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Coupling nutrient removal and biodiesel production by cultivation of *Chlorella* sp. in cafeteria wastewater: assessment of the effect of wastewater disinfection

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ABSTRACT

Cultivation of microalgae using wastewater as nutrient resource is a promising strategy to reduce the microalgae biodiesel production cost and increase nutrient recovery in one step. However, the inhibitive effect of indigenous bacteria on microalgae could negatively affect microalgal growth. Thus, in this study, the effect of wastewater disinfection methods on the growth and biochemical composition of *Chlorella* sp. and the nutrient removal efficiency in cafeteria wastewater was evaluated. Results showed a significant increase in the microalgal density and a reduction in bacterial abundance in the disinfected wastewater. Moreover, chemical oxygen demand removal ranging from 83.53%–87.16% was achieved in 15-d incubation and complete removal of total nitrogen and total phosphorus was achieved after 6 d of incubation. The total carbohydrate, protein and fatty acid contents in the harvested biomass were 128.49–139.33, 43.24–56.14 and 17.34–23.58 mg·g^{-1} on dry weight basis, respectively. Palmitic acid, palmitoleic acid, stearic acid and oleic acid accounted for more than 90% of the total fatty acids in the biomass, indicating great potential as an alternative feedstock for biodiesel production. This study provides a simple and efficient disinfection strategy to enhance *Chlorella* sp. growth and biomass production for biodiesel production.

Keywords: Chlorella sp.; Cafeteria wastewater; Nutrient removal; Biodiesel

1. Introduction

Energy depletion and environmental pollution are two main challenges to the sustainable development of the world. Biodiesel is a well-recognised renewable and clean energy that could be directly used in the inner combustion engine together with fossil-based diesel without modification [1]. Currently, biodiesel is generally produced from vegetable oil or waste/used cooking oil. However, production of biodiesel by using vegetable oil could compete with household consumption, where the supply of waste/used cooking oil is unstable.

Microalgae are unicellular photosynthetic microorganisms that could use solar energy nutrients to synthesise biochemicals, such as carbohydrate, protein and lipids. The neutral lipids in microalgae cells could be chemically converted into biodiesel. In addition, microalgae have the advantage of no competition with human beings in fresh water and arable land as they could survive in saline water and wastewater [2]. Thus, microalgae are considered very promising renewable alternative feedstock for biodiesel production. However, large-scale production of microalgal-based biodiesel is greatly hampered by high cost, especially in the microalgal biomass production process, in

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which the fertilizer supply accounts for 52% of the energy demand [3]. The use of residual N and P was also confirmed to show a potential to reduce up to 61% in energy demand, 84% in carbon footprint and 37% in financial cost compared with using fertilizer [3]. In this context, wastewaters rich in N and P, such as livestock wastewater, fertilizer production wastewater and landfill leachate, have been extensively evaluated for microalgal cultivation.

The number of cafes, canteens and restaurants have increased rapidly in the past decade [4], leading to the generation of a large volume of cafeteria wastewater. The wastewater from cafeteria activity contains a high concentration of organic material, such as proteins, carbohydrates, animal fat and vegetable oil [5]. Cafeteria wastewater also contains abundant nutrients, such as N and P. Improper management of cafeteria wastewater not only reduces the water-passing capacity of the pipeline or even blocks it but also increases the load of wastewater treatment plants and deteriorates the water quality [6]. Meanwhile, N and P could serve as nutrients for microalgal growth and consequently reduce the microalgal biomass production cost and relieve the negative effect on the water environment and ecology. Compared with the wide concern to industrial wastewater, very few research works have been reported regarding the production of microalgal biomass by using cafeteria effluent as nutrient source [7]. However, the growth patterns and biochemical composition of microalgae are species-specific. In addition, a notable detail is that the presence of bacteria in cultivations is not avoidable and maintaining axenic cultures in open systems is impossible [8] when scaling up microalgal cultivation. Not only symbiotic interactions could occur between microalgae and bacteria but also antagonistic interactions as the decay of dead microalgae competes with bacteria for oxygen. Besides, microalgae and bacteria could produce a wide range of inhibitory compounds that are harmful to their partners [9]. Thus, excessive bacterial populations in wastewater may result in competition with the desired microalgae and failure to produce a bulk volume of microalgal biomass at low cost given that biological contamination is a big constraint in the mass cultivation of microalgae [10,11]. Consequently, strategies on biological contamination control are in great demand for the mass cultivation of microalgae.

So far, few approaches have been proposed and applied to reduce the effect of indigenous bacteria on microalgae, including flocculation [12], hydrodynamic cavitation [13]; chemical additives, such as formaldehyde, chlorine, ozone [14] and hydrogen peroxide [15,16]; and by changing the environmental conditions, such as pH, temperature and illumination [10]. For example, Kim et al. [14] applied ozonation to treat anaerobically digested piggery effluent prior to *Scenedesmus quadricauda* cultivation and increased microalgal biomass from around 1,000 mg·L^{-1} to more than 3,000 mg·L^{-1}. Disinfection strategies using sodium hypochlorite in outdoor closed horizontal tube photobioreactor were studied by Fei et al. [16] to reduce the contaminants during outdoor culture and ensure the stability of large-scale astaxanthin production. Hydrodynamic cavitation was recently applied as a bacterial disinfection method for medium recycling, by which up to 100% of the bacteria was disinfected [13]. However, analysis of the reported research results showed that the disinfection effects on bacterial activity, microalgal growth and their biochemical compositions are species-specific. In addition, citric acid-modified PF (composed of 1 mg·L^{-1} Fe^{2+}, 25 mg·L^{-1} H_2O_2 and 17.5 mg·L^{-1} citric acid) can generate hydroxyl radicals, which have been confirmed to have strong disinfection effect on the organisms. To the best of our knowledge, wastewater disinfected by citric acid-modified PF (composed of 1 mg·L^{-1} Fe^{2+}, 25 mg·L^{-1} H_2O_2 and 17.5 mg·L^{-1} citric acid) for microalgal cultivation has not been reported yet.

Therefore, in the current work, cafeteria wastewater from a campus canteen was disinfected by 2% NaClO and citric acid-modified PF separately and then employed as nutrient source for *Chlorella* sp. cultivation to evaluate their effect on bacterial and *Chlorella* sp. growth, their performance on bioremediation of cafeteria wastewater nutrients and biochemical compositions.

2. Materials and methods

2.1. Cafeteria wastewater samples

The cafeteria wastewater samples used in this study were collected from the settling tank receiving effluent from a campus canteen at Shaoguan University, Guangdong Province, China. The obtained cafeteria wastewater samples were firstly subjected to oil removal, followed by filtration using gauze and gravity precipitation overnight to remove large particles. In addition, vacuum filtration (f11 quantitative filter paper) was applied to remove the remaining suspended solids in the wastewater prior to water quality analysis and microalgal cultivation. The obtained wastewater was designated as raw cafeteria wastewater. The representative physicochemical compositions of the raw cafeteria wastewater were analyzed and they are listed in Table 1.

2.2. Microalgal specie and cultivation

The microalgal specie used in this study is *Chlorella* sp. which was isolated from the water in a lake on the campus of Shaoguan University, Guangdong Province, China. The microalgae were subjected to pre-culture in BG11 medium in a 1 L Erlenmeyer flask until logarithmic growth phase before being cultivated in cafeteria wastewater. Raw cafeteria wastewater pre-treated with 2% sodium hypochlorite (w/w, designated as 2% NaClO) and citric acid-modified PF (composed of 1 mg·L^{-1} Fe^{2+}, 25 mg·L^{-1} H_2O_2 and 17.5 mg·L^{-1} citric acid; designated as PF) for 10 h were used as culture media to study the effect of disinfection to wastewater on

Table 1
Physico-chemical properties of cafeteria wastewater

Parameters	Values (mean value ± standard deviation)
pH	6.60 ± 0.23
Total nitrogen (mg·L^{-1})	8.73 ± 0.71
Total phosphorus (mg·L^{-1})	2.15 ± 0.01
Chemical oxygen demand (mg·L^{-1})	1,099.85 ± 52.65

microalgal growth and algal biomass biochemical compositions. For comparison, raw cafeteria wastewater was employed as the control (designated as untreated). During cultivation, all treatments were carried out under environmental conditions (18°C–25°C) and continuously illuminated with an LED light (Sunsun Group Co., Ltd., China) fixed at approximately 20 cm away from the flasks. The illumination intensity was determined to be 3,000 ± 100 lux on the surface of the flasks. Continuous aeration was provided to the culture broth to avoid culture sedimentation. During incubation, the microalgal and bacterial abundances and the chemical oxygen demand (COD) and nutrient concentrations in the wastewater were determined every 3 d. The incubation lasted for 15 d before the biomass was harvested by gravity sedimentation, followed by centrifugation (5,000 rpm, 10 min). Biomass pellets were freeze-dried at −80°C for total lipid, protein, carbohydrate content and fatty acid profile analyses.

2.3. Determination of microalgal and bacterial abundances

The abundance of microalgae during incubation was counted under a light microscope using a haemocytometer. The bacterial number during cultivation was determined by colony counting in accordance with the procedures described by Higgins et al. [17].

2.4. Wastewater sampling and quantity analysis

For evaluation of the COD and nutrient removal efficacy of microalgae, approximately 50 mL culture of each treatment was sampled every 3 d during cultivation for COD, total nitrogen (TN) and total phosphorus (TP) concentration analyses. A notable detail that distilled water was supplemented to the cultures for the compensation of water loss via evaporation before each sampling. The obtained samples were centrifuged at 5,000 rpm for 5 min and the supernatants were subjected to filtration through 0.45 μm membrane syringe filters. The obtained filtrates were appropriately diluted for COD, TN and TP concentration measurements in accordance with the corresponding Chinese National Standard methods [18]. The COD and nutrient removal percentage (RP) and removal rate (RR) were calculated using Eqs. (1) and (2), respectively, as follows:

$$\mathrm{RP}(\%) = \frac{C_i - C_f}{C_i} \times 100\% \tag{1}$$

$$\mathrm{RR}\left(\mathrm{mg}\cdot\mathrm{L}^{-1}\cdot\mathrm{d}^{-1}\right) = \frac{C_i - C_f}{t} \tag{2}$$

where C_i and C_f are the concentration at the beginning and completion of cultivation, respectively, mg·L^{-1}; and t is the incubation time, d.

2.5. Biochemical composition analysis

The total carbohydrate content of the harvested biomass was determined by phenol-sulfuric method [19]; the total lipids were measured following the procedures stated by Bligh and Dyer [20]; and the protein content in the biomass was analysed using Bradford method [21], with minor modification. Specifically, approximately 20 mg of freeze-dried biomass and 5 mL of 0.5 N NaOH aqueous solution were introduced into a sealed 15 mL screw glass centrifuge tube. The mixture was incubated in 80°C water bath for 20 min, followed by centrifugation (3,000 rpm, 5 min) to extract protein. The extraction procedure was repeated twice. The protein concentration in the obtained extracts was determined using a Bradford protein kit (Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China). The fatty acid profiles of the harvested biomass were analyzed as described previously [22].

2.6. Statistics analysis

All the experiments were conducted in duplicates and the average values with standard deviations were reported (except lipid determination). One-way ANOVA using SPSS software (version 11.0) with $p < 0.05$ was carried out for data validation.

3. Results and discussion

3.1. Effect of disinfection on microalgal growth and bacterial abundance

Apart from the synergistic relationship with microalgae, the bacteria in cafeteria wastewater could possibly limit the growth of microalgae by competing with microalgae for inorganic nutrients [23], producing variable algicidal compounds [24] and even switching the stoichiometry of microalgae [25]. Meanwhile, microalgae could also suppress bacterial growth by secreting antibiotic compounds [26]. Thus, in the present work, the variations of *Chlorella* sp. and bacterial abundance during the 15-d cultivation were determined and the results are illustrated in Fig. 1. No lag phase occurred during cultivation, suggesting that *Chlorella* sp. could rapidly adapt to the cafeteria wastewater in all treatments and no dramatic inhibition was found on the growth of *Chlorella* sp. by the disinfection chemicals and the indigenous bacteria. After 3 d of incubation, a significant increase in *Chlorella* sp. abundance in all treatments was observed, whereas an obvious reduction in bacterial abundance was detected at the same cultivation period, as shown in Fig.1b. After 6 d of incubation, the bacterial abundance in the disinfected wastewater was dramatically lower than that in untreated. This finding could possibly be explained by the generation of free chloride and $OH^{\bullet}$ when NaClO and PF mixed with water, respectively, which could provide lasting disinfection effect and minimize external contamination during microalgal cultivation [15]. Furthermore, the much larger abundance of microalgae than bacteria in the culture could lead to decreased bacterial growth. The highest growth rate of *Chlorella* sp. was also achieved in cafeteria wastewater disinfected by PF, followed by that obtained in 2% NaClO. The lowest growth rate of *Chlorella* sp. was determined in the raw cafeteria wastewater. This phenomenon is well consistent with the bacterial abundance during cultivation.

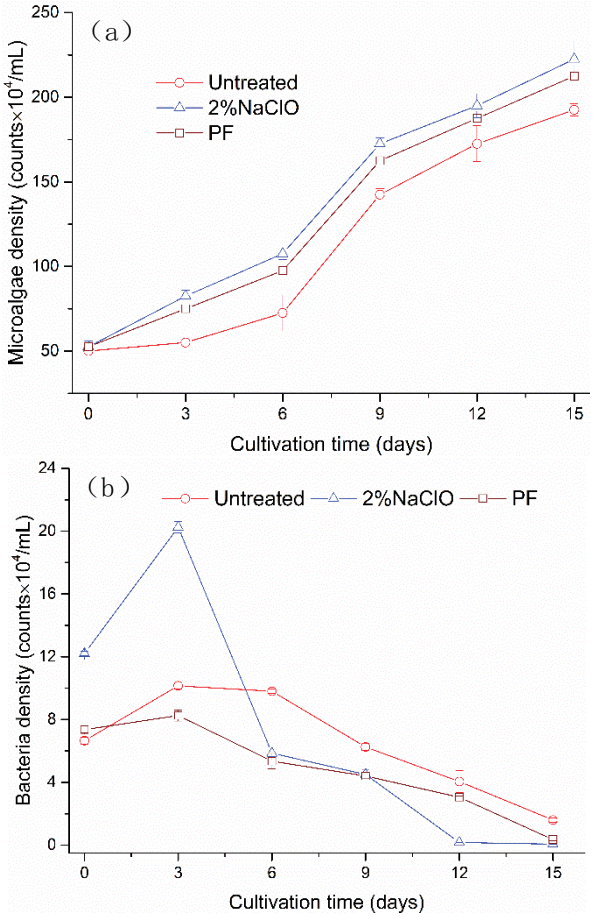

Fig. 1. Microalgal (a) and bacterial (b) abundances in cultures during cultivation.

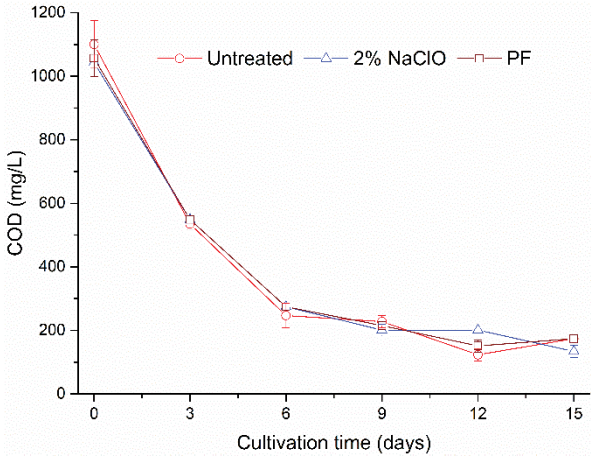

Fig. 2. Variation of chemical oxygen demand during incubation.

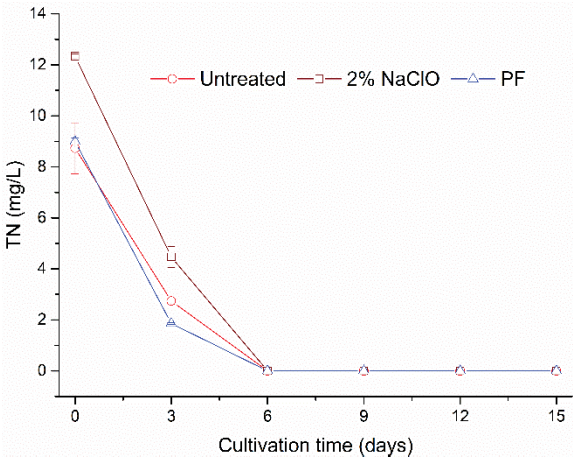

Fig. 3. Variation of total nitrogen during incubation.

3.2. Nutrients and COD removal

Improper management of wastewater rich in nutrients (i.e., N and P) not only could result in destroying the ecological balance of natural water body but also result in the waste of valuable resources [27]. Cafeteria wastewater contains abundant C, N and P elements, thus a promising alternative to the fossil-based culture medium usually used in massive cultivation of microalgae. In the present study, the variations of COD, TN and TP concentrations in wastewater are depicted in Figs. 2–4, respectively.

As shown in Fig. 2, a significant reduction in COD concentration could be observed in the initial 6 d of cultivation in all treatments. This finding could be ascribed to the assimilation of indigenous bacteria in the cafeteria wastewater and respiration by the microalgae. However, after 6 d of cultivation, a much slower decrease rate of COD concentration was observed in all treatments, especially in untreated and PF, in which a slight increase in COD concentration was detected after a minimum value occurred on day 12. This phenomenon could be attributed to the direct lysis of dead algal cells by the indigenous bacteria and the subsequent release of dissolved organic C [28,29]. After 15 d of cultivation, the maximum COD RP and RR were 84.18% and 61.72 mg·L^{-1}·d^{-1}, 87.16% and 60.59 mg·L^{-1}·d^{-1} 83.53% and 58.83 mg·L^{-1}·d^{-1} for untreated, 2% NaClO and PF, respectively. This result could be attributed to the assimilation of organic C by the remaining indigenous bacteria in the cafeteria wastewater [11]. The COD removal efficiency in the current work was higher than that reported by Cheng and Tiang [30], in which a COD removal percentage of 73.36% was achieved by cultivating *Scenedesmus* sp. in cafeteria wastewater. The better performance of COD removal efficiency in the current work could possibly be due to the higher microalgal numbers achieved during the cultivation. In addition, advanced oxidation with NaClO or PF also reduces the dark colour and improves the light transmission efficiency in the culture by breaking the C–C bond and convert refractory high-molecular-weight organics to low-molecular-weight organics [31], which, in turn, facilitates the assimilation of the dissolved organic substances by the indigenous bacteria and microalgae [15].

N is a dispensable nutrient in algal protein and nucleic acid synthesis. As indicated in Fig. 3, with the increase in microalgal density, a continuously rapid decrease in TN concentration was observed in the cafeteria wastewater. By the end of 6-d incubation, a complete TN removal was

achieved in all the treatments. Moreover, 1.76–2.55 mg·L^{-1}·d^{-1} of N was removed in all the treatments. Since the wastewater used for *Chlorella* sp. cultivation was not totally sterilized, therefore, small amount of bacteria must exist in the wastewater during microalgae cultivation. In this sense, some species of bacteria, such as nitrifying bacteria, denitrifying bacteria could assimilate nitrogen and phosphorous compounds in their metabolite process. Thus, the significant removal of nitrogen in the wastewater could be attributed to the synergistic effect of microalgae-bacteria consortia, including nitrifying and denitrifying bacteria. High nitrogen removal rate in the current work demonstrating that *Chlorella* sp. cultivation in disinfected cafeteria wastewater is a very promising approach to mitigate the environmental effect caused by overloaded N discharge to natural water body. The RP and RR of N were higher than those reported by Hu et al. [15], who obtained an N RP of 75%–80% by cultivation of microalgae in pre-treated meat processing wastewater with 0.2 mg·L^{-1} free chlorine.

P is another essential nutrient participating in microalgal metabolic activities, such as signal transduction, energy conversion and photosynthesis [32]. The variations of TP concentration in the raw and disinfected cafeteria wastewater during *Chlorella* sp. cultivation are presented in Fig. 4. A notable reduction in TP concentration ($p < 0.05$) and complete removal of P could be observed after 3 d for all treatments. The high P removal rate obtained in this work could be attributed to the combined effect of biological assimilation and chemical precipitation because of high pH values in the microalgal culture as carbon dioxide was continuously consumed during microalgal cultivation [33,34].

3.3. Chemical composition of biomass

The biochemicals in the microalgal biomass are mainly composed of carbohydrate, protein and lipids. Their distribution is one of the crucial indicators used to determine their commercial application potential. In addition, the biochemical components of microalgae may vary significantly from species to species and their growing environment conditions within the same species. For instance, as stated by Danger et al. [25], indigenous bacteria in wastewater could limit the growth of microalgae by switching the stoichiometry of microalgae, thereby altering the chemical composition of the obtained biomass. Algal carbohydrate and protein are sustainable and promising feedstock for bioactive substances in food and healthcare product production. Moreover, carbohydrate content could directly reflect the COD removal capacity of microalgae when applied in the bioremediation of organic C-abundant wastewater. Meanwhile, the neutral lipids in microalgal biomass could be applied in biodiesel production. Thus, in this work, the contents and productivities of total carbohydrate, protein, lipids and fatty acids and the fatty acid distribution in the harvested biomass from different treatments were analysed and compared to evaluate the effect of wastewater disinfections on the biochemical compositions in the harvested biomass. Fig. 5 shows that the carbohydrate and protein contents in the obtained biomass were 128.49–139.33 and 43.24–56.14 mg·g^{-1} on dry weight basis, respectively. No significant difference in the carbohydrate and protein contents in the biomass could be found in all the treatments, implying that disinfection to the cafeteria wastewater did not obviously affect the carbohydrate and protein synthesis in the microalgal cells. The slightly lower protein content in the biomass harvested from the untreated cafeteria wastewater could be attributed to the much higher indigenous bacterial density in the raw cafeteria wastewater compete N with *Chlorella* sp., leading to lower N availability for protein synthesis by *Chlorella* sp.

The lipid content and fatty acid profiles in microalgal biomass are critical indicators for the potential evaluation when serving as feedstock for biodiesel production. As shown in Fig. 5 and Table 2, the total lipid and fatty acid contents in the harvested biomass in all treatments were 86.96–188.98 and 17.34–23.58 mg·g^{-1}, respectively. The fatty acid contents in the biomass of the current study (less than 7%) were lower (17.2%–19.8%) than that achieved by Li [35] and da Rosa et al. [36], who used human urine as medium to cultivate *Chlorella* sp. The difference could be due to the microalgal species used in the current work being a wild one. However, higher lipids and fatty acid contents were detected in the microalgal biomass harvested from the treatment of 2% NaClO, with 35% increase in fatty acids and

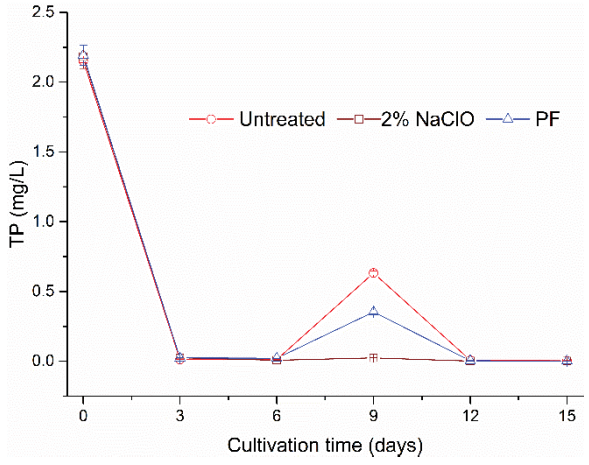

Fig. 4. Variation of total phosphorus during incubation.

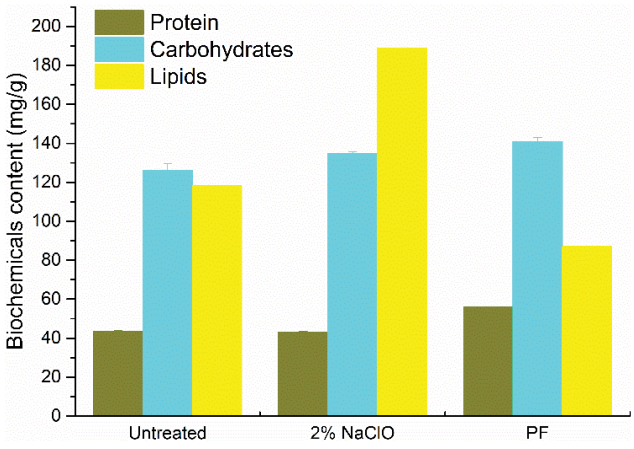

Fig. 5. Biochemical distribution of harvested biomass.

Table 2
Fatty acid composition of harvested microalgae cultivated in cafeteria wastewater pre-treated by different approaches

Components	Treatments		
	Untreated	2% NaClO	PF
		$mg \cdot g^{-1}$	
C14:0	0.12 ± 0.04	0.06 ± 0.01	0.05 ± 0.01
C16:0	6.82 ± 0.38	8.91 ± 1.54	7.07 ± 1.40
C16:1	0.46 ± 0.11	0.21 ± 0.01	0.17 ± 0.06
C18:0	2.88 ± 0.50	2.95 ± 0.47	2.64 ± 0.32
C18:1	6.03 ± 0.45	9.07 ± 1.32	7.29 ± 1.57
C18:3	0.87 ± 0.69	2.20 ± 0.23	1.75 ± 0.54
C20:1	0.17 ± 0.00	0.19 ± 0.01	0.14 ± 0.04
Total fatty acid esters ($mg \cdot g^{-1}$)	17.34 ± 1.12	23.58 ± 3.54	19.10 ± 3.30
Saturated fatty acid (%)	56.68 ± 3.16	50.47 ± 0.96	51.36 ± 3.14
Unsaturated fatty acid (%)	43.32 ± 3.16	49.53 ± 0.96	48.64 ± 3.14

esters. Furthermore, higher unsaturated fatty acids (49.53%, 48.64% and 43.32% for 2% NaClO, PF and untreated, respectively) were recorded in the microalgae cultivated in the disinfected cafeteria wastewater. These results were well in line with those reported by Li et al. [37], who confirmed that co-culture of microalgae and bacteria markedly increased the ratio of saturated/unsaturated fatty acids in microalgae. In addition, the fatty acid components in the harvested biomass were dominated by C16 to C18 methyl ester. Specifically, palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1) accounted for more than 90% of the total fatty acids in the biomass, indicating great potential as feedstock for biodiesel production. More interestingly, the quantity of palmitic acid and stearic acid indicated better fuel property and even ignition [38]. In the C18:1 distributions were 34.78%, 38.46% and 38.17% for untreated, 2% NaClO and PF, respectively, indicating higher oxidative stability for longer storage and lower cold filter plugging point [39]. Finally, a low distribution of C18:3 was observed, with values of 5.02%, 9.33% and 9.16% in the biomass obtained in untreated, 2% NaClO and PF, respectively. This finding complies well with the European Standard EN 1424 for biodiesel, in which the maximum limit of C18:3 is 12%. Therefore, the harvested microalgal biomass cultivated in the disinfected cafeteria wastewater has great potential for biodiesel production.

4. Conclusions

The results showed significantly better growth of *Chlorella* sp. in the cafeteria wastewater pre-treated using NaClO and PF. Disinfection of the indigenous bacteria in the cafeteria wastewater did not reduce the COD, TN and TP RPs. Furthermore, an increase in total carbohydrate, protein and fatty acid contents was achieved in the biomass harvested from the disinfected cafeteria wastewater. These results indicated that coupling biodiesel production and nutrient bioremediation by cultivating *Chlorella* sp. in NaClO-disinfected cafeteria wastewater is technically feasible. Disinfection of wastewater-borne bacteria is a promising approach to increasing the fatty acid productivity through *Chlorella* sp. biomass production, which could facilitate reduction in microalgae-based biodiesel production cost. However, the long-time effect of disinfection approaches on the growth of *Chlorella* sp. and bacteria remains to be clarified and techno-economic issues in scaling up of the proposed process must be thoroughly evaluated in the subsequent work.

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