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Biomass and Lipid Production of Chlorella Sp. Using Municipal Wastewater under Semi-continuous Cultivation

Yi-Chun Yang[1], Jhong-Fu Jian[2], Chiu-Mei Kuo[3], Wen-Xin Zhang[4] and Chih-Sheng Lin [5]

Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan

[1] **Abstract.** The stable-growth performance and CO2 fixation of Chlorella sp. cultivated in municipal wastewater were investigated. The biomass concentration and biomass productivity of Chlorella sp. cultured with the addition of nutrients in municipal wastewater were 6.48 g/L and 0.883 g/L/day, respectively. The biomass concentration and biomass productivity of Chlorella sp. cultured with nutrient addition in municipal wastewater were both approximately three fold higher than those observed in cultures without extra nutrients. In semi-continuous cultures, the half volume of culture broth was replaced every 2, 3 and 4 days during a period of 12 days, and the average biomass productivities with nutrient addition in municipal wastewater were 4.15, 4.62 and 5.43 g/L, and 1.071, 0.809 and 0.718 g/L/day, respectively. The accumulated biomass production in the 1-L photobioreactor in 2-day, 3-day and 4-day replacements of the semi-continuous culture for 12 days were 14.6, 11.6 and 10.8 g. Compared with the batch culture, the average biomass productivity and accumulated biomass production were 1.7- to 2.5-fold and 1.6- to 2.1-fold higher in the semi-continuous cultures, respectively. The higher biomass production in a semi-continuous culture was obtained because Chlorella sp. was maintained in an exponential phase. The average lipid content of Chlorella sp. in semi-continuous cultures (19.7%) was lower than that of batch culture (21.1%). Due to the higher biomass productivity observed in the semi-continuous culturing system, the maximum lipid productivity (0.209 g/L/day), which was obtained in the 2-day replacement, was 2.3-fold higher than that in batch culture. Furthermore, the content of C16:0, C18:0, C18:1 and C18:2 of Chlorella sp. was more than 75% of the totalfatty acids, regardless of whether the semi-continuous or batch culture was performed.

Keywords: biomass, Chlorella, lipid, municipal wastewater, semi-continuous cultivation

1. Introduction

Third-generation biofuel feedstock derived from microalgae can play an important role in alternative energy resources because it not only can decrease concentrations of GHGs (since carbon dioxide (CO2) is used as carbon source during autotrophic growth), but the abundant energy-rich components from microalgal biomass can also be converted to various types of biofuels, such as ethanol, butanol, methane and biodiesel [1] [2]. Moreover, the microalgal biomass can be extracted to the functional components, such as polysaccharides, phycobilins, lutein, β-carotene, astaxanthin, docosahexaenoic acid, and eicosapentaenoic acid, to apply in high-value products for animal feeds, cosmetic, nutritional supplements, among others [3]-[5]. However, the high cost of biodiesel production from microalgae in microalgal cultivation is unable to compete with fossil fuels. The total medium costs of microalgae cultivation were approximately 80% because it requires considerable consumption of nutrients and water to grow [6]. Nitrogen and phosphorus in the wastewater, such as ammonia (NH4+), nitrite (NO2-), nitrate (NO3-) and phosphates (PO43-), are useful ingredients for microalgal cultures, and they can be utilized for microalgal growth to obtain wastewater purification simultaneously [7]-[9].

Large quantities of municipal wastewater are produced because of the increasing urbanization and expansion of urban populations. When Scenedesmus sp. was cultured with 2.5% CO2 in municipal

[+] Corresponding author. Tel: +886-35712121 ext. 56940
E-mail address: dorothy324047@gmail.com

wastewater, the maximum biomass productivity and lipid content were 196 mg/L/day and 33.3%, respectively [10]. The genera Chlorella and Dictyochloris were isolated from three municipal wastewater treatment samples and grew faster than other isolates [11]. In municipal wastewater with glycerol addition, a maximum biomass productivity of Chlorella vulgaris was 56 mg/L/day, which was achieved for 12 days [12]. These studies indicate that municipal wastewater can be used for microalgal cultivation to replace a large amount of freshwater. The aim of this study is to establish an efficient and stable semi-continuous culturing system for long-term cultivation of Chlorella sp. using municipal wastewater.

2. Materials and Methods

2.1. Microalgal Cultures, Medium and Chemicals

The freshwater microalgal strain, Chlorella sp., was originally obtained from the collection of Taiwan Fisheries Research Institute (Tung-Kang, Taiwan). According to our previous report [13], Chlorella sp. was screened for potential microalgal growth ability and biomass production at the National Chiao Tung University, Taiwan. To maintain the Chlorella sp. strain, it was cultured in solid agar (1.5%, w/v) modified freshwater medium and transferred to fresh growth medium once per month at 26 ± 1 ℃ with 100 μmol/m2/s of light intensity. A stock culture of Chlorella sp. was subcultured in an Erlenmeyer flask containing modified freshwater medium at 26 ± 1 ℃ with approximately 300 μmol/m2/s of surface light intensity that was provided by a continuous cool-white fluorescent light. A modified freshwater medium, which was used for the Chlorella sp., was composed of the following (per liter): 1.25 g KNO3, 1.25 g KH2PO4, 1 g MgSO4·7H2O, 83.5 mg CaCl2·2H2O, 0.1142 g H3BO3, 49.8 mg FeSO4·7H2O, 88.2 mg ZnSO4·7H2O, 14.4 mg MnCl2·4H2O, 10 mg CuSO4, 7.1 mg Na2MoO4 and 4 mg CoCl2·6H2O. The initial pH of the initial medium was adjusted to 6 with NaOH. The "1× nutrient medium" was modified freshwater medium.

2.2. Source and Pretreatment of Municipal Wastewater

The municipal wastewater was collected from the National Chiao Tung University (Hsinchu, Taiwan) between April and October 2016. Large insoluble particles in the municipal wastewater were centrifuged at 3000 × g for 10 min for separation, and the resulting supernatant was stored at 4 ℃ for subsequent experiments.

2.3. General Culture Conditions of Indoor Photobioreactors

The column-type glass-fabricated photobioreactors used for the cultivation of Chlorella sp. had working volumes of 1 L (ϕ 6 cm × 80 cm high), which were incubated at 26 ± 1 ℃ with approximately 300 μmol/m2/s of surface light intensity provided by a continuous cool-white fluorescent light. The CO2-containing gas was premixed with air and supplied from the bottom of the photobioreactor with an aeration rate of 0.05 to 0.3 volume per volume per minute (vvm). The initial biomass concentration of the Chlorella sp. fresh cultures was approximately 0.3 g/L. The Chlorella sp. in difference culture conditions was sampled at 24 h intervals to determine biomass concentrations.

2.4. Batch Cultivations

The *Chlorella* sp. was cultured in municipal wastewater supplied with 2% CO_2-supplemented air at an aeration rate of 0.2 vvm for 7 days. The growth and CO_2 fixation efficiency of the microalgal cells was enhanced by adding 0, 0.25, 0.5, 0.75 and 1× nutrient medium for 7 days.

2.5. Semi-continuous Cultivations

There were two stages employed in the semi-continuous culturing system. First, a prepared inoculum of *Chlorella* sp. was incubated in 1× nutrient-added municipal wastewater to start with an initial biomass concentration of 0.3 g/L. When the biomass concentration of the logarithmic phase Chlorella sp. reached approximately 3.6−3.8 g/L after nearly 3 days of incubation, half the volume (0.5 L) of the culture broth was replaced with 1× nutrient-added municipal wastewater. Subsequently, the initial biomass concentration of the culture reached approximately 1.8−1.9 g/L, after which half the volume of the culture broth was again replaced with nutrient-added municipal wastewater at intervals of 2, 3, and 4 days, and the cultures were allowed to grow for a period of 12 days.

2.6. Determination of Microalgal Cell Biomass and Specific Growth Rate

A calibration curve equation considering the dry weight of Chlorella sp. and the optical density at 682 nm was established, as shown in formula (1).

Biomass concentration (g/L) = 0.3101 × A682nm − 0.0065 (1)

Ultrospec 3300 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, UK) was used for optical density (A682nm) measurement of microalgal culture, and the biomass concentration could be precisely calculated ($R2 = 0.9981$; $p < 0.001$). The absorbance of sample needs to be diluted in the range of 0.1 to 1.0 if the optical density was over 1.0.

2.7. Lipid Extraction, Lipid Transesterification, and Fatty Acid Profile Assay

Lipid extraction was performed according to our previous report [13]. Extraction of lipids from the dried microalgal biomass (200 mg) with 4 mL of a methanol/chloroform solution (1/2, v/v) was sonicated for 1 h. Subsequently, a 0.9% NaCl solution was added to give the mixture a 2:2:1 ratio of methanol, chloroform and water. After centrifugation, the chloroform phase was collected and allowed to evaporate. The remaining solution was weighed as a lipid. The methods used for the transesterification and fatty acid profile assay of the microalgal lipids were based on the previously reported procedures [14]. The FOCUS Gas Chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) is used for determining the fatty acid composition.

3. Results and Discussion

3.1. *Chlorella* sp. Cultivated in Municipal Wastewater with Nutrient Addition

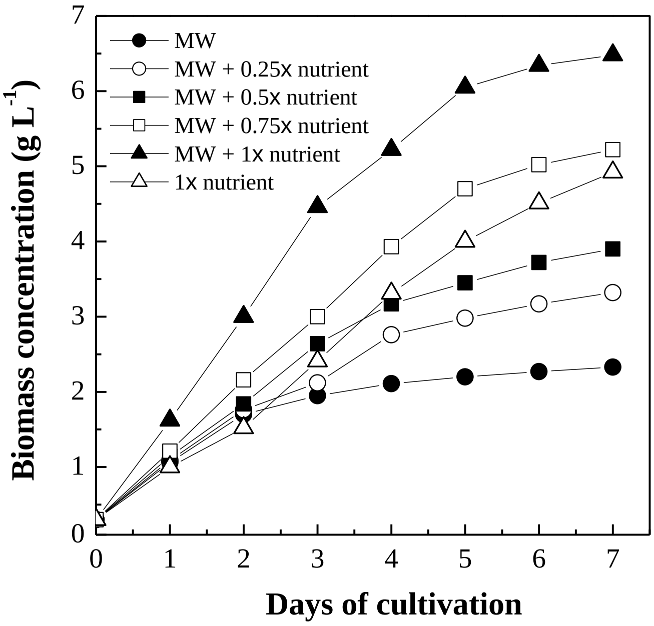

Fig. 1: Growth profiles of Chlorella sp. cultured in municipal wastewater with the addition of different ratios (0.25, 0.5, 0.75, and 1×) of nutrients. The cultures with pure municipal wastewater and 1× nutrient medium were plotted for comparison. The cultures were operated at 26 ± 1 ℃ with 300 μmol/m2/s light intensity and with a 2% CO2 aeration rate of 0.2 vvm for 7 days.

When Chlorella sp. was cultured in municipal wastewater with 2% CO2 aeration, the maximum biomass productivity was 0.551 g/L/day at 3 days because the microalgal culture was entering stationary phase. For 7 days of culture, the biomass concentration was 2.41 g/L, and biomass productivity was 0.301 g/L/day. Additionally, the total phosphorous content in municipal wastewater was almost depleted, and the total nitrogen (TN) content was rapidly decreased from 54 to 11 mg/L within 3 days of cultivation. Approximately

90% of the TN was utilized, and the residual TN content in the wastewater broth was only 5.6 mg/L. In contrast to industrial and agricultural wastewater, there is less nitrogen and phosphorus in municipal wastewater. To enhance the efficiency of CO2 fixation by increasing the microalgal biomass concentration, Fig. 1 shows that the biomass concentration and productivity of Chlorella sp. cultured with the addition of 0 (i.e., municipal wastewater without nutrients), 0.25, 0.5, 0.75 and 1× nutrients in municipal wastewater were 2.33, 3.32, 3.91, 5.22 and 6.48 g/L, and 0.291, 0.431, 0.515, 0.703 and 0.883 g/L/day, respectively. The biomass concentration and productivity of microalgae growing with 0.25−1× nutrient addition in municipal wastewater were 1.4- to 3.1-fold higher than those of microalgae growing without extra nutrients. The biomass productivity of Chlorella vulgaris cultured with 2 g/L glycerol addition in municipal wastewater was 30-fold higher than that of without glycerol [12].

The lipid content and lipid productivity of Chlorella sp. harvested in municipal wastewater with the addition of different ratios (0.25, 0.5, 0.75, and 1×) of nutrients for 7 days is shown in Fig. 2. The lipid content of Chlorella sp. in municipal wastewater was approximately 21% with the nutrient added or not. Compared with 1× nutrient in freshwater, the lipid content of Chlorella sp. cultured with the nutrient addition in municipal wastewater was 1.4-fold higher. Because the biomass productivity increased significantly with the nutrient ratio added to the microalgal cultivation in municipal wastewater, the maximum lipid productivity (0.178 g/L/day) was obtained in municipal wastewater with a 1× nutrient addition. The lipid productivity of Chlorella sp. cultured with a 1× nutrient addition in municipal wastewater was approximately 2-fold higher than that of a 1× nutrient in freshwater. Several studies show that the biomass and lipid production in heterotrophic or mixotrophic culture was higher than autotrophic culture [15]. In this study, the results indicate that the lipid of Chlorella sp. for biodiesel production could be produced in microalgal cultivation using municipal wastewater.

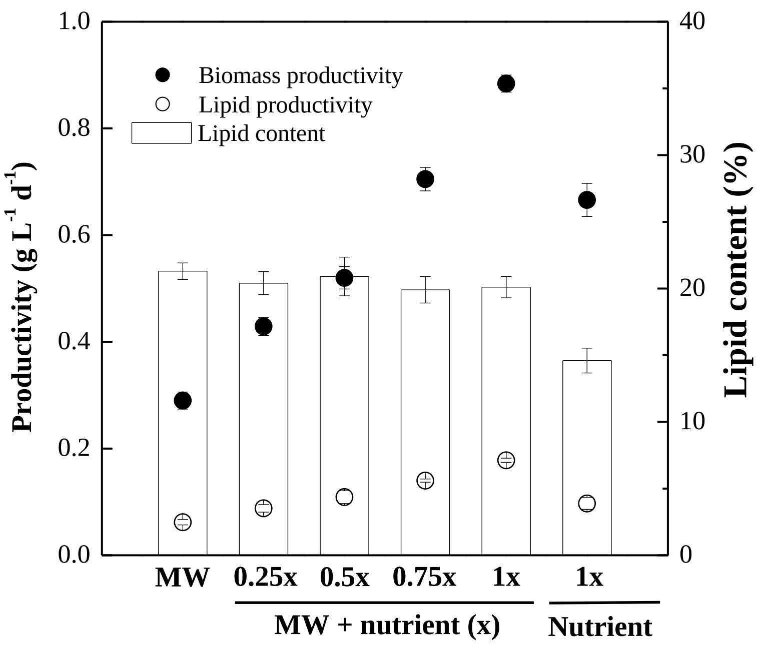

Fig. 2: Biomass productivity, lipid content and lipid productivity of Chlorella sp. cultured in municipal wastewater with the addition of different ratios (0.25, 0.5, 0.75, and 1×) of nutrients. The cultures with pure municipal wastewater and 1× nutrient medium were plotted for the comparison.

3.2. *Chlorella* sp. Cultivated in Nutrient-added Municipal Wastewater with Semi-continuous Culture Operation

In semi-continuous cultures, the half volume of culture broth with replacement performed every 2, 3 and 4 days (during a period of 12 days), the average biomass concentration and biomass productivity with 1× nutrient addition in municipal wastewater were 4.15, 4.62 and 5.43 g/L, and 1.071, 0.809 and 0.718 g/L/day, respectively, for each of the days (Fig. 3). The accumulated biomass production in 1-L photobioreactor in 2-day, 3-day and 4-day replacements of the semi-continuous culture for 12 days was 14.6, 11.6 and 10.8 g, respectively (Fig. 4). Compared with the batch culture, the average biomass productivity and accumulated biomass production were 1.7- to 2.5-fold and 1.6- to 2.1-fold higher in the semi-continuous cultures, respectively. The microalgae in semi-continuous culture had a significantly higher growth performance than that in the batch culture because the Chlorella sp. was maintained in an exponential phase during the semi-continuous culture. In addition, when the higher biomass concentration was obtained, an increase of self-shading phenomenon and a decrease of light penetration were caused during microalgal cultivation [15].

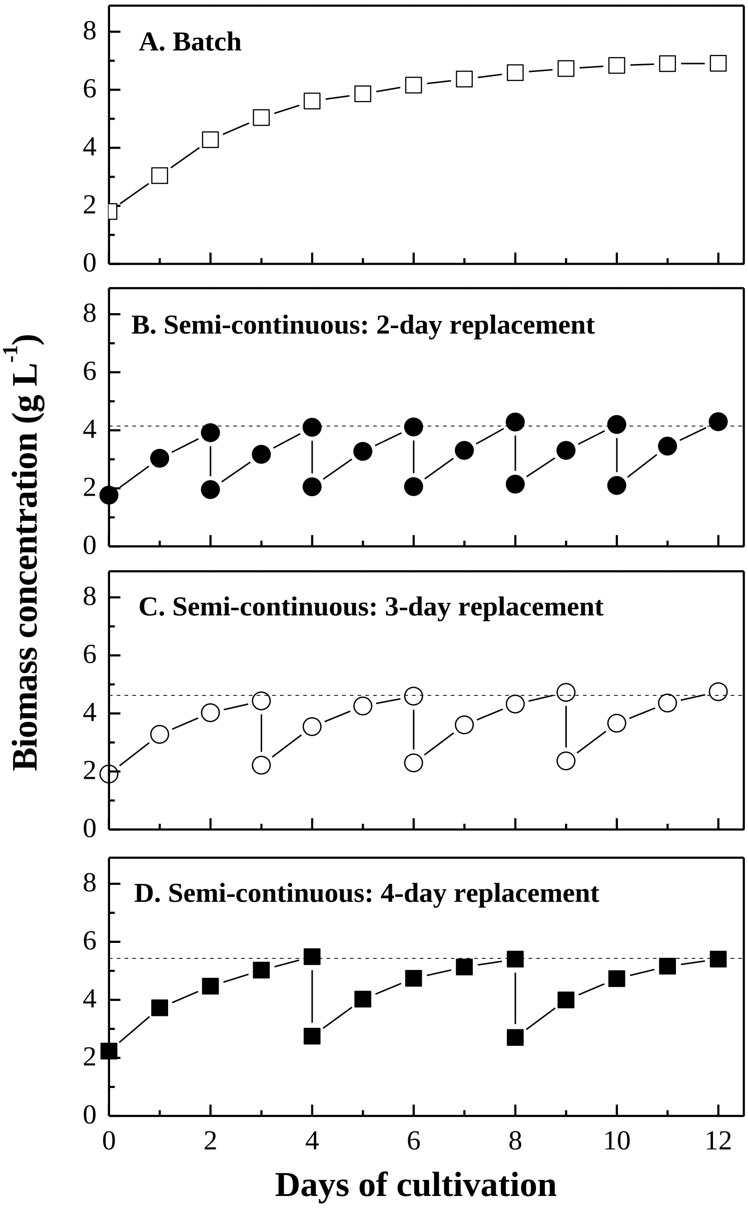

Fig. 3: Growth profiles of Chlorella sp. cultured with 1× nutrient in municipal wastewater batch culture (A) and semi-continuous cultures with 2-day (B), 3-day (C) and 4-day (D) replacement for a period of 12 days. A half volume of the culture broth was replaced by fresh nutrient-added municipal wastewater. A starting biomass concentration of approximate 1.8 g/L was used by replacing half the volume of the microalgal broth at logarithmic phase.

Furthermore, the total amount of wastewater used in 2-day, 3-day and 4-day replacements of the semi-continuous culture was 3.5, 2.5 and 1.5 L, respectively. In the batch culture, the wastewater only used 1 L for 12 days of microalgal cultivation. These results show that higher accumulated biomass production was obtained, and a larger amount of wastewater could be reutilized to replace fresh water usage, in a semi-continuous culture compared with that obtained using a batch culture. These results indicate that semi-continuous replacement culture strategy could achieve a stable growth performance of long-term microalgal cultures. In other words, optimization of a semi-continuous replacement culture strategy was observed to be most feasible for a large-scale microalgal cultivation and a practical approach [16].

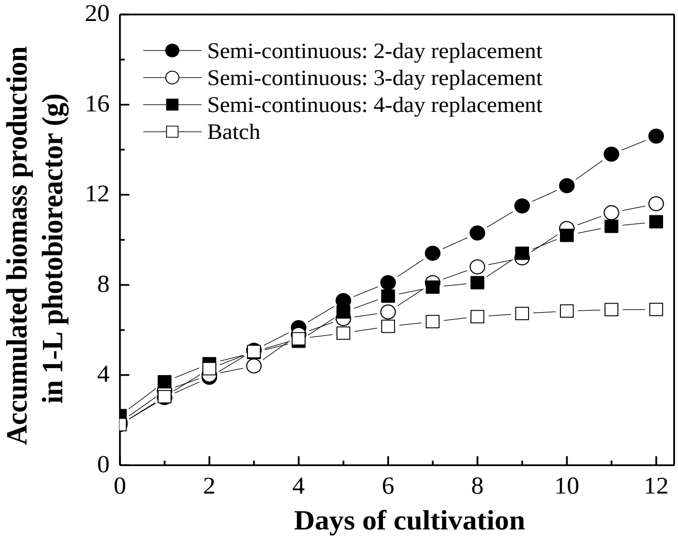

Fig. 4: Accumulated biomass production profiles of Chlorella sp. cultured in nutrient-added municipal wastewater with the aeration of 2% CO2 in a batch culture, and a semi-continuous culture with 2-day, 3-day and 4-day replacement for a period of 12 days in a 1-L photobioreactor.

The lipid contents and lipid productivities of 2-day, 3-day and 4-day replacements in semi-continuous culture were 19.5, 19.6 and 20.1%, and 0.209, 0.159 and 0.144 g/L/day, respectively. The average lipid content of 2-day, 3-day and 4-day replacement in semi-continuous cultures was 19.7%, which was slightly lower than that of the batch culture (21.1%). Due to the higher biomass productivity in semi-continuous culturing system, the maximum lipid productivity (0.209 g/L/day) was obtained in the 2-day replacement and it was 2.3-fold higher than that obtained in the batch culture. Fatty acids C16:0, C18:0, C18:1 and C18:2 are considered to be good for producing biodiesel [14]. The primary fatty acids of Chlorella sp. were C16:0, C18:0, C18:1 and C18:2 which accounted for more than 75% of the total fatty acids under cultivation, regardless of whether the semi-continuous or the batch culture was used (Fig. 5). Among the fatty acids, the C16:0 (24.9−34.4%), C18:1 (16.6−26.1%) and C18:2 (20.5−28.2%) were more abundant than others. C16:0 was often the most abundant fatty acid with microalgal wastewater cultivation in other studies [12], [13], which was consistent with our results. Higher contents of saturated fatty acids were associated with higher oxidative stability of biodiesel [14].

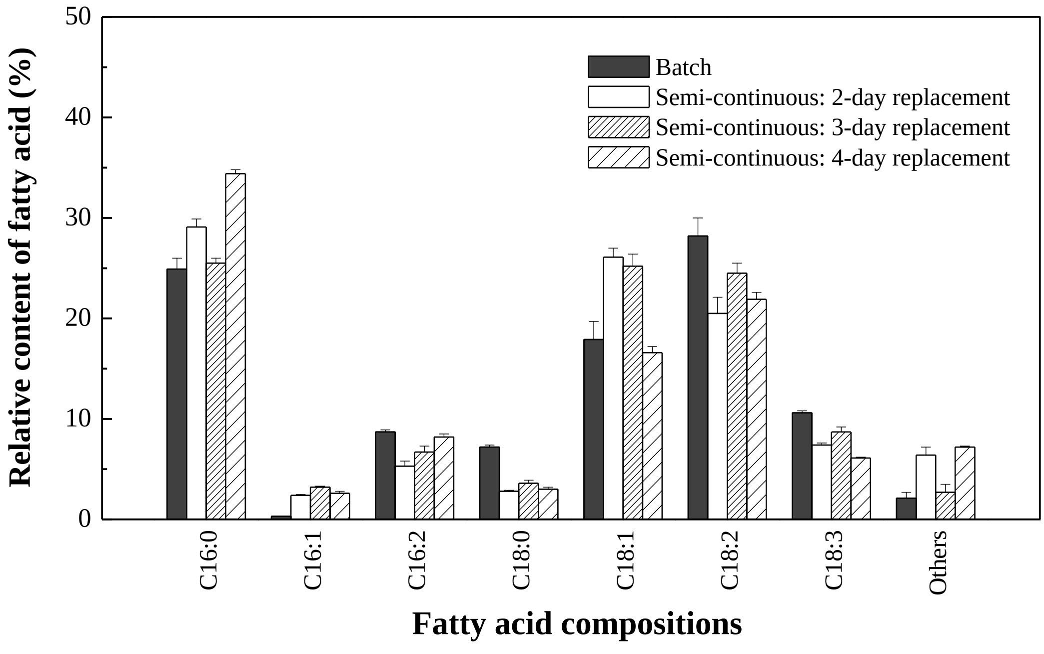

Fig. 5: Primary fatty acid profiles of Chlorella sp. cultured with 1× nutrient in municipal wastewater by batch culture and semi-continuous cultures with 2-day, 3-day and 4-day replacement for a period of 12 days.

4. Conclusions

This study indicates that the biomass productivity and CO2 fixation efficiency of Chlorella sp. was enhanced when it was cultured in nutrient-added municipal wastewater. In a semi-continuous culturing system for long-term cultivation of microalgae with wastewater replacement, stable growth performance was achieved. The lipid productivity was increased with the benefit of enhancing total lipid production along with higher biomass productivity. The lipid of Chlorella sp. cultivated in municipal wastewater with nutrient addition was suitable for biodiesel production. However, using wastewater for outdoor cultivation and avoiding microorganisms, heavy metals and biotic pollution warrant further investigation.

5. Acknowledgement

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