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Research article

Freshwater microalgae-based wastewater treatment under abiotic stress

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Abstract: Wastewater treatment by microalgae is an eco-friendly and sustainable method for pollutant removal and biomass generation. Microalgae production under abiotic stress (such as salinity/salt stress) has an impact on nutrient removal and fatty acid accumulation. In this study, a freshwater microalgal strain (*Desmodesmus communis* GEEL-12) was cultured in municipal wastewater with various NaCl concentrations (ranging from 25–150 mM). The growth kinetics and morphological changes of the microalgae were observed. The nutrient removal, salinity change, fatty acid composition, and biodiesel quality under various groups were also investigated. The maximum growth of *D. communis* GEEL-12 was observed in the control group at 0.48 OD_{680nm}. The growth inhibition was observed under high salt conditions (150 mM), which showed poor tolerance with 0.15 OD_{680nm}. The nitrogen (N) and phosphorus (P) removal significantly decreased from 99–81% and 5.0–5.9% upon the addition of 100–150 mM salt, respectively. Palmitic acid (C16:0) and stearic acid (C18:0) were the most common fatty acid profiles. The abundance of C18:0 enhanced from 49.37%–56.87% in *D. communis* GEEL-12 upon high NaCl concentrations (100–150 mM). The biodiesel quality index of *D. communis* GEEL-12 under 50–75 mM salt concentrations reached the levels advised by international standards.

Keywords: microalgae; salinity; wastewater treatment; nutrient removal; biodiesel

1. Introduction

The salt-containing wastewater is mainly released from living activities, raw wastewater, and agents added in the wastewater treatment processes [1]. Wastewater rich in salt tremendously pollutes the soil and groundwater environment, causing soil salinization and destroying groundwater quality [2]. The salt-rich wastewater generally increases the nitrogen (N), phosphorus (P), and heavy metals content in water [3]. The physical and chemical techniques (including electrodialysis, incineration, evaporation, and membrane separation) have been largely deployed for salt-rich wastewater treatment [4]. The major drawbacks of these techniques include expensive equipment investments, high running costs, secondary contamination, and challenges with large-scale practical implementation [5,6]. The biological methods (such as intermittent activated sludge process, membrane bioreactor process, anaerobic process, and Anoxic/Oxic (A/O) process) can reduce the challenges associated with the aforementioned processes [6]. However, an excessive salt concentration hinders the metabolic activity of microbes and drifts the microbial community, which significantly reduces the ability of microbes to treat wastewater [7,8].

Microalgae is a potential candidate for wastewater treatment due to its ability to remove N and P, inorganic or organic carbon, and other elements from water [9]. Utilization of microalgae in sewage treatment has several advantages over conventional methods, including low cost, reduction in greenhouse gas emissions, decrease in salt concentration, and removal of heavy metals [10]. Microalgae cultivation combined with wastewater treatment has many advantages in the production of biofuels. However, in such a conjunction, much efforts are still remains to be directed to accelerate wastewater treatment. Thus, it is necessary to screen the various microalgal strains with a high rate of growth and effective N, and P removal efficiencies for wastewater treatment and biofuel production [11,12]. The abiotic stress (such as salt) to the microalgae can effectively enhance biomass productivity and N, P removal [13]. Salinity has been reported to increase ionic transport through nutrient (N and P) uptake for osmotic regulation of microalgae cells [14]. Salinity also up-regulated the expression of genes encoding reductase, dehydrogenase, transferase, and carboxylase, which can enhance the degradation of organic pollutants by microalgae [15]. Therefore, this study was designed for the cultivation of microalgae in municipal wastewater with varying salt contents to observe their growth. The microalgal growth kinetics and specific growth rate were evaluated. The changes in the salinity and TN, and TP removal were detected to provide scientific support for the biological treatment of saline wastewater from microalgae. The fatty acids composition with biodiesel quality estimation of harvested biomass was also investigated.

2. Materials and methods

2.1. Wastewater collection and characterization

The municipal wastewater was collected from YanChang Wastewater Treatment Plant (Lanzhou China Railway Water Co., Ltd.) (103.86° E and 36.08° N) in Lanzhou City, China. The physicochemical properties of wastewater (including pH, salinity, total dissolved solids, conductivity, TN, TP, and COD) were performed according to the previous protocols [16].

2.2. Experimental methods

The freshwater microalga *Desmodesmus communis* GEEL-12 (Accession number: MZ219090) was selected from previously isolated microalgae strains based on the high growth and N and P removal [16]. A 250 mL conical flask was filled with 200 mL of filtered wastewater followed by the addition of various doses of NaCl (Control, 25, 50, 75, 100, 125, 150 mM). The initial inoculum of *D. communis* GEEL-12 was adjusted to 1.5 OD_{680nm} and 2 mL was added into each conical flask. All the flasks were placed in the shaker under a temperature of 25±2 °C, light intensity of 40 µmol m^{-2} s^{-1}, and rotation speed of 150 rpm for 12 days. The morphology and appearance of microalgae growing in various salinities were studied under an optical microscope (oil immersion lens, 100 ×) after the experiment and the salinity concentration in the wastewater culture media was determined.

UV 5500, Metash, a spectrophotometer was used to detect the microalgal growth optical density at 680_{nm} on alternative days The specific growth rate (µ) was calculated by Eq. (1):

$$\mu=(lnX_1-lnX_0)/(t_1-t_0) \tag{1}$$

Where X_1=final biomass concentration, X_0=initial biomass concentration, t_1=final time, t_0=initial time.

The amount of N and P removal in wastewater was measured after every 4th day. Removal of nutrients was calculated by Eq. (2):

$$W(\%)=(W_0-W_1)/W_0 \times100 \tag{2}$$

Where W_0 and W_1 represent the initial and final nutrient concentrations.

2.3. Quality estimation

The methanol-chloroform was employed to extract lipids from the harvested dried 40 mg microalgal biomass [17]. The Lepage and Roy's method was applied for the transesterification of extracted lipids for analyzing fatty acid composition and biodiesel quality estimation [18]. 1 mL methanol and 0.3 mL H_2SO_4 were added to 1 mL of the extracted crude lipid layer. Following 3–5 mins of vortexing, the mixture was incubated at 100 °C for 10 mins. Then 1 mL of dH_2O was added, vortexed for 3–5 mins, and then centrifuged at 4000 rpm for 10 mins. The separated layer was collected in a black glass vial. The gas chromatography was operated based on the conditions mentioned previously [16]. Based on the FAMEs profile, the following equations (3–8) were used to compute the properties of the biodiesel:

$$DU=\sum[MUFA+(2\times PUFA)] \tag{3}$$

$$SV=\sum[(560\times N\%)/M] \tag{4}$$

$$IV=\sum[(254\times N\%\times D)/M] \tag{5}$$

$$CN=46.3+(5458/SV)-(0.225\times IV) \tag{6}$$

$$LCSF=(0.1\times C16:0)+(0.5\times C18:0)+(1\times C20:0)+(1.5\times C22:0)+(2\times C24:0) \tag{7}$$

$$CFPP=(3.1417\times LCSF)-16.477 \tag{8}$$

where MUFA and PUFA represent mono-unsaturated and poly-unsaturated fatty acids, DU is degree of unsaturation (%), SV demonstrates the saponification value (mg KOH g^{-1}), N% is each type of fatty acid, M is the molecular weight of the fatty acid, IV represents iodine value (g I$_2$/100 g oil), D demonstrates the number of double bonds, CN is the cetane number, LCSF is the long-chain saturation factor, and C16:0, C18:0, C20:0, C22:0, and C24:0 represent the weight percentage of the corresponding fatty acids, CFPP stands for cold filter plugging points (°C) [19,20].

2.4. Statistical analysis

All experiments were performed in triplicate and data are expressed as mean ± standard deviation. Graphpad 8.0 was used to draw the data. The statistical software SPSS 26.0 was used to inspect differences in the experimental data. The significant difference level was set at $p < 0.05$ confidence level.

3. Results and discussion

3.1. Physicochemical parameters of collected wastewater

The appearance of the collected wastewater sample was cloudy having a foul smell with a pH of 6.94. The initial salt concentration in wastewater was 251.33 mg L^{-1}. The total organic solid, conductivity, and total suspended solid were 351.67 ng L^{-1}, 516.33 µS cm^{-1}, and 0.91 g L^{-1}, respectively. The COD, TN, and TP of the collected wastewater were 45.65, 12.14, and 3.19 mg L^{-1}, respectively.

3.2. Growth kinetics of microalgae in saline wastewater

The microalgae growth in salt-rich wastewater was slow during the initial days indicating the adaptation phase, which started to increase after the log phase (Fig. 1a) There was a general drop in OD$_{680nm}$ value when the NaCl concentration increased from 25 mM to 75 mM. The OD$_{680nm}$ variations between the control group and the treatment groups in *D. communis* GEEL-12 cultures of saline wastewater increased rapidly starting on the 4th day of culture. The control group achieved the highest growth on the 12th day (0.48 OD$_{680nm}$), while the microalgal growth in the highest salt treatment (150 mM) group was 0.15 OD$_{680nm}$. The low concentration of salt had no appreciable impact on the development of GEEL-12. However, under the high salt conditions, the microalgal growth was severely restricted. The results of this study were similar to the previously isolated strain, indicating that excessive salinity impeded microalgae's normal ability to grow [21].

The specific growth rate (µ) was applied to evaluate the microalgal growth during the cultivation time in the wastewater (Fig. 1b). The growth of microalgae and their specific growth rate depends on the initial inoculation and culture parameters (such as temperature, light, nutrients availability, and pH) [22]. *D. communis* GEEL-12 showed the highest µ value for all treatment groups on day 2. The addition of 25 and 75 mM NaCl decreased the specific growth rate owing to increased salinity and nutrient depletion during its rapid growth phase. The specific growth rate of GEEL-12 remained essentially unchanged from the 6th to the last day of culture at high salinity conditions (100–150 mM), indicating that the growth of microalgae was sluggish and hindered under high NaCl. The high salt concentration could exceed the osmotic pressure which will act on the Na$^+$

and Cl$^-$ pumps on the cell membrane, resulting in cell destruction, and the accumulation of reactive oxygen species (ROS) in cells which causes the inhibition of microalgae growth [23,24].

In the first four days of microalgae cultivation, the pH of the saline wastewater increased rapidly (Fig. 1c). The pH of the saline wastewater increased rapidly during the first four days of microalgae cultivation (Fig. 1c). In the subsequent stages, the pH value of the medium varied steadily under different salt concentrations, indicating a gradual growth of the microalgae. The difference in pH value between the treatment groups with different salt concentrations was significant, especially in the control and 100–150 mM treatment groups. The photosynthesis of microalgae will absorb CO_2 and H$^+$ from outside the cell and simultaneously release OH$^-$ which results in enhanced pH [25]. The high salt concentration of 100 mM or higher decreased the pH of the wastewater showing that the increased salinity impeded the development of the microalgae and reduced photosynthesis. The salt tolerance capacity of microalgae highly depends on species level and the growth of microalgae depends upon the salt concentration [26].
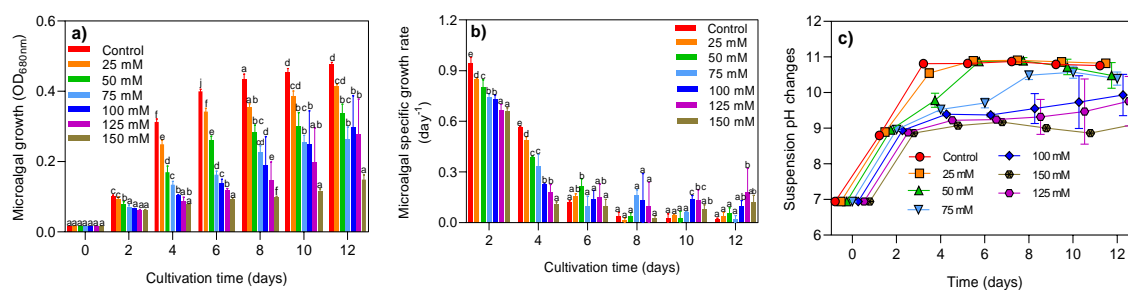

Figure 1. The microalgal growth (a), specific growth rate (b), and pH (c) of *D. communis* GEEL-12 in wastewater under different salinity concentrations.

3.3. N and P removal under salinity stress

The 12.14 mg L^{-1} content of N was found in wastewater used in this study. The content of N was significantly removed after 12 days under various salinity stress concentrations (Fig. 2). The 99% removal of N was observed in the control group, while 81% N was significantly removed under high salt concentration (150 mM) in wastewater. The slow progress of microalgal cells under high salt concentration might be attributed to the reduced N removal rate compared to the control group. The effect of salt concentration might vary according to species type. The *Chlorella* sp. removed 95% of the nitrate nitrogen (NO_3-N) in synthetic media under 1% salt concentration within 2 days, demonstrating strong N removal efficiency [27]. The removal of N in saline wastewater is significantly influenced by the specific type of microalgae. The N removal effect of Chlorella spp. was reported positively connected with high salt concentration [28]. In contrast, *D. communis* GEEL-12 had the opposite removal effect under high salt conditions, demonstrating the importance of the selection of the microalgal strains.

The wastewater had an initial P content of 3.19 mg L^{-1}. The P removal under high salt concentration was low. When the NaCl was between 100 and 150 mM, P was substantially less uptake via microalgae. The microalgae were able to reduce 5.9% of P under high salt conditions (150 mM), suggesting the potential of *D. communis* GEEL-12 for P removal under salinity stress. The results of this study were comparable to the previously disclosed microalgae strain *Chlamydomonas* sp. JSC4 [29]. In this study, microalgae could rapidly take P from wastewater under low salt

concentration (≤75 mM). N and P can be used by microalgae to regulate metabolic processes and growth [30]. A study reported enhanced biomass productivity with high N and P removal during the cultivation of microalgae in wastewater [31]. The wastewater treatment by microalgae and the generation of fatty acids under abiotic stress are currently receiving attention due to dual applications (wastewater treatment and biomass productivity) [12,32].
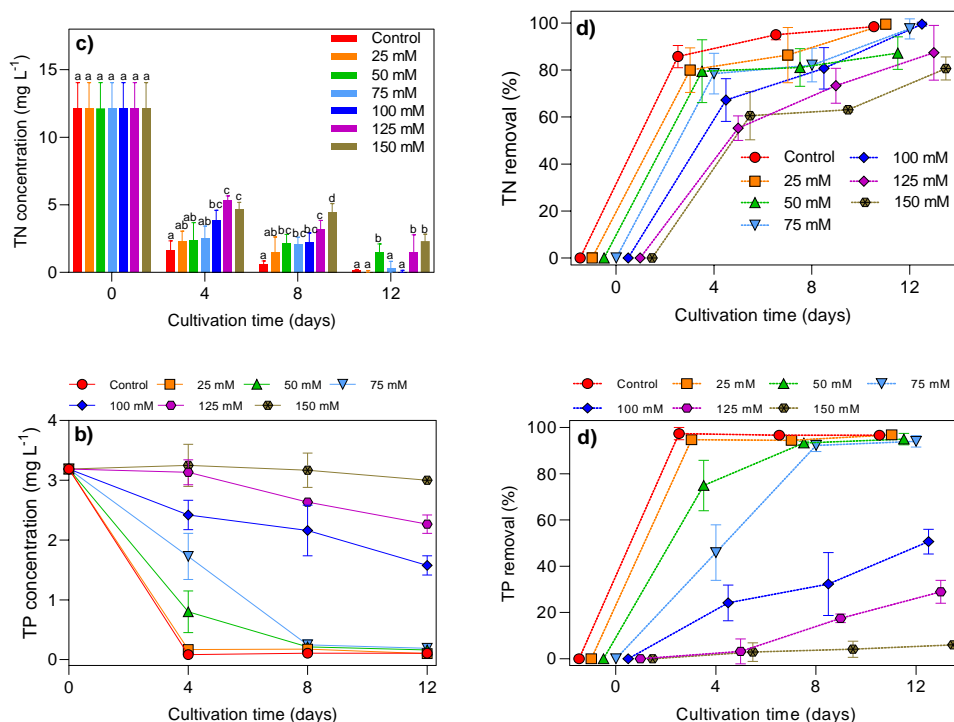
Figure 2. Removal of nutrients in wastewater with different salinity concentrations. The concentration and removal of N (a and b) and the concentration and removal of P (c and d).

3.4. Morphological variations

The morphological traits of *D. communis* GEEL-12 after 12 days of culture at different NaCl concentrations are displayed in Figure 3. Under the NaCl concentration below 50 Mm, the appearance features of the microalgal cells were observed with whole flagella. The cells developed some vacuolar structures as a result of the salt stress. The volume of the cell also grew along with the salinity (75 and 125 mM), and the flagella were disorganized, showing that *D. communis* GEEL-12 could still adapt to the salt concentration. The microalgal strain died upon high salt addition (150 mM) as the volume of the microalgal cells reduced and the shape and structure changed drastically.

The high salinity can affect the morphological structure of microalgae cells and cause the secretion of substances, such as extracellular polysaccharides (EPS), which protected the integrity of their structures [33,34]. In some previous studies, the microalgal cell development was inhibited and cell degeneration was observed upon excessive salt concentration [33,35]. *D. communis* GEEL-12's cell structure has also changed upon high loading of NaCl in wastewater.
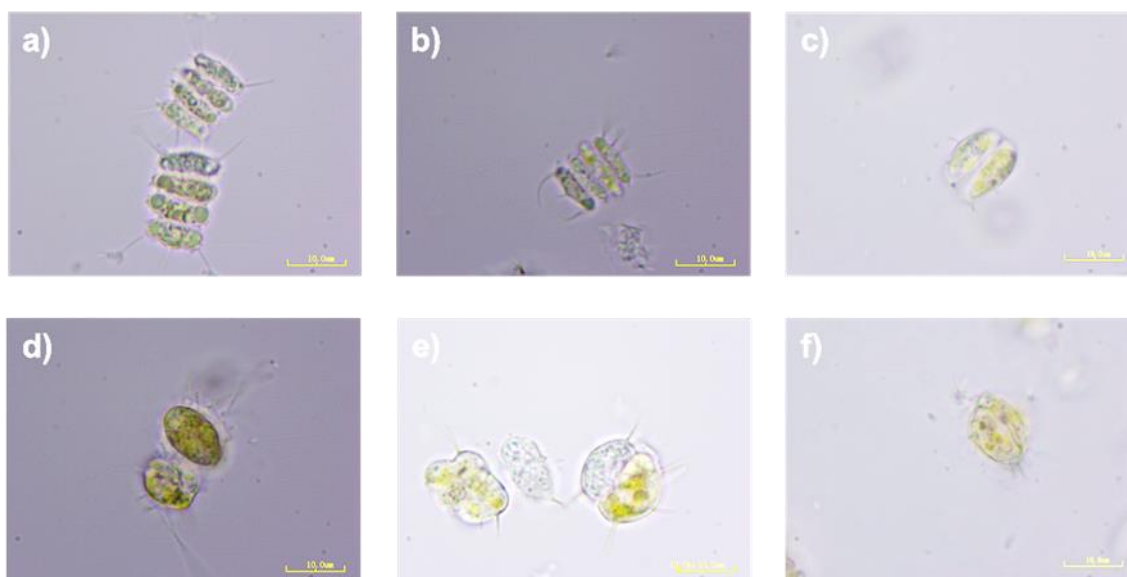
Figure 3. Removal of nutrients in wastewater with different salinity concentrations. The concentration and removal of N (a and b) and the concentration and removal of P (c and d).

3.5. Salinity variation and fatty acid composition

The salt concentration in all the groups decreased significantly within 12 days. The salt stress causes a reduction in microalgal growth and decreases chlorophyll content, photosynthesis inhibition, and oxidative damage [28]. In this study, the reduction in the salt concentration might be attributed to the utilization or adaptation of microalgal cells under salt stress. A significant reduction was observed even under high salt concentrations, indicating the potential of microalgal strain toward salt stress. The findings discussed above demonstrated that microalgae may reduce the amount of salt in the environment and increase their tolerance to salt through associated mechanisms, opening the possibility that they can be utilized to treat saline wastewater.

The fatty acid composition of microalgae is also a crucial indicator for assessing the quality of microalgal biofuel [36]. The physical-chemical characteristics of microalgae, the elements of culture medium, temperature, light, and nutrient quantity and proportion are considered to influence the fatty acid composition of microalgae [33]. Salinity stress increases the ROS in the microalgae, which further causes oxidative stress [37]. To combat the ROS accumulation, the microalgal cells express lipid production genes and upregulate the lipid biosynthesis pathway which mainly forms higher neutral lipids [38]. Thus, the proportion of saturated fatty acids production is comparatively higher than polyunsaturated fatty acids under salinity stress which also indicates high biodiesel quality [39]. *D. communis* GEEL-12 exhibited a 98.10% accumulation of saturated fatty acids in BBM media [16]. The saturated fatty acid content was less than its saturation in the BBM medium (98.10%). Similarly, studies have demonstrated that during salt stress, the eicosapentaenoic acid (EPA) of *C. vulgaris* increased [21]. These findings show that salt stress may be utilized to control microalgal development and produce microalgal products with high fatty acid content.

Palmitic acid (C16:0) and stearic acid (C18:0) were the predominant fatty acids when cultivation occurred in saline wastewater (Fig. 4). The content of C16-C18 is often used to evaluate the yield of biodiesel owing to their degree of saturation [40]. The amount of C16:0 in the fatty acid fraction of *D. communis* GEEL-12 was reasonably high when the NaCl concentration was <75 mM. However, the amount of C18:0 significantly increased from 49.37%–56.87% under 100–150 mM salt concentration.
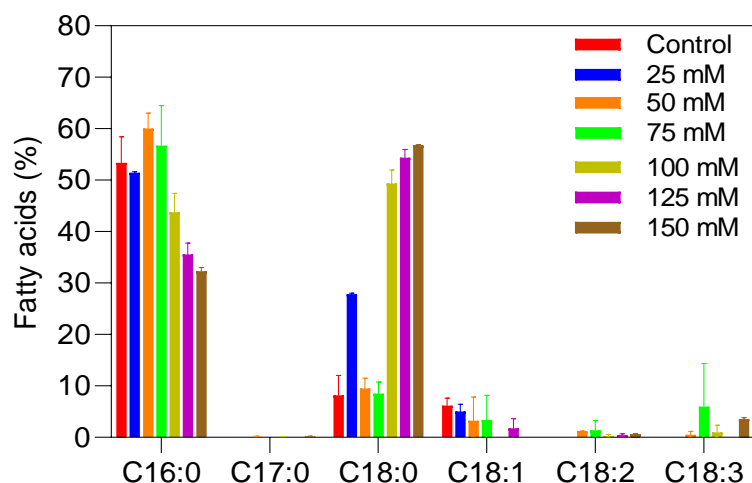

Figure 4. The main fatty acid components of *D. communis* GEEL-12 are cultured in wastewater with different salinity concentrations.

3.6. Microalgal-derived biodiesel quality

The quality of biodiesel produced from microalgal biomass was evaluated using fatty acid methyl esters (FAMEs). The main metrics used in the present investigation were unsaturation degree (UD), saponification value (SV), iodine value (IV), cetane number (CN), long-chain saturation factor (LCSF), and cold filter plugging point (CFPP). Fatty acid structure and content have a simple impact on SV, IV, and LCSF. The emission of toxic gases (such as nitrogen oxide (NOx), and carbon monoxide) and low hydrocarbons, is due to high UD values of the microalgal-derived biodiesel [41].

The IV represents the content of unsaturated FAs in microalgae. The biodiesel CN is also directly related to LCSF, depending on the length of saturated FAs [42]. However, LCSF has a negative impact on CFPP as long-chain FAs have a higher temperature of precipitation than short-chain FAs. The CFPP is a crucial indicator for determining if the fuel is appropriate for use in cold climates, and higher CFPP values of biodiesel result in problems in filter plugging and the flow of fuels [43]. The CFPP values were 17.30 and 14.73, respectively, when *D. communis* GEEL-12 was cultivated in 50 mM and 75 mM saline wastewater, which were significantly lower than those of other treatments. The results of this study indicated that the microalgal strain *D. communis* GEEL-12 can be deployed as a source of biofuels since all the biodiesel quality falls near to the standard ($\leq$–5/$\leq$–20).

4. Conclusions

The microalgal strain *D. communis* GEEL-12 exhibited favorable outcomes under low NaCl stress (25–75 mM), however, excessive concentration (100–150 mM) restricted the growth. The nutrient (N and P) removal was decreased (>81%, >5.9%) due to microalgal failure to acclimatize at higher salinity range. The cell structure shrank and normal cell structure was also disrupted under high salinity stress (150 mM). The salinity in each treatment group drastically decreased after 12 days. The *D. communis* GEEL-12 strain exhibited a significant amount of saturated fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0). Moreover, the biodiesel quality indicators showed favorable results under 50–75 mM salt content. The high salinity removal and high fatty acid content make it a viable option for use as feedstock for biofuel production and to treat high-salt wastewater. However, to ensure the economic viability of microalgae-based wastewater treatment at a commercial scale, there is a requisite to explore high salt tolerant microalgal species. The extensive research on synergistic treatment methods that integrate microalgae to improve the performance of wastewater bioremediation is also required.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

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