07	0.44 ± 0.05
C15:0	Pentadecylic acid	0.52 ± 0.02	0.27 ± 0.15
C15:1	Pentadecenoic acid	1.20 ± 0.04	0.56 ± 0.10
C16:0	Palmitic acid	12.50 ± 0.82	17.12 ± 0.47
C16:1ω7	Palmitoleic acid	1.05 ± 0.14	1.47 ± 0.08
C18:0	Stearic acid	16.65 ± 1.23	12.15 ± 0.40
C18:1t	Elaidic Acid	2.96 ± 0.04	5.93 ± 0.01
C18:1c	Oleic acid	1.20 ± 0.06	1.96 ± 0.05
C18:3n6	γ-linolenic acid	13.09 ± 0.38	21.50 ± 0.18
C20:0	Arachidic acid	31.90 ± 1.14	19.96 ± 0.04
Saturated		66.41 ± 1.92^a	54.14 ± 0.64^b
Monounsaturated		7.26 ± 0.16^b	10.34 ± 0.07^a
Polyunsaturated		13.09 ± 0.38^b	21.5 ± 0.18^a
Not determined		13.24 ± 1.70	14.02 ± 0.53
Total		100	100

Equal letters on the same line indicate no statistical difference between treatments ($p < 0.05$) ANOVA and Tukey's test at a 95% confidence level

In contrast to the PW 30% treatment, the major FA in the control was C20:0. This difference was attributed to the composition of the culture, which consisted of only BG11 only, which has a higher nitrate concentration than PW 30%. Costa et al. [45] similary reported 22.62% C20:0 when cultivating *Chlorella minutíssima* in BG11 medium and attributed the high SFA content to a high nitrate concentration in the medium. The diversity and profile of FAs found in the PW 30% treatment reinforce the possibility of obtaining high-quality biodiesel from the produced biomass.

Evaluation of Biodiesel Properties

Table 4 presents the quality indices of the theoretical biodiesel produced using biomass from the PW 30% treatment as the substrate. The iodine value (IV) indicates the degree of fatty acid unsaturation. High unsaturation levels result in the deposition and deterioration of lubricating oil. In the present study, the IV was adequate for both Brazilian and European standards (< 120 g I_2/100 g). The PW 30% treatment resulted in 68.48 g I_2/ 100 g due to the higher SFA content (54.14%) than unsaturated fatty acids. Cardoso et al. [15]

Table 4 Theoretical biodiesel properties of fatty acids obtained from *Chlorella vulgaris* biomass grown in BG11 medium supplemented with 30% PW or the control

Biodiesel properties	Tratament	
	Control	PW 30%
Iodine value (IV) (g I$_2$ 100/g)	42.77	68.48
Saponification value (SV)	177.69	182.07
Cetane number (CN)	67.36	60.84
Long-chain saturation factor (LCSF) (wt.%)	41.48	27.75
Degree of unsaturation (DU) (wt.%)	33.44	53.34
Cold filter plugging point (CFPP) (^{o}C)	113.83	70.7

also found results within the recommended IV limit from the cultivation of *Spirulina* sp. LEB18 in different proportions of aquaculture wastewater (87.78–103 g I$_2$/100 g), as did Jawaharraj et al. [39] in the cultivation of *Oscillatoria* sp. (65.6 g I$_2$/100 g) and *Synechocystis* sp. (50.5 g I$_2$/ 100 g) in industrial wastewater.

The saponification value index (SV) indicates the amount of oil needed to saponify 1.0 g of oil. The saponification reaction interferes with the transesterification reaction during biodiesel production. Therefore, low saponification level imply better biodiesel quality. Although there are no standardized SV parameters, these values were compared with biodiesel from vegetable oils, such as palm oil (192–202); soybean oil (189–195), and sunflower oil (188–194) [23]. In this study, the PW 30% treatment presented values close to those of the vegetable oils (182.07). The SV result found in this study were similar to those reported by Jawaharraj et al. [39] for the cultivation of *Synechocystis* sp. (193.4), *Oscillatoria* sp. (202.8), and *Jatropha* sp. (199.7) in industrial wastewater.

The cetane number (CN) defines the ignition quality of the fuel. The CN range indicated by American standards require a minimum of 47, while European standards require a minimum value of 51 [23]. The CN found in this study (60.85) was attributed to the high SFA concentrations and was above the level recommended by international standards. High cetane values promote good engine performance and reduce smoke formation, which are parameters used to evaluate combustion quality and ignition delay time [44]. Zhang et al. [44] found similar results (CN = 59.24) from the cultivation of *Chlorella sorokiniana* in kitchen wastewater.

The long-chain saturation factor (LCSF) determines the behavior of biodiesel at low temperatures. It represents long-chain saturated fatty acids (C16; C18; C20; C22, and C24) [44]. The PW 30% treatment showed a higher value (27.75%) than that indicated for biodiesel quality, which is different from what was reported by Cardoso et al. [23] from the cultivation of *Spirulina* sp. LEB18 in aquaculture wastewater (4.81 to 5.52%). High LCSF values decrease

the quality of biofuels. Saturated FAs tend to crystallize at engine operating temperatures; therefore, the crystals rapidly clump together when the temperature is reduced, causing clogged filters and fuel lines [46].

The degree of unsaturation (DU) is related to the number of double bonds and the IV and describes the oxidation stability of biodiesel [39]. The DU value found in this study (53.34%) was proportional to the IV. However, both DU and IV were lower than those reported by Cardoso et al. [23] for *Spirulina* sp. LEB18 cultivation in different proportions of wastewater (74.54–86.83%). The DU was measured based on the mass of monounsaturated and polyunsaturated fatty acids. Therefore, the greater the number of unsaturations, the higher the DU and IV [46]. Low DU values are desirable, particularly for polyunsaturated and long-chain fatty acids. Linoleic and linolenic acids are more susceptible to oxidation than monounsaturated fatty acids because of the bis-allylic position at C11 in linoleic acid and at C11 and C14 in linolenic acid [46].

The cold filter plugging point (CFPP) is an index used to observe biodiesel performance at low temperatures. CFPP is related to the SFA content, as SFAs tend to crystallize at low temperatures and consequently clog filters, pumps, injectors, and pipes due to changes in viscosity and inadequate cold flow properties [44]. The Brazilian Petroleum Agency considers a maximum of 19 °C for this parameter. The CFPP found in the present study (70.70 ºC) exceeded the recommended value by nearly fourfold. This high index indicates low induction at low temperatures [44]. However, additives can be used to inhibit crystal agglomeration [23]. Jawaharraj et al. [39] measured CFPP values ranging from -8.1 to 3.1 °C in the cultivation of *Oscillatoria* sp., *Synechocystis* sp. and *Jatropha* sp. in industrial wastewater. The quality of biodiesel is related to its oxidative stability, which depends on peroxides, light, air, heat, and trace metals in the compounds [46]. Therefore, *Chlorella vulgaris* biomass has appealing properties and a high potential for biodiesel production.

Chlorophyll a, Chlorophyll b and Total Carotenoids

Chlorophyll a (chl a), and chlorophyll b (chl b) values in the PW 30% treatment and control groups were significantly different ($p < 0.05$) (Fig. 3). Both treatments resulted in appropriate levels of pigments, which could be related to high biomass production and luminosity. High biomass concentrations result in high pigment content associated with the growing conditions [47]. Luminosity affects the quantity and quality of energy used for the metabolic activities of photosynthetic organisms and stimulates the growth and accumulation of biocompounds such as pigments [11].

The chl a and chl b of the PW 30% treatment group were measured at 6.64 and 10.57 µg/mL, respectively. The control treatment had 15.81 µg/mL (chl a) and 24.21 µg/mL (chl b).
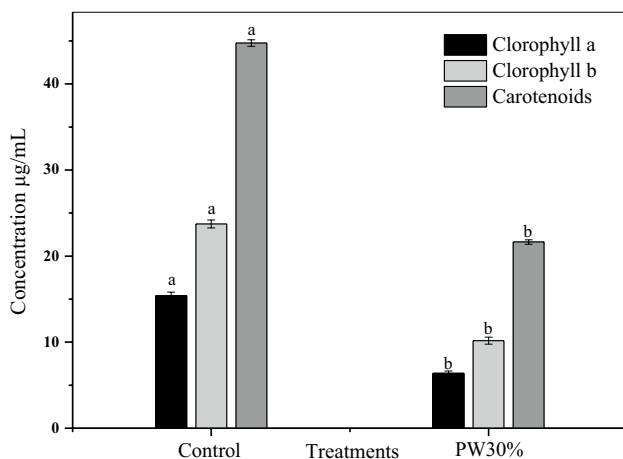
Fig. 3 Chlorophyll *a*, and *b* and total carotenoids concentration of *Chlorella vulgaris* biomass grown in BG11 medium supplemented with PW 30% or the control. Equal letters in the same parameter indicate no statistical difference between treatments ($p < 0.05$) ANOVA and Tukey's test at a 95% confidence level

The lower chlorophyll content found with the PW 30% treatment is attributed to the stress conditions under which the microalgae were subjected, resulting in microalgae degradation and reducing in pigments such as chlorophyll [10]. Gao et al. [40] reported a total chlorophyll content of approximately 18 mg/L after 20 d of cultivation of *Chlorella* sp. in seafood wastewater. The chlorophyll content found in this study was similar to that of the present study (17.21 µg/mL).

The carotenoid contents in PW 30% and control groups was 21.38 µg/mL and 44.37% µg/mL, respectively. The high levels found in both treatments were attributed to continuous exposure to light. Microalgae produce carotenoids as an antioxidant that inhibit the formation of peroxides and reactive oxygen species. These orange pigments are present in the chloroplast membrane and protect photosynthetic systems against the stress of lighting [10]. Ge et al. [47] obtained 12.9 µg/mL of carotenoids from cultivating *Chlorella vulgaris* in municipal wastewater, which is lower than that obtained in this study (21.38 µg/mL). High concentrations of carotenoids are related to the synergistic effect of high luminosity and pollutants in PW. Carotenoids are sensitive biomarkers for contaminants in aqueous media. The higher the carotenoids content, the greater the pollution in the culture medium [15, 23].

Theoretical Conversion of Carbohydrates from Microalgae Biomass to Ethanol

The theoretical production of ethanol from the carbohydrates in the *C. vulgaris* biomass was 17.02 mL/100 g in the PW 30% treatment and 15.15 mL/100 g in the control. These considerable theoretical ethanol values represent an

alternative for the production of biofuels because microalgae biomasses has a high carbohydrate content. The high levels of starch and cellulose in biomass make it an excellent raw materials for bioethanol production, mainly due to the absence of lignin. In addition, microalgal biomass does not contain recalcitrant cells that efficiently convert sugars into ethanol, reducing the cost of processing and pre-treatment [26].

Bioethanol is promising sustainable and renewable fuel. In addition, it has a high octane number and lower CO_2 emissions, despite its lower energy content compared to oil [26]. Biomass has great advantages as a substitute for fossil fuels because of its high organic content, low cost, and high yield, and it can be transformed into chemical products through biological and thermochemical processes [41].

Duarte et al. [26] found a theoretical ethanol value of 25.84 mL/100 g in the cultivation of *Spirulina* sp. LEB18 in brackish groundwater. The theoretical ethanol value in this study was similar to that reported by Duarte et al. [26]. Thus, the results showed that biomass has potential for use in bioethanol production.

Thermogravimetric Analysis (TGA)

The PW 30% and control treatments presented two and three thermal events, respectively. Figure 4a shows the TG profiles, and Fig. 4b shows the derivative (DTG) profiles of both samples. The first mass loss occurred in the temperature range of 30 to 190 °C and was attributed to the loss of free and crystallized water [18, 20] for the PW 30% treatment (12.48%) and control (13.18%). The second mass loss occurred between 190 and 520 °C, representing the devolatilization of the main components of the microalgae such as proteins, polysaccharides, and lipids, starting with unsatured fatty acids and followed by saturated fatty acids as they have a higher melting point [18, 20].

Among the polysaccharides, pyrolysis occurs first in cellulose, a crystalline, linear material composed of glucose, with pyrolysis at 250–350 °C, and then in hemicellulose, an amorphous polysaccharide composed of xylose, galactose and arabinose, with pyrolysis between 325 and 400 °C. The main by-product of pyrolysis, l-glucan, can be used in the production of fiber ethanol in the biogas industry [41]. The third mass loss occurred from 520 to 800 °C and was observed only in the control. This was attributed to the decomposition of carbonaceous residues from macromolecules and inorganic compounds, which is characterized by the decomposition of C–C and C-H bonds. These differences in treatments may be related to the presence of chemical compounds, such as Na, K, Mg, and silicates, which affect the degradation via pyrolysis of the biomass material as well as the char yield [44, 48].
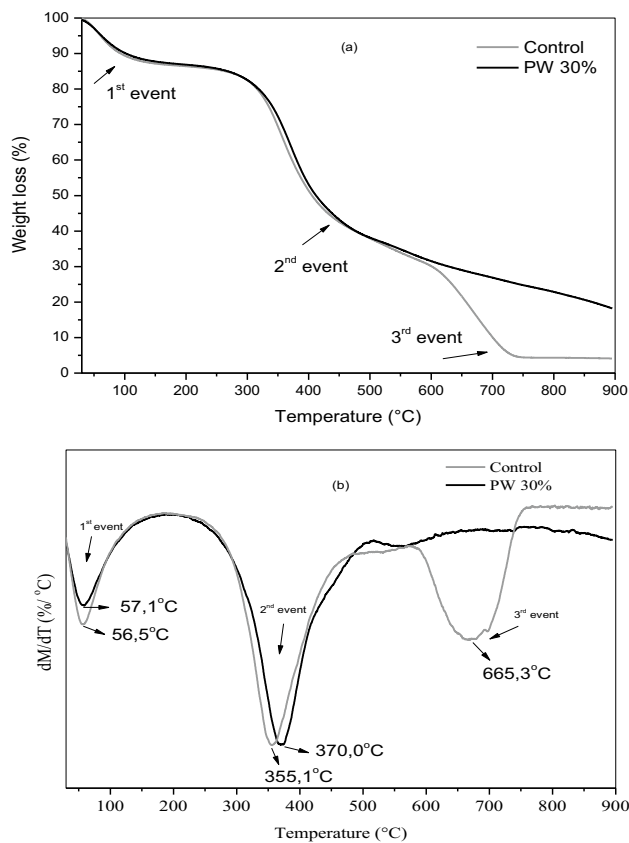
Fig. 4 Thermogravimetric analysis (**a**) and thermogravimetric derivative (**b**) of *Chlorella vulgaris* biomass grown in BG11 medium supplemented with PW 30% or the control

Fig. 5 Fourier transform infrared spectroscopy of *Chlorella vulgaris* biomass grown in BG11 medium supplemented with PW 30% or the control

Andrade et al. [20] reported three thermal decomposition events during cultivation of *Spirulina* sp. LEB18 in reused Zarrouk's medium, similar to what was observed in this study. The first represented mass loss up to approximately 190 °C, the second at approximately 290 °C, and the third thermal event after 500 °C. Gai et al. [48] also reported three thermal events in cultivating *Chlorella pyrenoidosa* and *Spirulina platensis*. The first, second, and third events occurred from 25 to 127 °C, 127 to 327 °C, and 427 to 727 °C, respectively. The *onset* and *endset* temperatures were close to those reported in the present study.

Fourier Transform Infrared Spectroscopy (FTIR) Coupled to Attenuated Total Reflectance (ATR) Device

The spectroscopic profiles of the PW 30% and control treatments showed differences in the intensity and shape of some spectral bands (Fig. 5). The association of wavenumber with some functional groups is shown in Table 5. The PW 30% and control treatments presented a band between 3400 and 3275/cm referring to the –OH group, which mainly
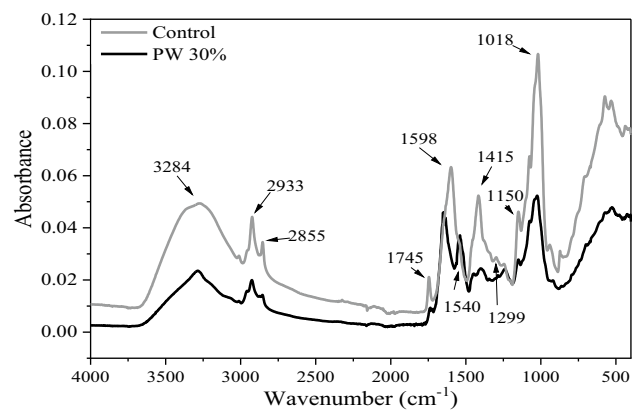
represents residual water [49]. A band between 2934 and 2915/cm was also observed, representing the elongation of symmetrical and asymmetrical vibrations of saturated C–H chains [49, 50]. Spectral bands from 2860 to 2847/cm can also be attributed to –CH$_2$ and –CH$_3$ groups, which form the lipid backbone.

PW 30% displayed shifted bands from 1745–1410/cm. According to Kumar et al. [9], this spectral region represents the C=O methyl esters groups from algal oil. Li et al. [49] and Bataller and Capareda [50] reported that the range between 1774 and 1583/cm is related to the type-I amide band in the C=O stretching vibrations, while bands from 1583 to 1484/cm are associated with type-II amide, resulting in a curvature N–H and C–N, therefore confirming the presence of a peptide bond between amino acids [48]. Protein side-chain and amide III COO⁻ were observed in the spectrum between 1426–1377/cm and 1329–290/cm [49, 50]. The treatments also produced an intense band between 1031 and 1014/cm. These regions

Table 5 Functional groups were obtained by FTIR from the *Chlorella vulgaris* biomass grown in BG11 medium supplemented with 30% PW

Spectral bands (1/cm)	Wavenumber (1/cm)	Functional group
3400—3275	3284	-OH
2934—2915	2933	C-H
2860—2847	2855	-CH$_2$; -CH$_3$
1774—1583	1745	(R-CO)NH$_2$
1745—1410	1598	C=O
1583—1484	1540	(R-CO)$_2$NH
1426—1290	1415	COO-; (R-CO)$_3$N
1031—1014	1018	C-O; C–C

represent the elongation of C–O and C–C, typical of carbohydrates [50].

Li et al. [49] reported a spectral profile similar to that of the present study when cultivating *Tetraselmis suecica* at different CO_2 concentrations. Spectral bands at 3402/cm (referring to the –OH bond), 3325/ cm (N–H bonds), 2998–2854/cm (C–H of lipids), 1734/cm (C = O of the esters), 1583/cm (amide I), 1521/cm—amide II (N–H and C–H), and 1423/cm (–CH$_3$ and –CH$_2$) were associated with proteins, and 1059/cm (C–O–C) was associated with polysaccharides. Bataller and Capareda [50] also reported a similar spectral profile in the cultivation of *Spirulina platensis*, 3960–3340/cm (–OH), 2981–2767/ cm (C–H of lipids), 1762–1719/cm (C = O of esters), 1583–1484/cm (amide II), 1482–1456/cm (–CH$_2$ absorption band of proteins), 1426–1377/cm and 1329–1290/cm (protein side chain and amide III), in addition to 1186–955/cm (referring to carbohydrates). These similarities show that the spectral cell model is applicable for predicting biomass composition.

Conclusion

The cultivation of microalgae in produced water (PW 30%) showed viability and high biomass production among the treatments tested. The pre-adaptation of the inoculum to the continuous photoperiod, additional stress from the 24 h photoperiod, and chemical stress acted synergistically to favor biomass production and biomolecules synthesis by *Chlorella vulgaris*. The obtained biomass has great potential for use as a raw material in the biodiesel and bioethanol industries because of its fatty acid profile and high concentration of carbohydrates. Furthermore, microalgae also showed the environmental benefit of high removal efficiency of the analyzed chemical compounds, including petroleum hydrocarbons and heavy metals found in the medium, as a wastewater treatment method. Therefore, using 30% PW and a constant photoperiod stimulated the production of biomass-derived biomolecules with high added value that can be converted into bioproducts of interest for the petrochemical industry.

Declarations

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