

Article

Transforming Wastewater into Biofuel: Nutrient Removal and Biomass Generation with *Chlorella vulgaris*

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Abstract: This study investigates the potential of *Chlorella vulgaris* for nutrient removal and biomass production in synthetic wastewater. The experiments were conducted in 2 L photobioreactors under controlled aeration, agitation, and lighting conditions for 19 days. Despite a moderate growth rate (0.137 d^{-1}), *C. vulgaris* achieved efficient pollutant removal, with 97% of nitrate, 90% of nitrite, and 90.6% of COD eliminated. Additionally, the biomass was processed to extract fatty acids, yielding a 20% extraction rate, indicating its potential as a biofuel feedstock. These results demonstrate *C. vulgaris*'s dual function in wastewater remediation and biofuel production, presenting a sustainable and economically viable approach to addressing environmental challenges.

Keywords: *Chlorella vulgaris*; nutrients removal; microalgae growth; wastewater; algal biomass



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1. Introduction

The combined effects of record-high energy prices, fuel shortages, rising poverty, slowing economies, and climate change have created an urgent need to identify alternative energy sources that are both economically viable and environmentally sustainable. Biomass has emerged as a versatile renewable resource, capable of producing solid, liquid, and gaseous fuels, making it a key player in the search for sustainable energy solutions [1]. Biofuels, derived from renewable feedstocks such as biomass, have gained significant scientific attention due to their potential to provide energy and serve as alternative fuels [2].

Among various feedstocks, microalgae, particularly *Chlorella vulgaris*, stand out as promising candidates for biofuel production, thanks to their rapid growth rates and high lipid content [3]. Additionally, microalgae can thrive in wastewater, using the nutrients present while simultaneously purifying the water [4]. This dual functionality positions microalgae as a sustainable option for addressing both energy production and environmental remediation.

The excessive presence of nutrients, especially phosphorus (P) and nitrogen (N), in wastewater leads to the overgrowth of algae and other aquatic plants, triggering harmful algal blooms and the deterioration of water quality—a process known as eutrophication [5]. Conventional wastewater treatment methods involve various physical, chemical, and biological processes such as filtration, sedimentation, chemical coagulation, and activated sludge systems [6]. While effective, these methods are often chemically intensive, expensive, and complex. Advanced treatment options like ozonation, chlorination, and filtration through activated carbon or membranes are used to meet stringent environmental regulations [7]. However, these approaches may not always be environmentally sustainable.

In contrast, microalgal bioremediation presents a more eco-friendly and cost-effective alternative, capitalizing on the algae's ability to absorb inorganic nitrogen and phosphorus for growth, while generating valuable biomass [8,9]. Several studies have highlighted the potential of microalgae as biofuel feedstock and for the biological removal of pollutants from wastewater [10,11]. Microalgae require water, CO₂, and nutrients to grow, making

wastewater an ideal source of nutrients [12]. Among the species, *Chlorella* sp. has shown remarkable efficacy due to its rapid growth and resilience in adverse conditions [13,14]. For instance, Znad et al. [15] achieved 100% phosphorus removal and 80% nitrogen removal using *Chlorella vulgaris* in primary wastewater, with a maximum biomass concentration of 1.6 g/L. Moreover, this microalga can thrive in different types of sewage and establishes symbiotic relationships with various bacterial species [16], further enhancing its role in both sustainable biofuel production and wastewater treatment [12].

Building on this, the present study investigates the growth and nutrient removal capacity of *Chlorella vulgaris* in synthetic wastewater. Unlike previous research [17], this study employs wastewater with significantly higher concentrations of key contaminants, including nitrates, nitrites, phosphates, sulfates, chlorides, and chemical oxygen demand (COD). By analyzing the effects of various physicochemical parameters on the microalga's growth and its efficiency in contaminant removal under high-load conditions, this study aims to provide a more realistic assessment of *Chlorella vulgaris*'s potential for nutrient removal in real-world scenarios and challenging environments. The results are expected to contribute to the development of integrated systems for wastewater treatment and biofuel production, transforming waste into a valuable resource while addressing the urgent need for renewable energy.

2. Materials and Methods

2.1. Microalga Strain

C. vulgaris strains were provided by the Algae Collection of the University of Vigo at Marine Science Station (ECIMAT) (Spain). Before the experiments, the strains were maintained in a nutrient medium with the following chemicals: NaNO_3 , KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, Na_2CO_3 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, H_3BO_3 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, ZnCl_2 , $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$. The microalgae were cultivated under fluorescent light with 14/10 h light/dark periods at a temperature of 21 ± 1 °C.

2.2. Experiment Conditions

The microalgae were inoculated into cylindrical photobioreactors (PBRs) with dimensions of 11.5 cm (diameter) $\times$ 24.5 cm (height) and a working volume of 2 L. The initial concentration of the microalgae biomass in the photobioreactor was 118 ± 10 cell/L. Synthetic wastewater was used as culture medium, with the following composition: 1000 mg/L of $\text{C}_6\text{H}_{12}\text{O}_6$, 95.5 mg/L of NH_4Cl , 56.3 mg/L of $\text{CH}_4\text{N}_2\text{O}$, 22.6 mg/L of KH_2PO_4 , 12.6 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 309 mg/L of NaHCO_3 , and 35 mg/L of yeast extract. The cultures, conducted in triplicate, were illuminated by LED lamps (MASLIGHTING 20W 6000K 1900 lm, Maslighting, Spain) under a 14/10 h light/dark cycle, with two lamps positioned horizontally and parallel to the photobioreactors. The reactors were maintained at 25 ± 3 °C (Checktemp[®] 1 Laboratory Thermometer, HANNA Instruments, Smithfield, RI, USA) and aerated using an atmospheric air pump (MARINA 300, Hagen, Germany) filtered at 0.20 μm to ensure complete mixing conditions. Temperature was monitored daily. A schematic of the cultivation process is presented in Figure 1.

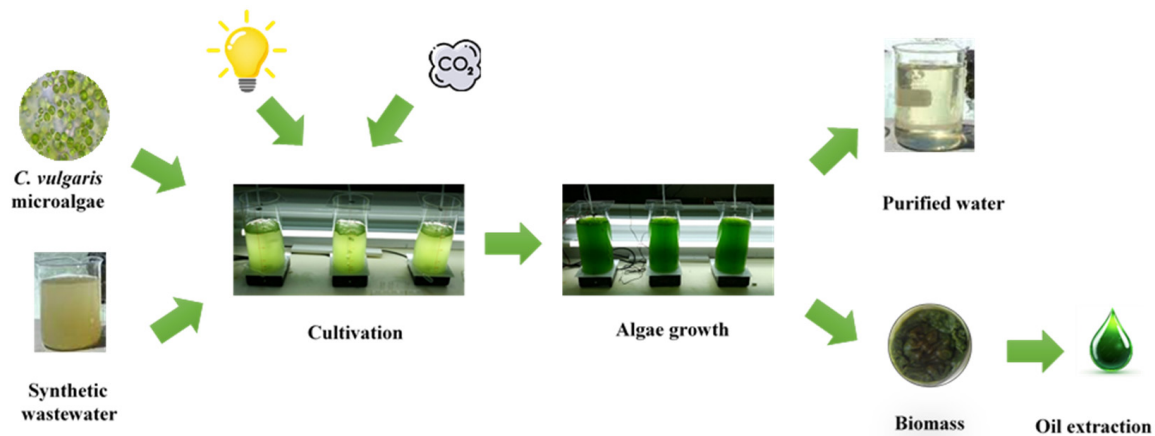


Figure 1. Schematic of cultivation process.

2.3. Analytical Methods

2.3.1. Biomass Determination

The evolution of microalgae biomass was determined by two different methods. On the one hand, algae concentration was directly measured using a hemocytometer. Thus, microalgae cells were counted with an improved Neubauer chamber through an optical microscope (WPI model T-29033, Sarasota, FL, USA). Counting was performed in the middle quadrant of the chamber, where the camera contained 25 small squares [18]. The results were expressed in cell/L. On the other hand, the microalgal biomass was estimated by measuring the optical density at a given wavelength. The wavelength was obtained by scanning a culture sample between 600 and 800 nm [19], as shown in Figure 2, using an ultraviolet spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan). Maximum absorbance was calibrated at 680 nm, indicating the wavelength of maximum sensitivity for quantifying *C. vulgaris* samples.

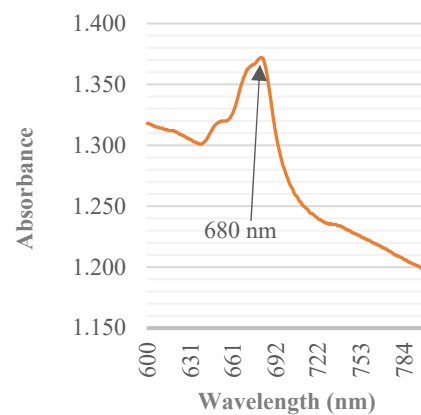


Figure 2. Pattern of light absorbance of *C. vulgaris* between 600 and 800 nm.

The indirect measurement of optical density was correlated with dry cell weight (DCW) by spectrophotometry. At the end of the culture, the dry weight determination was carried out in triplicate. Samples of 25 mL were filtered through a dried (105 °C, 1 h) and weighed glass fiber filter (Millipore nominal pore size 0.45 µm, Merck, Burlington, MA, USA). Finally, they were placed in a drying oven (Raypa Model Incuterm I-40, RAYPA, Barcelona, Spain) at 105 °C for 24–48 h to achieve constant weight. Next, the specific growth rate was calculated using the following equation [20]:

$$\mu \text{ (day}^{-1}\text{)} = (\ln N_2 - \ln N_1) / (t_2 - t_1) \quad (1)$$

where N_1 and N_2 are the biomass concentration (g/L) at time t_1 (day) and time t_2 (day).

2.3.2. Physico-Chemical Water Characteristics

Some physical-chemical parameters of the wastewater were determined before introducing the microalgae and were then monitored during the culture. Before performing the analysis, the samples collected from the medium were harvested by centrifugation at 4000 rpm for 10 min (Selecta Model Mixtasel, Barcelona, Spain). The phosphate, nitrite, nitrate, chloride, sulphate, and fluoride were analyzed at the Centre for Scientific and Technological Support to Research (CACTI) of the University of Vigo using ion chromatography. Analysis of chemical oxygen demand (COD) was performed using Hanna test kits (HI 94754C). All measurements were conducted in triplicate. The percentage reduction of each parameter was calculated as follows:

$$\% \text{ reduction} = (\text{Initial value} - \text{Final value}) / \text{Initial value} \times 100 \quad (2)$$

2.3.3. Lipid Extraction Method

Ultrasound-assisted extraction (US) was employed to extract lipids from *Chlorella vulgaris* microalgae. To further enhance lipid extraction efficiency, microwave pretreatment was applied. In both extraction methods, a solvent mixture of methanol and chloroform (2:1 v/v) was used, with methanol added at a rate of 5 mL per gram of dry biomass. For microwave pretreatment, 24.5 g of microalgal biomass (with 80% moisture content) was combined with methanol and chloroform, and then irradiated with microwaves for 10 min at a power of 140 W. The microwave pretreatment was carried out using a BeckenEasycook Digital 2 microwave oven (Becken, Hamburg, Germany), which operates at a rated power of 700 W and a frequency of 2450 MHz. Following pretreatment, the mixture underwent ultrasound-assisted extraction. In this process, 10 g of microalgae were used in each experiment. Lipid extraction was conducted in a 500 mL flask. The mixture was subjected to ultrasonic treatment using an Elmasonic Model S 300H ultrasonic bath (Elma Schimdbauer, Singen, Germany) operating at 37 kHz for 60 min. After extraction, the mixture was filtered to separate the residue by vacuum filtration, and the solvent was evaporated at 60 °C. The lipid fraction was then dried to a constant weight in an oven.

2.3.4. Lipid Analysis

Following lipid extraction, the algal oil was characterized. Gas chromatography was employed to analyze the composition of Fatty Acid Methyl Esters (FAMES) derived from the *Chlorella vulgaris* microalgal oil. The conversion of free fatty acids into FAMES was carried out in accordance with the UNE-EN ISO 12966-2:2017 standard [21].

3. Results and Discussion

3.1. Evaluation of Algal Growth

Biomass was monitored for 19 days to determine optical and cell density. The optical density was correlated with the dry weight by the following equation:

$$\text{Dry cell weight (g/L)} = 0.3120 \times \text{OD}_{680\text{nm}} \quad (3)$$

Thus, the evolution of algal growth according to these two parameters is shown in Figure 3. An adaptation phase to the culture conditions (lag phase) was observed during the first three days by both methods. After the lag phase, the microalgae experienced rapid growth until the fifteenth day (exponential phase) due to the availability of biodegradable carbon for its growth [22]. The maximum cell density was achieved at the end of this phase, reaching 640 cells/L. Regarding the specific growth rate, a value of 0.137 d^{-1} was reached. However, a specific growth rate of 0.38 d^{-1} was observed when *C. vulgaris* microalgae was grown in urban sewage [2]. Based on these results, this difference could be attributed to the poorer acclimatization of the strain to the culture medium used. After the fifteenth day, the decrease of the main nutrients showed an almost nil-specific growth rate.

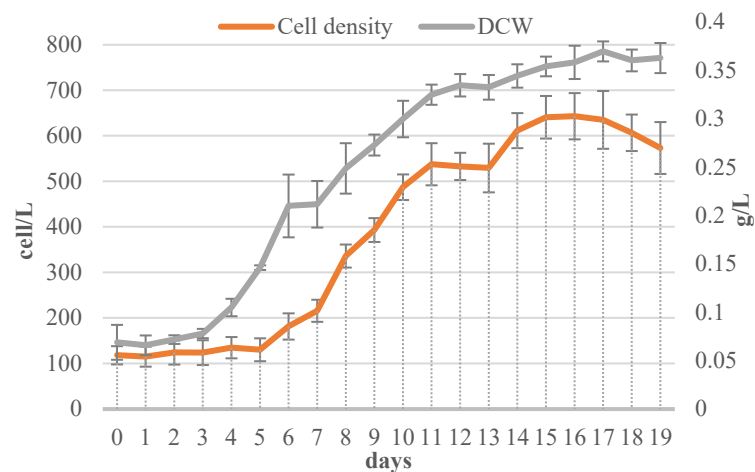


Figure 3. Monitoring of algal growth of *C. vulgaris* in terms of cell density and DCW.

As shown in Figure 3, the growth curves from both methods displayed different slopes. According to previous studies, this could be due to a possible opacity and turbidity interference in the medium, which may alter the cell measurement by optical density [23].

When the number of cells was plotted against absorbance, a linear correlation was observed with an R^2 of 0.9299 (Figure 4). Therefore, the algal biomass can be determined through the absorbance (ABS) using the following linear expression:

$$\text{Algal biomass} \left(\frac{\text{cell}}{\text{L}} \right) = \frac{\text{ABS} - 0.1519}{0.0017} \quad (4)$$

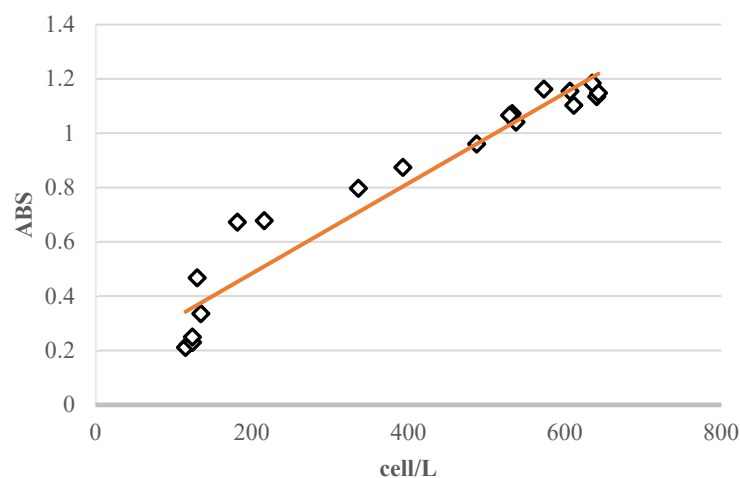


Figure 4. Relationship between absorbance and cell density for *C. vulgaris*.

3.2. Removal of Nutrients

Microalgae require diverse nutrients for their growth, with nitrogen and phosphorus being among the most important, as they are the main contributors to eutrophication [24]. In addition to nitrates and phosphates, other nutrients can also support the growth of microalgal strains [25]. Thus, the removal capacity of the microalga *C. vulgaris* has been analyzed. An evaluation of six nutrients was conducted.

3.2.1. Nitrogen

In general, microalgae are capable of completely eliminating nitrogen. In wastewater, nitrogen can exist in various oxidation states: nitrate, nitrite, ammonium, molecular nitrogen, and other organic compounds [26]. The absorption capacity of this nutrient

depends on the species of the microalga studied [27]. However, few studies consider the environmental importance of each nitrogen form.

Although nitrogen is available in several forms, nitrate is one of the most common and stable forms in water. The influence of the nitrate ion on *C. vulgaris* growth is shown in Figure 5a. During the lag phase, nitrate concentrations did not show significant alterations. However, at the beginning of the exponential phase, the microalgae exhibited a high rate of nitrate uptake in the first seven days of cultivation. A substantial reduction in nitrate levels was observed from 6.68 ± 0.36 to 0.15 ± 0.02 mg/L. Thus, this green microalga was able to remove more than 97% of the nitrate ions from synthetic wastewater during that period.

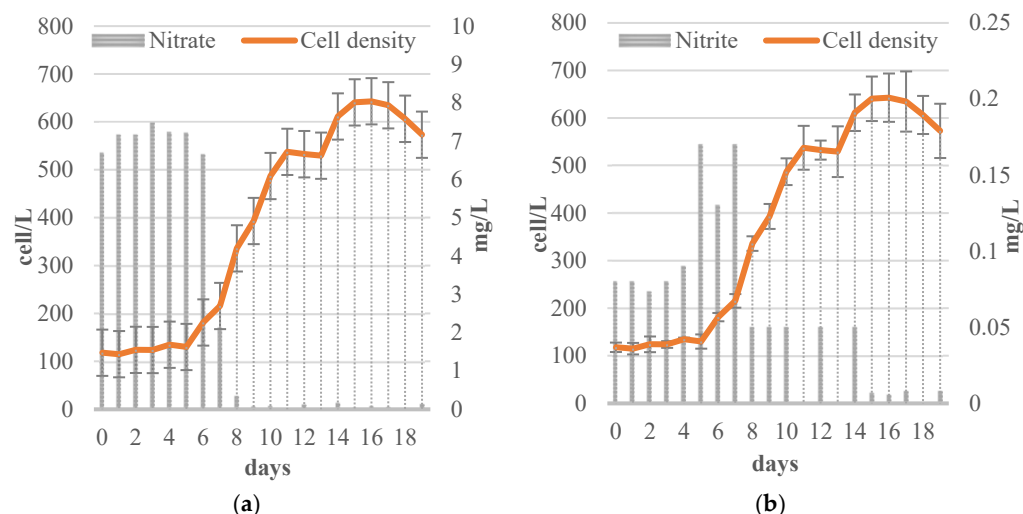


Figure 5. Different forms of nitrogen and cell density evolution of *C. vulgaris* in wastewater. (a) Nitrate concentration and (b) Nitrite concentration.

On the other hand, the removal of nitrite during culturing is presented in Figure 5b. Low concentrations of this parameter were observed. Although this unstable form of nitrogen exhibited several concentration peaks, a 90% nitrite removal efficiency was achieved by the end of the cultivation. In addition, the figures show a preferential elimination of nitrates (from 6.94 mg/L to 0.06 mg/L) over the nitrites (from 0.09 mg/L to 0.01 mg/L).

Thus, high levels of nitrogen elimination from wastewater, in the form of nitrite and nitrate, were achieved when *C. vulgaris* culture was conducted. Similar results have been reported by other authors [28].

3.2.2. Phosphate

The data presented in Figure 6 show the variation of the phosphate content over 19 days. The results of this research demonstrate a clear reduction of this compound starting on the sixth day. A 55.22% phosphate removal efficiency was achieved on day 6, reaching 69% six days later. Consequently, the microalgae had reduced the phosphate content from 29.12 ± 1.45 to 8.82 ± 2.81 mg/L by day 12. Similar results were obtained when an algal–bacterial culture was grown in wastewater, achieving phosphate removal efficiencies ranging from 54.5% to 72.6% [28]. Higher phosphorus removal, up to 90%, was reported by Wang et al. [29] when the *Chlorella* sp. microalga was cultivated in different wastewaters. An inverse relationship between the phosphate concentration and algal cell concentration is shown in Figure 6, where phosphate levels did not drop below 8.82 ± 2.81 mg/L. Towards the end of the culture period, an increase in phosphate concentration was observed; this phenomenon could be due to cellular leakage [26].

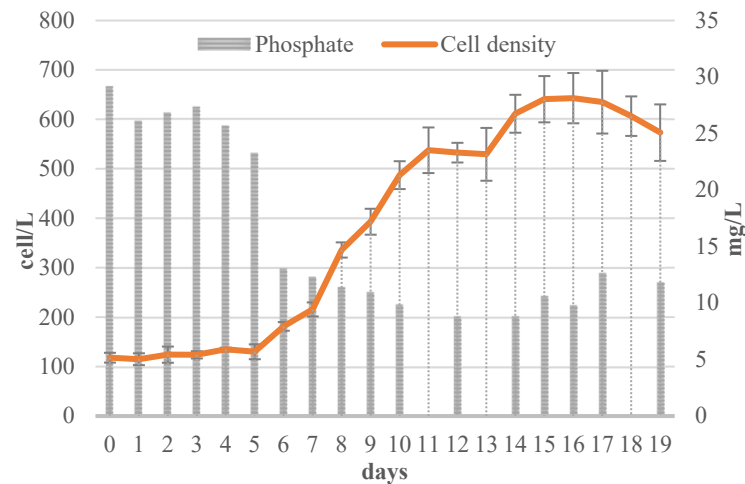


Figure 6. Phosphate concentration and cell density evolution of *C. vulgaris* cultivated in wastewater.

3.2.3. Sulfate

Sulfur is adsorbed by the autotrophic organisms in the form of sulfate, as it is an essential nutrient for their growth [30,31]. The influence of algal cell density on sulfate absorption capacity is depicted in Figure 7. A slight decrease in the sulfate levels was observed during the lag phase. In the following days (exponential phase), significant sulfate variations were recorded. Nonetheless, maximum sulfate removal was achieved at a cell density of 643.22 cells/L, with a concentration of 0.33 mg/L during the death phase. Therefore, sulfate adsorption by *C. vulgaris* ranged from 5.33 ± 0.31 mg/L to 0.33 ± 0.08 mg/L, with a maximum removal efficiency of 93.8%. This demonstrates that *C. vulgaris* show good sulfate elimination ability. It should be noted that the sulfate concentration used in this research was lower than the typical concentration found in domestic wastewater (20–60 mg/L) [32].

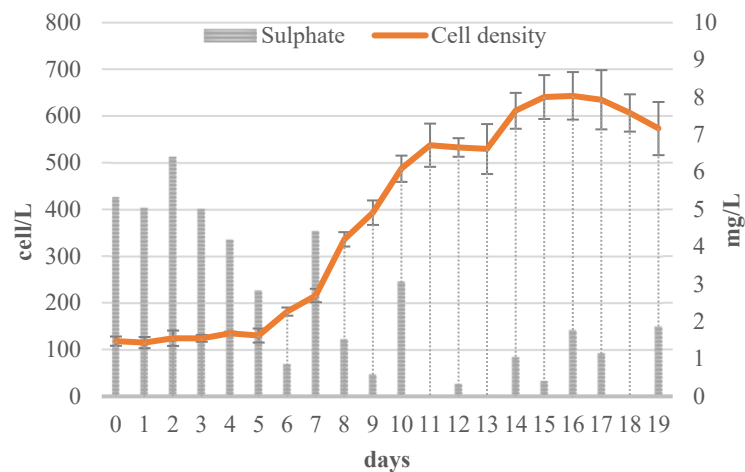


Figure 7. Sulfate concentration and cell density evolution of *C. vulgaris* cultivated in wastewater.

3.2.4. Chloride

A slight decrease in chloride concentrations was detected throughout the culture period (Figure 8). The greatest reduction in chloride levels occurred between the third and fourth days, dropping from 171.94 ± 1.79 to 130.26 ± 2.02 mg/L. This variation coincided with the last days of the lag phase, where the microalgae population remained stable at around 125 cells/L. In the following days, only minor variations in chloride concentration were observed. At the end of the experiment, the highest chloride removal achieved was 32%. This study suggests that the microalgae *C. vulgaris* exhibited low assimilation of chloride, which could be attributed to the inhibitory effect of chloride on growth [33].

However, other species, such as *Chlamydomonas polypyrenoides* microalgae, have achieved a 61% reduction in nutrient levels when grown in industrial wastewater [34].

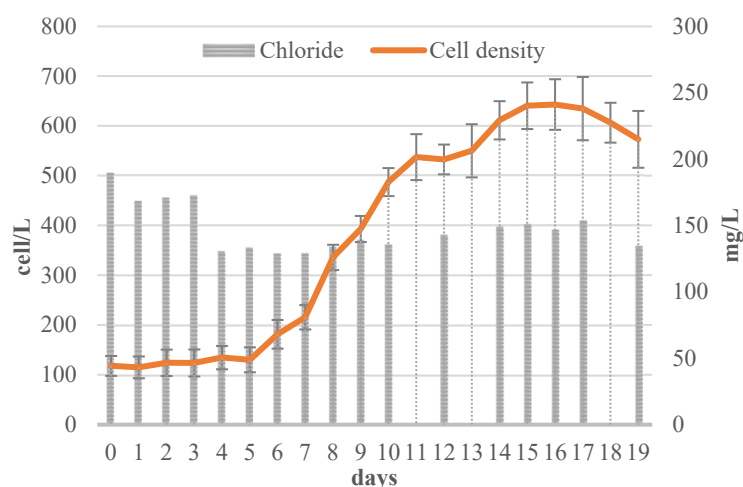


Figure 8. Chloride concentration and cell density evolution of *C. vulgaris* cultivated in wastewater.

3.2.5. Fluoride

Fluoride ions occur naturally in water and the environment, but high concentrations can be harmful to both human health and ecosystems [35]. Figure 9 illustrates fluoride concentration and cell density over 19 days of cultivation. Low fluoride concentrations, around 0.05 ± 0.01 mg/L, were detected in the synthetic wastewater used. Previous studies conducted by Ali [36] and Venkata Mohan et al. [37] showed fluoride accumulation in microalgae, leading to a decrease in fluoride concentrations in water during purification. However, in the current study, no significant decrease in fluoride levels was observed. Therefore, *C. vulgaris* did not exhibit a notable fluoride removal capacity in the sewage.

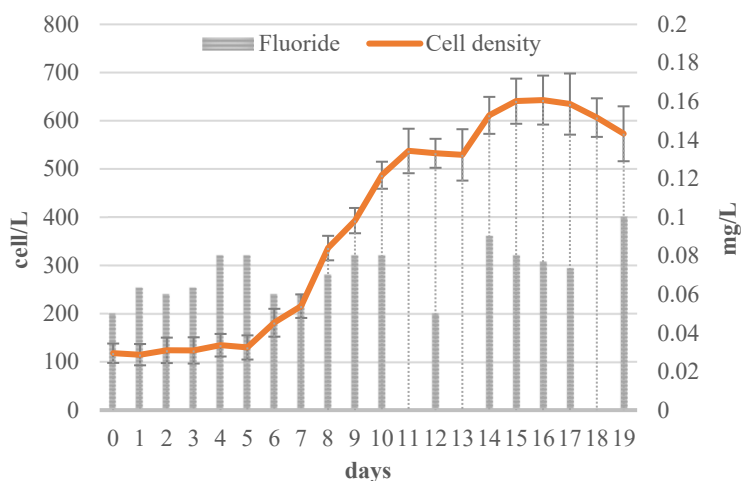


Figure 9. Fluoride concentration and cell density evolution of *C. vulgaris* cultivated in wastewater.

3.2.6. COD

Chemical oxygen demand (COD) is one of the most important water quality parameters, as it reflects the organic load present in water [38]. This parameter is commonly used as an indicator of water quality when wastewater is discharged into the environment. High COD levels indicate an increased amount of organic material, which can reduce dissolved oxygen levels and potentially create anaerobic conditions detrimental to aquatic ecosystems.

A significant reduction in COD after the adaptation phase is shown in Figure 10. COD concentration decreased from $3018 \pm 2.81 \text{ mgO}_2/\text{L}$ to $283 \pm 1.41 \text{ mgO}_2/\text{L}$ within the first 7 days, achieving a maximum COD removal efficiency of 90.6% on the seventh day. This indicates that COD can be reduced in a relatively short period. Similar results were found in a study of Ren et al. [39], where COD removal reached 80.5% in co-culture of anaerobic sludge and oleaginous microalgae in wastewater. Nevertheless, lower removal efficiencies, ranging from 60 to 70%, were reported by Wang et al. [40] when *C. vulgaris* microalgae was cultivated in swine wastewater.

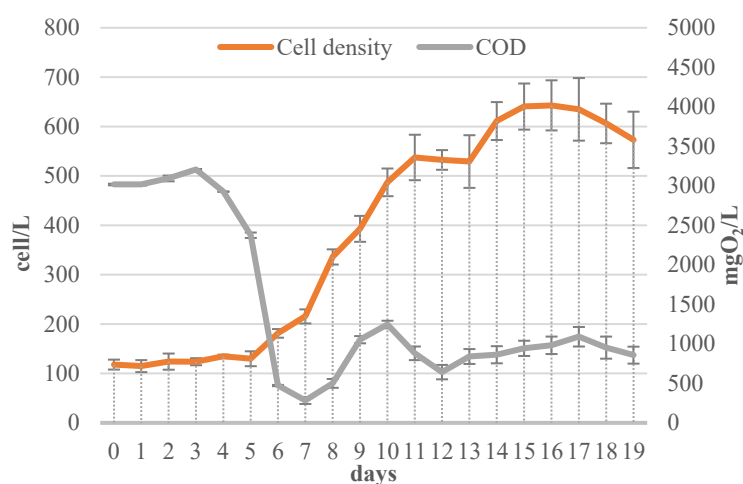


Figure 10. COD and cell density evolution of *C. vulgaris* cultivated in wastewater.

As shown in Figure 10, an increase in COD levels was recorded after the seventh day of culture. This phenomenon may be attributed to both the heterotrophic and autotrophic growth exhibited by *Chlorella* microalgae. Similar behavior was observed in another study, which confirmed this pattern when *Chlorella* was grown in wastewater [29].

3.3. Lipid Extraction

In this study, an extraction yield of $19.80 \pm 2.01\%$ was achieved using a combination of microwave and ultrasound techniques for lipid extraction from *Chlorella vulgaris*. This result is comparable to yields reported in previous studies utilizing similar methods. For example, Rezende et al. [41] reported a yield of 17% using ultrasound-assisted extraction for the same algal species using the same solvents. Similarly, a lipid yield of 17.5% using microwave-assisted extraction is presented in the research by Yeong et al. [42], and similar values (18.14%) were reported in a study by Vijay et al. [43] when microwave technology was applied as an extraction method. Thus, the results of this study align with the general range of lipid yields reported in the literature for *C. vulgaris*.

Variations in lipid extraction yield can be attributed to several factors, including differences in the operational parameters such as microwave power, ultrasound frequency, and extraction duration, as well as variations in the algal biomass composition and pre-treatment methods used. It is important to note that the combination of microwave and ultrasound can be highly effective, but achieving optimal results requires precise control over process parameters. Further optimization of extraction conditions could improve yield and efficiency, contributing to the more effective utilization of *Chlorella vulgaris* for biodiesel production.

The algal oil obtained from the extraction process was analyzed using gas chromatography. The fatty acid profile of *Chlorella vulgaris* microalgae revealed that palmitic acid (C16:0) was the most abundant, constituting $51.08 \pm 0.70\%$ of the total fatty acids. Stearic acid (C18:0), as well as oleic and elaidic acids (C18:1), were also present in lower proportions, accounting for $17.51 \pm 0.32\%$ and $18.34 \pm 0.13\%$, respectively. Additionally, minor amounts of lauric acid (C12:0), myristic acid (C14:0), linolelaidic acid (C18:2), and linoleic

acid (C18:3) were detected in the oil. In a study conducted by Ma et al. [44] on *C. vulgaris*, palmitic acid was also found to be one of the predominant fatty acids, though at a lower percentage compared to the findings of this research ($20.63 \pm 1.28\%$).

The results of this study suggest that integrating wastewater treatment and biofuel production systems can optimize resource recovery, leading to more sustainable environmental practices and renewable energy solutions.

4. Conclusions

This study highlights the effectiveness of *Chlorella vulgaris* in synthetic wastewater treatment, emphasizing its role in nutrient removal and biomass production. The microalgae demonstrated significant pollutant removal capabilities, achieving high efficiencies that meet regulatory standards (91/271/EEC) [45]. Furthermore, the processing of biomass for fatty acid extraction underscores the versatility of *C. vulgaris* as a potential biofuel feedstock. These results emphasize the dual functionality of *C. vulgaris* in both wastewater remediation and biofuel production, offering an eco-friendly and economical approach to wastewater treatment.

Future work should focus on optimizing culture conditions to enhance growth rates and further improve removal efficiencies, paving the way for large-scale applications that integrate wastewater treatment with renewable energy generation. This strategy not only addresses environmental challenges but also contributes to sustainable biofuel production.

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Conflicts of Interest: The authors declare no conflicts of interest.

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