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Research Article

Cultivation of Mixed Microalgae Using Municipal Wastewater: Biomass Productivity, Nutrient Removal, and Biochemical Content

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Background: Microalgal biotechnology has gained much attention previously. Monoculture algae cultivation has been carried out extensively in the last decades. However, although the mixed microalgae cultivation has some advantageous over pure cultures, there is still a lack of knowledge about the performance of mixed cultures.

Objective: In this study, it has been tried to investigate all growth aspects of marine and freshwater microalgal species in a mixed culture and their biological effects on biomass growth and composition based on wastewater nutrient consumption.

Material and Methods: Three algal species of *Chlorella vulgaris, Scenedesmus obliquus,* and *Nannochloropsis* sp. were cultivated in saline wastewater individually, then the effects of mixing the three strains on biomass productivity, nutrient removal efficiency, chlorophyll, carotenoid, and lipid content were investigated.

Results: The obtained results revealed that the mixed culture of three strains showed the highest biomass productivity of 191 mg. $L^{-1}.d^{-1}$. Also, while there were no significant differences between the performance of mono and mixed culture of algal species in the removal efficiency of wastewater nutrients, the three-strain microalgal mixed culture showed the highest values of 3.5 mg.$L^{-1}.d^{-1}$ and 5.75 mg.$L^{-1}.d^{-1}$ in the removal rate of phosphate and nitrate, respectively. In terms of total chlorophyll and carotenoid per produced biomass, however, the mixed culture of three species showed the lowest values of 4.08 and 0.6 mg. g $biomass^{-1}$, respectively.

Conclusions: The finding proves the potential of attractive and economically feasible mixed microalgae cultivation for high percentage nutrient removal and microalgal biomass production.

Keywords: Biomass production; Mixed microalgae; Nutrient removal; Saline wastewater.

1. Background

The more population of the world and, consequently, the more demand for energy in the next years will result in a higher cost of energy (1). Biofuels are a sustainable source of energy and offer pragmatic solutions to the world's growing energy problems. Recent studies have demonstrated that microalgae can be the best candidate to be used as a biofuel feedstock (2-4). Microalgal biotechnology has gained much attention during the last decades because different microalgal species diminish vast quantities of carbon dioxide and greenhouse gas emissions through the photosynthesis process (5). Although the cultivation of algae is technically viable, there are economic obstacles. Limitations for mass production of algae include: 1-nutrient supply in this process could be expensive, 2- contamination with other microorganisms, especially bacteria, often exists in large-scale algal cultivation, 3- downstream process demand nearly 30% of the whole investment, and 4- a large amount of water is required for the cultivation of microalgae (6).

Water is a necessary resource for algal culture. Depending on species, microalgae can be grown in freshwater, including domestic wastewater, brackish water, and seawater. Existing studies of microalgae using freshwater media are of little relevance to any future large-scale production of algal oils for fuels (7). This is because fresh water is in short supply globally and its use for producing microalgae will undoubtedly compete with its existing applications in the production

of food and fodder (8). Biological contamination has also become a considerable restraint in massive microalgal cultivation in freshwater culture media, especially when cultured in wastewater (9-11). Shen *et al.* (11) reported that the cultivation of *S.obliquus* in real wastewater was challenging mainly because of the inevitable contamination of bacteria. The concept of using saline wastewaters as a culture medium for the cultivation of microalgae can be a wise decision with benefits for both the environment and energy sector (6, 12). Various types of wastewater such as industrial, municipal, agricultural, and anaerobic digestion effluent, have been studied previously for microalgae cultivation (13-16). Municipal wastewater contains necessary nutrients for microalgae cultivation such as N-ammonium, N-nitrate, and P- phosphate (17). Secondary treated wastewater has been used for the cultivation of microalgae since the 1970s due to the lower amount of organic carbon in the effluent of the second stage and, therefore, the lower likely contamination of bacterial microorganisms.

Chlorella vulgaris and *Scenedesmus obliquus* grown in freshwater media have been used for different applications but are not still economically viable for mass production. Extensive studies on freshwater cultivation of *C.vulgaris* and *S.obliquus* have been reported (18-21), but there are insufficient investigations on brackish media. However, in a study carried out by Luangpipat and Chisti (22), it was shown that microalgae *C.vulgaris* could grow exceptionally well in saline water with 20 g. L^{-1} NaCl. Their study also demonstrated that the biomass growth rate of both freshwater algal species of *C.vulgaris* and *S.obliquus* did not differ significantly in brackish water compared with freshwater. Meanwhile, there have been few reports on the evaluation of the performance of marine microalgae *Nannochloropsis* sp. in urban wastewater (23, 24). Marine microalgae *Nannochloropsis* are capable of being used industrially due to their ability to accumulate high levels of polyunsaturated fatty acids and also lipid up to 60-70% of their dry cell weight in the nitrogen depletion conditions (23).

Pure culture algae cultivation has been carried out extensively in the last decades. However, pure culture may enhance the risk of contamination by existing unintentional non-target algae (competitors) or grazers (predators) which may, then, lead to a decrease in biomass productivity as well as decrease the economic viability of algal biofuels (25). In this regard, the risk of contamination even in the saline environments is high in which the freshwater algal species that are tolerable to salinity may be able to grow in part of the cultivation period and reduce the overall efficiency of the system**.** On the other hand, mixed culture biotechnology has some advantages over pure culture. For example, microbial diversity in most cases cause an adaptive condition in which there wouldn't be any severe concerns about contamination, and also there is more possibility of a continuous process (13, 16, 26, 27). However, there is a gap of knowledge about the feasibility of integrating the cultivation of freshwater and marine algal species in a mixed culture with the treatment of saline wastewaters.

2. Objective

This study aims to investigate the performance (including biomass growth, nutrient removal rate, specific nutrient consumption rate, lipid, chlorophyll, and carotenoid content) of freshwater (*C.vulgaris* and *S.obliquus*) and marine (*Nannochloropsis* sp.) mixed microalgae with salinity as well as competition stress. This research is novel since these algal species have not been utilized in mixed culture in order to treat saline wastewaters. It can also contribute to the sustainable development of the algal biomass industry.

3. Material and Methods

3.1. Microalgae Strains and Algal Cultivation

The studied microalgae strain *C.vulgaris* (INACC 50026), *S.obliquus* (INACC 50028), and *Nannochloropsis* sp. (INACC 50024) were purchased from Iranian National Algae Culture Collection (INACC), Tehran, Iran. Algal cells were cultivated in a flat plate photobioreactor (PBR) with a working volume of 1 L at 10 % concentration ($V_{inoculum}/V_{wastewater}$) for 7 days to reach the stationary phase. Also, a high-pressure sodium lamp (Osram 250W, DAYLIGHT E40, Germany) provided monotonic light irradiance of 104 µmole. m^{-2}. s^{-1}. The air was fed to the PBR through a porous sparger with an airflow rate of 0.5 vvm, which was placed close by to the base of the PBR.

3.2. Wastewater Collection and Analysis

The secondary treated urban wastewater was collected from Ekbatan Wastewater Treatment Plant, Tehran, Iran, filtered with a 0.2µm filter (Accu-Jet pro filter BR 26530 India) to remove suspended particles, autoclaved at 121°c for 15 min to remove any biological contamination, and stored at 4 °c in the refrigerator. The collected wastewater characteristics are summarized in supplementary data. The amounts of NO_3-N and PO_4-P were determined using the spectrophotometric standard method for the examination of water and wastewater (28). In order to

measure the nutrient content of the culture, 15 mL of the daily effluents centrifuged at 4000 rpm for 10 min and filtered with a 0.45 µm filter to prevent microalgal contribution in the analysis. 1 ml HCl 1 N was added to 5 mL of the filtered sample, mixed thoroughly, and its absorbance was read at an optical density (OD) = 220 nm and OD = 275 nm. Then, the nitrate (NO_3^-) content of the liquid was measured from the calibration curve. Besides, in order to measure the phosphate (PO_4^{-3}) content of the liquid, 0.2 mL ammonium molybdate ($(NH_4)_6Mo_7O_{24}$) solution was added to the 5 mL of the filtered sample. While it was mixing thoroughly in a tube, 20 µL stannous chloride ($SnCl_2$) solution was added to the tube, and after 8 min, its absorbance was read at OD = 650 nm. The phosphate content of the liquid was measured from the calibration curve.

3.3. Analytical Methods

3.3.1. Biomass Growth

Biomass concentration was measured daily by reading OD at 680nm using a UV-spectrophotometer (Vis 2100 Unico China/USA). The biomass growth curve was determined from the calibration between absorbance and the weighted dry biomass (29). The biomass growth rate has been measured by the following equation:

$$\mu\ (d^{-1}) = \frac{\mathbf{Ln\,C - LnC_0}}{\mathbf{t - t_0}} \quad (1)$$

Where C and C_0 are the biomass concentration at the beginning and the end of the biomass log phase, respectively. t-t_0 is the period of the log growth phase (30). To calculate volumetric biomass productivity, which represents the produced biomass per day and the reactor volume unit Equation2 has been used.

$$\mathbf{P\ (g.L^{-1}.d^{-1})} = \frac{\mathbf{X_2 - X_1}}{\mathbf{t - t_0}} \quad (2)$$

Where P signifies the biomass productivity, X_2 and X_1 represent the biomass concentration at the end and on the first day of the cultivation, and t-t_0 is the period of the growth phase.

3.3.2. Calculation of Culture Kinetic Parameters

The nutrient removal rate (Ri) was measured using Equation 3 (31):

$$\mathbf{Ri\ (mg.L^{-1}.d^{-1})} = -\frac{\mathbf{S_0 - S}}{\mathbf{t_0 - t}} \quad (3)$$

Where S_0 and S represent the initial and final nutrient concentration, respectively, and t is the time when S consumed utterly. Thus, the specific nutrient consumption rate was calculated as Rx_i = Ri/ (the concentration of biomass in which S consumed completely) (31).

The biomass yield on phosphate ($Y_{X/P}$, $mg.mg^{-1}$) was calculated using the following equation (8):

$$\mathbf{Y_{X/P}} = \frac{X_f - X_i}{P_i - P_f} \quad (4)$$

In the above equation, X_i represents the initial biomass concentration, and X_f signified the biomass concentration when phosphate consumed utterly.

The biomass yield on nitrate ($Y_{X/N}$, $mg.mg^{-1}$) was calculated using the following equation (8):

$$\mathbf{Y_{X/N}} = \frac{X_f - X_i}{N_i - N_f} \quad (5)$$

Where X_i is the initial biomass concentration, and X_f is the biomass concentration when nitrate consumed utterly.

3.4. Biochemical Composition of Microalgal Biomass

Lipid content was assessed using the modified method of Bligh and Dyer (32). Lipid was extracted from the microalgal biomass using a mixture of water, methanol, and chloroform. Lipid productivity was calculated by Equation 4 that was formerly proposed by (33).

$$\mathbf{Lipid\ productivity(mg.\ L^{-1}.d^{-1})} = \text{biomass productivity}(mg.L^{-1}.d^{-1}) \times \text{lipid content (\%)} \quad (6)$$

Lichtenthaler and Wellburn (34) proposed a method for measurement of chlorophyll. In this regard, 2 mL of microalgal suspension was centrifuged at 4000 rpm for 15 min; the supernatant was removed, then 2 mL of methanol was added to the microtube and put it in the refrigerator (4 °C) for 24 h. After that,the chlorophyll content was calculated by measuring the absorbance according to the following equations:

$$\mathbf{Chl_a\ (mg.\ L^{-1})} = 15.65 \times OD_{666} - 7.34 \times OD_{653} \quad (7)$$

$$\mathbf{Chl_b\ (mg.\ L^{-1})} = 27.05 \times OD_{653} - 11.21 \times OD_{666} \quad (8)$$

$$\mathbf{Carotenoid\ (mg.L^{-1})} = \frac{1000 \times OD_{470} - 2.27 \times Ch_a - 129.2 \times Ch_b}{221} \quad (9)$$

3.5. Microscopic Analysis

Cell counting through microscopic analysis using a hemocytometer (Sigma-Aldrich BR717805-1EA) was carried out two times in the whole period of each experiment in order to monitor relative algal dominance in the mixed culture (at the beginning and end of the cultivation period).

3.6. Statistical Analysis

All the experiments were conducted in triplicate, and the average values have been reported. Also, in order to assess the significance of the obtained data, a 2-sample t-test has been undertaken using Minitab 18 software.

4. Results

4.1. Environmental Conditions and PBR Operational Parameters

The environmental and operational parameters in the flat panel PBR over the 7 day culture period are shown in **Table 1**. The temperature was almost constant at different experiments and the average value was 32 °C. The average day-time dissolved oxygen was 6.8, 6.7, 6.6, and 6.5, mg.L^{-1}, for *C.vulgaris, S.obliquus, Nannochloropsis* sp., and mixed culture, respectively, which showed that there were no significant differences among different algal species. The pH value was significantly higher in *Nannochloropsis* sp. and mixed culture than *C.vulgaris* and *S.obliquus*.

4.2. Biomass Growth of Microalgal Cells in Wastewater

Growth characteristics of each microalgal species are depicted in **Table 2**. In this regard, the monocultures of algal species of *C.vulgaris, S.obliquus,* and *Nannochloropsis* sp. reached the maximum biomass concentration of 1.05, 0.837, and 1.29 g.L^{-1}, respectively. While the mixed culture of three strains reached the maximum biomass yield of 1.42 g.L^{-1}. Also, as illustrated in **Table 2**, the mixed culture of algal species presented the biomass productivity of 191 mg.L^{-1}. d^{-1}, which showed that biomass productivity was improved by 78%, 45%, and 12% compared with the monocultures of *S.obliquus, C.vulgaris*, and *Nannochloropsis* sp., respectively.

4.3. Algal Abundance

Relative algal dominance in a system is changed by various operational (pH, nutrient content and composition, Hydraulic Retention Time), environmental (light and temperature), and biological (algal pre-adaptation and inoculum, grazers, and parasites) parameters (35, 36). The relative abundance of algal cells in the mixed culture is depicted in **Figure 1**. The cell population of *Nannochloropsis* sp. increased from almost 33% to 48%, while the dominance of freshwater algal species of *C.vulgaris* and *S.obliquus* decreased to 28% and 24%, respectively. This phenomenon may attribute to the fact that environmental condition (especially salinity) was more favorable for *Nannochloropsis* cells to grow than other two algal species.

4.4. Nutrient Removal

Figure 2 shows the nutrient consumption by various microalgal strains. In this regard, all of the algal species consumed all of the available nutrients within the culture

Table 1. Daytime pH, temperature, and dissolved oxygen (DO) in the flat panel photobioreactor (PBR). Data are means ± standard deviation.

Parameter		*C.vulgaris*	*S.obliquus*	*Nannochloropsis* sp.	Mixed
Temperature (c)	Mean ± s.d.	32.0 ± 1.0	32.0 ± 1.4	32.0 ± 1.5	32.0 ± 0.8
DO (mg.L^{-1})	Mean ± s.d.	6.8 ± 0.3	6.7±0.2	6.6 ± 0.1	6.5 ± 0.4
pH	Mean ± s.d.	7.5 ± 0.3	7.6 ± 0.3	8.6 ± 0.5	8.6 ± 0.4

Table 2. Growth characteristics of different algal species. Data are means ± standard deviation.

Species	X_0 * (g.L^{-1})	X_m ** (g.L^{-1})	μ (d^{-1})	biomass productivity (g.L^{-1}. d^{-1})
Chlorella vulgaris	0.13 ± 0.02	1.05 ± 0.12	0.15± 0.02	0.13 ± 0.005
Scenedesmus obliquus	0.09 ± 0.01	0.84 ± 0.15	0.15± 0.03	0.11 ± 0.004
***Nannochloropsis* sp.**	0.10 ± 0.01	1.29 ± 0.2	0.20± 0.04	0.17 ± 0.009
Mixed culture	0.08 ± 0.01	1.42 ± 0.15	0.19± 0.05	0.19 ± 0.012

* biomass concentration at the beginning of cultivation
** Maximum biomass concentration during the cultivation period.

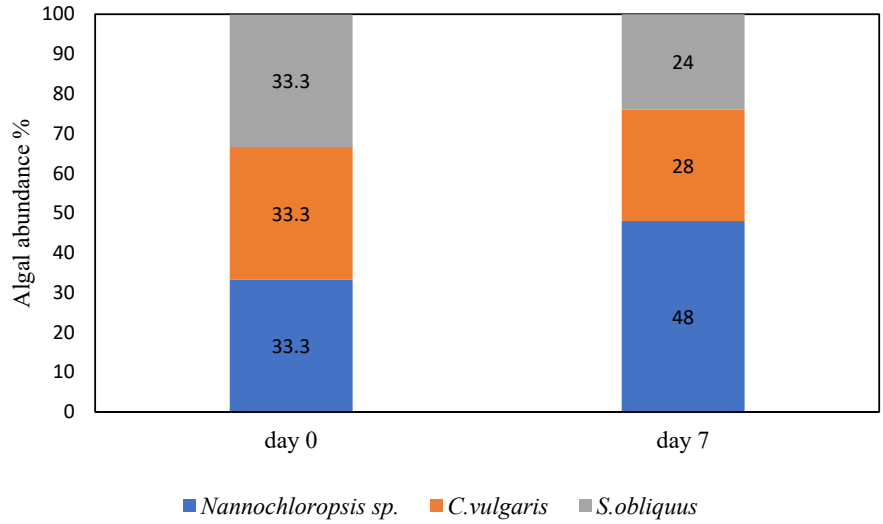

Figure 1. Relative algal abundance in the mixed culture at the first and end of the cultivation period.

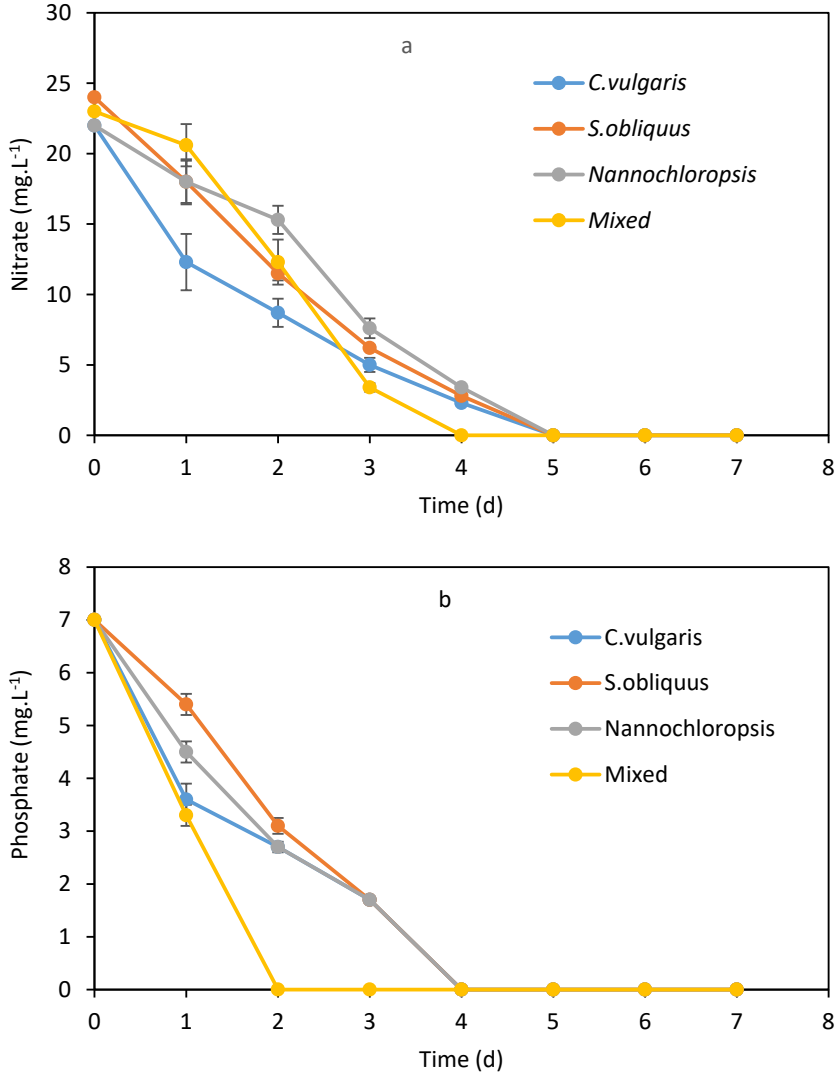

Figure 2. Microalgal uptake of Nitrate- N (a) and Phosphate-P (b) from secondary treated municipal wastewater. Error bars correspond to the standard deviation determined for three independent experiments.

Table 3. Kinetic parameters for the algae cultivation in secondary treated municipal wastewater. Data are means ± standard deviation.

Algal species	removal rate of Phosphate (mg. $L^{-1}.d^{-1}$)	biomass yield on phosphate ($Y_{X/P}$, mg. mg^{-1})	removal rate of Nitrate (mg.L^{-1}. d^{-1})	biomass yield on Nitrate ($Y_{X/N}$, mg. mg^{-1})	specific phosphate consumption rate (mg.g $cell^{-1}.d^{-1}$)	specific nitrate consumption rate (mg.g $cell^{-1}.d^{-1}$)
C.vulgaris	1.75 ± 0.3	0.079 ± 0.008	4.40 ± 0.3	0.028 ± 0.004	2.3 ± 0.3	5.7 ± 0.4
S.obliquus	1.75 ± 0.3	0.065 ± 0.005	4.80 ± 0.2	0.026 ± 0.006	2.9 ± 0.2	8.0 ± 0.8
***Nannochloropsis* sp.**	2.30 ± 0.5	0.055 ± 0.008	4.40 ± 0.2	0.037 ± 0.007	2.6 ± 0.2	4.8 ± 0.3
Mixed culture	3.50 ± 0.6	0.032 ± 0.004	5.75 ± 0.5	0.034 ± 0.008	4.0 ± 0.5	6.5 ± 0.4

Table 4. Chlorophyll a, b, carotenoid, and lipid content in various microalgal strains cultivated in secondary treated municipal wastewater. Data are means ± standard deviation.

Microalgae	Chlorophyll				Carotenoid		Lipid	
	Chl a (mg. L^{-1})	Chl b (mg.L^{-1})	Total chl (mg. L^{-1})	Total Chl / biomass (mg.g $biomass^{-1}$)	Carotenoid (mg. L^{-1})	Carotenoid/ biomass (mg. g $biomass^{-1}$)	Content (% of dried weight)	Productivity (mg.$L^{-1}.d^{-1}$)
C.vulgaris	2.21 ± 0.3	1.71 ± 0.3	3.92 ± 0.8	4.62 ± 0.6	0.64 ± 0.1	0.75 ± 0.08	24.0 ± 3	31.40 ± 1.6
S.obliquus	2.07 ± 0.5	1.48 ± 0.2	3.55 ± 0.9	4.24 ± 0.9	0.50 ± 0.05	0.71 ± 0.07	28.0 ± 4	29.90 ± 2.4
***Nannochloropsis* sp.**	2.42 ± 0.7	1.38 ± 0.5	3.80 ± 0.7	5.64 ± 0.9	0.54 ± 0.04	0.80 ± 0.06	39.3 ± 5	66.10 ± 3.8
Mixed culture	3.50 ± 0.8	2.30 ± 0.7	5.80 ± 0.6	4.08 ± 0.6	0.86 ± 0.08	0.60 ± 0.07	32.0 ± 3	61.12 ± 2.9

period and presented the same removal efficiency of 100% for N-nitrate and P-phosphate. However, as illustrated in **Table 3**, the mixed culture of *S.obliquus, C.vulgaris,* and *Nannochloropsis* sp. presented the highest N-Nitrate and P-Phosphate removal rate of 5.75 and 3.5 mg.$L^{-1}.d^{-1}$, respectively, which was significantly higher than that of the monoculture of algal species ($P < 0.05$). Precipitation of some quantity of phosphate contributed toward P removal (37), which might be in response to the increase of the pH value in the process of photosynthetic activity of algal cells (**Table 1**). Therefore, the removal of phosphate in this study was not entirely attributed to microalgal uptake.

The rapid decline in phosphate concentration to nearly zero in **Figure 2** might suggest an insufficiency of phosphate in the medium, but this was not so. In all media, the cells continued to grow right up to the point of harvest around day 7 despite a near-complete consumption of phosphate by day 2 to 4 (**Fig. 2**). The rapid decline in phosphate concentration as in **Figure 2** is typical for microalgae which are known to accumulate excess phosphorus as intracellular polyphosphate when growing in a phosphorus-rich medium (38-42). The intracellular accumulation of phosphate in the inocula used to initiate the cultures assured that the alga could continue unlimited growth for many generations in the absence of extracellular phosphate in the medium (22).

4.5. Chlorophyll a, b, Carotenoid, and Lipid Content of Biomass

The maximum Chlorophyll a, b, and carotenoid content of different microalgal strains cultivated in saline wastewater are shown in **Table 4** which indicates that, in terms of total chlorophyll and carotenoid per gram of produced biomass, mixed culture of *S.obliquus, C.vulgaris,* and *Nannochloropsis* sp. presented the lowest amount of 4.08 and 0.6 mg. g $biomass^{-1}$, respectively. Total chlorophyll and carotenoid of the microalgal mixed culture, however, reached the highest quantity of 5.8 and 0.86 mg.L^{-1}, respectively, which is mainly due to the higher biomass of the mixed culture. In this regard, total chlorophyll (mg.L^{-1}) of the mixed culture heightened 52, 48, and 64 % compared with the monocultures of *Nannochloropsis* sp., *C.vulgaris,* and *S.obliquus*, respectively. Moreover, in the mixed culture of these three strains, total carotenoid (mg.L^{-1}) enhanced 60, 34, and 72 %compared with the monocultures of *Nannochloropsis* sp., *C.vulgaris,* and *S.obliquus*, respectively.

The total lipid content of the biomass was measured at

the end of the cultivation period (day 7). The quantities of lipid content are shown in **Table 4**. *Nannochloropsis* sp. presented the highest amount of lipid content and productivity of 39% (of dry cell weight) and 66.1 $mg.L^{-1}.d^{-1}$, respectively. The lipid content of *S.obliquus* and *C.vulgaris* reached the maximum value of 28% and 24% on the basis of dry cell weight, respectively. Additionally, the mixed culture of the three strains showed the lipid content of 32% of the biomass.

5. Discussions

Salinity provides sodium ions that typically affects microalgal cells by 1) ion homeostasis mechanism, 2) changing the cellular ionic ratios due to the membrane selective ion permeability, 3) regulating of intracellular pH, 4) facilitating photosynthesis through inorganic nutrient uptake and alkalinity tolerance (43). *C.vulgaris* and *S. obliquus* are usually freshwater algae, but there are some strains that could tolerate the salty water. Luangpipat and Chisti (22) showed that the biomass productivity of *C.vulgaris* was 0.068 and 0.096 $g.L^{-1}.d^{-1}$ in freshwater and brackish water, respectively, while the biomass productivity of *S.obliquus* was 0.042 and 0.187 $g.L^{-1}.d^{-1}$ in freshwater and brackish water, respectively. Sanchez *et al*. (44) investigated the tolerance of *Scenedesmus almeriensis* in saline medium and reported higher biomass productivities at 0.1 M NaCl compared to the productivities observed in freshwater media. As NaCl concentration increases, biomass production of freshwater species generally declines (45). In this regard, the growth of *S. obliquus* was found to be inhibited at NaCl concentrations above 0.6 M NaCl (45). Kaewkannetra *et al*. (45) showed that at 0.05 M NaCl, the *S. obliquus* biomass was comparable to that found in the freshwater and was 0.63 $g.L^{-1}.d^{-1}$, slightly higher than 0.54 $g.L^{-1}.d^{-1}$ measured in the freshwater experiment. In another study, Pandit *et al*. (46) investigated the effect of salinity stress on growth and lipid productivity of *C.vulgaris*. They reported the biomass and lipid productivity of 31 and 19 $mg.L^{-1}.d^{-1}$, respectively, at NaCl concentration of 0.1 M, while these values in the control experiment (NaCl concentration of 0 M) were 28 and 11.3 $mg.L^{-1}.d^{-1}$. The marine microalgae *Nannochloropsis* can grow exceptionally well in salinities ranged from 8 to 68 PSU (47). Kim *et al*. (48) demonstrated that the fastest growth rate of *Nannochloropsis salina* occurred in salinities between 20 to 40 PSU (22 to 45 g. L^{-1} NaCl) and the highest lipid content obtained in salinities between 30 to 35 PSU (30 to 40 $g.L^{-1}$ NaCl). In the current study, however, the results showed that the monocultures of both freshwater and marine algal species could grow well in brackish water (wastewater with 20 $g.L^{-1}$ NaCl). Besides, the adequate performance of mixed algal culture indicted that the competition among different algal species did not inhibit their cell growth. Taskan (36) evaluated the performance of mixed algal culture cultivated in slaughterhouse wastewater. Biomass production and productivity in their study were 2.5 $g.L^{-1}$ and 0.29 $g.L^{-1}.d^{-1}$, respectively. Prathima Devi *et al.* (49) assessed heterotrophic cultivation of mixed microalgae, collected from a lake in India, using domestic sewage, and reported the biomass concentration of 0.98 $mg.mL^{-1}$. A comparison of the results of this study and the previous studies are shown in supplementary data.

Competition between algal cells as well as the saline environment provides a stressful condition for the mixed algal culture in which algal species could consume more nutrient values (16, 22). The obtained results in this study demonstrated that biomass yield on phosphate ($Y_{X/P}$, $g.mg^{-1}$) in the mixed culture is significantly lower than that of the monocultures, while the specific phosphate consumption rate (mg P. g $cell^{-1}.d^{-1}$) in the mixed culture is significantly higher than that of the monocultures. This act showed that algal species were interested in removing higher phosphate content in the mixed culture and storing it as intracellular compounds without dedicating the phosphate values to cell proliferation. This result is consistent with the study conducted by Luangpipat and Chisti (22). Nitrogen is one of the key elements for cell proliferation of algal species. In this regard, the biomass yield on nitrate ($Y_{X/N}$, mg N. mg^{-1}) in the mixed algal culture was significantly higher than that of the monocultures of *C.vulgaris* and *S.obliquss* which showed that the mixed culture of marine and freshwater algal species tended to allocate the nitrogen values for higher cellular proliferation. The biomass yield on nitrate ($Y_{X/N}$, mg N. mg^{-1}) in the algal mixed, however, did not differ significantly with the monoculture of *Nannochloropsis.* Therefore, mixing different algal species can be an appropriate strategy for stimulating algal species in order to improve nutrient removal efficiency. Taskan (36) evaluated the performance of mixed algae for treatment of slaughterhouse wastewater with TN of 102 and TP of 17.6 $mg.L^{-1}$ and reported nutrient removal efficiency of 70% and 96.2% for TN and TP, respectively. In another study, Devi *et al.* (49) cultivated mixed microalgae in domestic sewage with NO_3 of 115 $mg.L^{-1}$ and PO_4 of 48 $mg.L^{-1}$. While N and P removal rates were 37% and 33%, respectively. Álvarez-Díaz *et al.* (50) reported that *C.vulgaris* and *S.obliquus*, grown on secondary treated urban wastewater, have nearly equal nutrient removal

rate and could completely assimilate Nitrate-N and Phosphate-P during the first 6 and 3 days of cultivation. In comparison with *C.vulgaris* and *S.obliquus*, there were few reports on the cultivation of *Nannochloropsis* sp. in wastewater. However, Sirakov and Velichkova (51) cultivated *Nannochloropsis oculata* in aquaculture wastewater and reported nitrate-N and phosphate-P removal rate of 92 and 72 %, respectively.

It is also observed that although the mixed culture of three strains consumed almost all of the culture medium's nutrients, they did not allocate the nutrients for storing cellular pigments. Instead, the mixed culture of microalgal cells uptake nutrients for cellular proliferation. In this study, the chlorophyll and carotenoid content of microalgal cells were quite low which may be due to the low nutrient content in secondary treated municipal wastewater. Gupta *et al.* (52) cultivated *C.vulgaris* and *Nanochloropsis* sp. in raw municipal wastewater. The chlorophyll a, b, and carotenoid content of *C.vulgaris* were 10, 4, 2.2 µg. mL^{-1}, respectively, while these values for *Nannochloropsis* sp. were 0.5, 0.2, 0.05 µg.mL^{-1}, respectively. Pandit *et al.* (46) also investigated the effect of salinity stress on growth characteristics of *C.vulgaris* (KY436509.1) cultivated in BG11 culture medium under 75 µmol. m^{-2}. s^{-1} illumination with a pH value of 7.4 at 25 °C temperature. They showed that the total Chl and carotenoid content of the microalgae reached the highest value of 55 and 2 µg. mg biomass $^{-1}$, respectively, at NaCl concentration of 0.08 M. While these values in the control experiment (NaCl concentration of 0 M) were 32 and 0.5 µg. mg $biomass^{-1}$.

The lipid content of mixed algae culture is decreased slightly compared with the monoculture of marine algae *Nanochloropsis* sp. Although mono-culture algae cultivation for biofuel production has been studied extensively in the last decades, it has been shown that the risk of contamination in monocultures is high which may result in high costs of treatment and even could lead to shutting down the process (6, 10, 12, 53). While if biofuel is the target, cultivation of mixed culture is a promising scenario in which the risk of contamination is low, and the possibility of the continuous process is high (13-16). The obtained results in this study for the lipid content of the algal cells are consistent with the results provided in previous studies. Kaewkannetra *et al.* (45) investigated the effect of salinity on the cultivation of *S.obliquus* for biodiesel production. They observed a slightly increment in the biomass concentration up to 0.2~ 0.3 M NaCl and reported. a maximum oil accumulation of 36% at 0.3 M NaCl at which plasmolysis occurred. Microscopic observation of the algal isolates under salt stress conditions in their study also revealed changes in the morphology of *S.obliquus* and increment in algal isolate vacuolization. They concluded that further increase in NaCl concentration would not be an effective strategy, though, due to the noticeable decrease in biomass production. Álvarez-Díaz *et al.* (50) reported that the lipid content of *S.obliquus* and *C.vulgaris*, cultivated in secondary treated municipal wastewater with TN of 20 $mg.L^{-1}$, TP of 1.55 $mg.L^{-1}$, and COD of 70 $mg.L^{-1}$ reached 34 and 18% of produced biomass, respectively. Ji *et al.* (31) achieved the lipid content of 25% and 22 % of dry cell weight in the cultivation of *C.vulgaris* and *S.obliquus*, respectively, in tertiary treated municipal wastewater. The lipid content of *Nanochloropsis* sp. is generally ranged between 21 to 36% of dry cell weight (54). Similar results were stated by Cai *et al.* (24), where they cultivated *Nannochloropsis salina* in an anaerobic digestion effluent with a 3% loading ratio and reported that lipid content and productivity reached 36% of dry biomass and 29.2 $mg.L^{-1}.d^{-1}$, respectively.

6. Conclusions

Microalgal cells tend to have a regular performance in a monoculture, and a number of researches have been carried out in monocultures. While in a mixed culture, microalgae performance is more complicated due to their interactions. The obtained results in this study showed that the salinity stress of the environment, as well as the competition stress among microalgal species, stimulated algal cells to utilize more nutrient content of the culture medium, which, then, resulted in the higher proliferation rate of algal cells in the mixed culture. Meanwhile, total chlorophyll and carotenoid content of the microalgal mixed culture was increased compared with the monoculture of algal species. Also, the obtained results in this study suggested that it is possible to cultivate freshwater together with marine microalga in a mixed culture using brackish waters in order to produce microalgal biomass economically. Overall, more distinguished results can be achieved by investigating the interaction and also competition between other microalgal strains in a mixed culture.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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