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Isolation of microalgae with potential for integrated biomass production and nutrient removal from wastewater

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ABSTRACT

The study first investigates the diversity of microalgae in the constructed wetland. Then one of the potential microalgal species was isolated and cultivated in photoautotrophic and mixotrophic growth conditions to compare the performance on biomass production and to evaluate the ability of wastewater treatment and simultaneous biodiesel production. *Scenedesmus* sp. was an abundant strain in the constructed wetland during 1 year of monitoring. Under both photoautotrophic and mixotrophic cultivation conditions, an appropriate composition of each source was beneficial for respective biomass production, 8% (v v^{-1}) CO_2 and 40% piggery wastewater content. The specific growth rate and lipid productivity obtained from photoautotrophic growth were slightly higher than those obtained from mixotrophic growth. Both cultivation conditions led to different fatty acid compositions. Comparing the photoautotrophic and mixotrophic growth, the mixotrophic cultivation not only produced biomass but also could assimilate up to 81.5% total nitrogen (TN), 64.6% total phosphorous (TP), and 60.7% chemical oxygen demand (COD) from piggery wastewater, respectively. The highest biomass productivity was observed at 16.9:1.1:1 of COD/TN/TP of piggery wastewater.

Keywords: Biodiesel; Piggery wastewater; *Scenedesmus* sp.

1. Introduction

Microalgae have been considered as a potential source of third generation biodiesel, due to its higher biomass production and faster growth rate than other energy crops. However, the production cost of biodiesel from microalgae is usually higher than that from traditional crops. Because the high cost of chemical usage during algae cultivation and the high costs for the harvesting and drying process [1]. Moreover, algae production was higher than those for conventional energy crops in terms of energy use, greenhouse gas emissions, and water resources [2]. In order to cope with these problems, a more effective and economic way to reduce the biomass production cost is that microalgae cultivated using wastewater. This promising approach also provides the extra benefits of simultaneous wastewater treatment [3].

Biodiesel production from microalgae is significantly affected by factors such as cultivation conditions, culture system, algae species, biomass harvesting, and oil extraction. There are four major types of cultivation conditions, namely photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic cultivation. The cultivation condition significantly influences the growth characteristics and composition of microalgae. For example, the mixotrophic *Nannochloropsis* sp. grown in the presence of 2 g L^{-1} glycerol resulted in higher fatty acid methyl esters (FAMEs) productivity, an increase of over 72% compared with photoautotrophic culture [4]. Bohutskyi et al. [5] showed that *Scenedesmus acutus f. alternans* had higher growth rates, productivities, while supplementing with 5%–10% nutrient-rich anaerobic digestion centrate enhanced microalgal growth rates from 0.2–0.3 to 0.7–0.9 d^{-1} and biomass productivity (BP) from 10–20 to 40–60 mg L^{-1} d^{-1} with greater improvements for secondary effluents. However, Yeh and Chang [6] reported that higher

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biomass production (2–5 g L^{-1}) of *Chlorella vulgaris* ESP-31 was obtained using nitrogen-rich medium (basal medium and Modified Bristol's medium) under phototrophic (CO_2), photoheterotrophic, and mixotrophic conditions. Moreover, the highest lipid productivity (144 mg L^{-1} d^{-1}) was obtained from mixotrophic cultivation on Modified Bristol's medium.

Lipid accumulation in wastewater-grown microalgae for biomass production and wastewater treatment depends on its growth conditions, especially the nutrients content [7,8]. Sydney et al. [9] found that *Botryococcus braunii* was able to remove N and P nutrients (79.63%) from treated domestic wastewater, and accumulate oil with a dry biomass of up to 36%. Zhu et al. [10] reported that over 60% total nitrogen (TN), 88% total phosphorous (TP), and 66% chemical oxygen demand (COD) were removed by *Chlorella zofingiensis* from piggery wastewater, while producing the highest BP (296.16 mg L^{-1} d^{-1}) and lipid productivity (110.56 mg L^{-1} d^{-1}) in the 1,900 mg L^{-1} COD culture. Li et al. [11] reported that *Scenedesmus* sp. in an artificial medium at a 5–8:1 N/P ratio can efficiently remove both nutrients. This microalgae also led to the accumulation of lipids (30% vs 53%) when N or P was limited. However, a high lipid content being induced, the cell growth rate is often very low, thereby limiting lipid productivity [12]. Hence, high lipid productivity associated with biodiesel production needs both high lipid content and suitable nutrient ratio for microalgae growth in wastewater.

It has been suggested that indigenous mixotrophic strains have intrinsic characteristics lacking in type culture collections and genetically engineered organisms [13]. Therefore, the specific objectives of this study were to investigate first the variations of microalgae in the constructed wetland, which mainly receives domestic and piggery wastewater. Then one of the potential microalgal species was isolated and cultivated under photoautotrophic and mixotrophic growth conditions to compare the performance on biomass production and to evaluate the ability of wastewater treatment and simultaneous biodiesel production.

2. Materials and methods

2.1. Collection and isolation of microalgal sample

Water samples were collected from Dashu constructed wetland in southern Taiwan (22°39′30″N, 120°25′18″E) with a total open surface water area of 177 ha (Fig. 1). The whole Dashu constructed wetland consists of two parts. The first part is a fully vegetated zone with a four open-water surface zone and the second part is also a fully vegetated zone with a nine ecological open-water pond. The average water depth of Dashu constructed wetland was less than 1.2 m. A 1,500-mL of each sample was taken at two depths (0.15 and 0.5 m below the surface), totally 3 L composite water sample was collected. After thoroughly mixing, a final 1 L water sample was then collected and preserved by adding 3 mL of Lugol's iodine solution [14]. The water sample was stored in a transparent plastic bottle in a dark environment and sent to the laboratory within 24 h for algal cell identification and isolation.

The protocol for isolating the microalgal strains was as follows: biomass concentration concentrated by centrifugation, removing zooplankton by a 60 μm plankton net, enrichment of the cells by inoculating onto sterile tissue culture plate containing BG-11 medium, the microalgal strains obtained by sequential subculturing, and cell grown in liquid cultures containing sterile BG-11 medium by inoculating single colonies of purified strains from agar plates into conical flasks and placing on an orbital shaker at 100 rpm. Then the strains were identified for taxonomy by observation of morphological characters at the microscope according to Standard Methods, AWWA, and Hoek et al. [14–16].

2.2. Microalgal culture

The microalgae strain *Scenedesmus* sp. used was isolated from the sample water and cultivated in a medium according to a method given by Norris et al. [17]. The microalgae that reached enough seed culture, about ~10^6 cells mL^{-1}, then the cells of seed culture were collected by centrifugation, and the precipitated algal cells were inoculated in batch mode in a 1 L modified serum bottle containing 600 mL of pretreated piggery wastewater for mixotrophic growth, and Norris medium for photoautotrophic growth, respectively. The bottles were placed in the incubator, which continuously provided 5 kLux of illumination, 25°C of temperature, and 100 rpm of stirring speed. For mixotrophic and photoautotrophic cultivation, the organic carbon in piggery wastewater and CO_2 was used as carbon source, respectively. CO_2

Fig. 1. The image of the Dashu constructed wetland.

was supplied to the cultures by syringe injection every day. The piggery wastewater was collected from the effluent of a wastewater treatment plant of a local pig farm in southern Taiwan. The piggery wastewater effluent was diluted by deioned water according to the experimental design. The piggery wastewater was then filtered through a 0.45 μm membrane and sterilized before experiments. Before the start of the experiments, the strain was cultivated in piggery wastewater and Norris medium for three generations to obtain stable characteristics, respectively. The cultures were harvested in the log growth phase after 8 d for experiments.

2.3. Biomass concentration and lipid content

The dry weight of the microalgal biomass was determined gravimetrically. A known volume of microalgal culture was collected and dried at 90°C for 3 h. The growth rate (μ) was calculated according to the equation $\mu = (\ln A_1 - \ln A_0)/(T_1 - T_0)$, where A_1 and A_0 are the dry weights of the microalgal biomass at times T_1 and T_0, respectively. The dry weight of cells was obtained using an analytical balance with a precision of 0.1 mg. The biomass concentration (mg L^{-1}) is expressed as the dry weight of the microalgal biomass.

A stock culture of *Scenedesmus* sp. cells was collected by centrifugation at 2,000 rpm for 10 min (CR22G III, Hitachi, Japan). The precipitated algal cells were washed and resuspended in deionized water in triplicate.

Cells were collected by centrifugation and then dried in a freeze dryer at –80°C at about 30 Pa. The microalgal total lipids were extracted with *n*-hexane/methanol (2/1, v v^{-1}) in a Soxhlet extractor and quantified gravimetrically. The lipid content (g g^{-1}) is expressed as the dry weight of the microalgal biomass. The BP was calculated from cultivation time and biomass concentration, expressed as $BP = (W_1 - W_0)/(T_1 - T_0)$, in which W_1 and W_0 are dry biomass concentration (mg L^{-1}) at time T_1 and T_0 (d), respectively.

2.4. Nutrients analysis

The samples were first filtered through a 0.45-μm filter and then the filtrate was properly diluted for the determination of COD, TN, and TP. All of the measurements were conducted according to the Methods for Examination of Water and Wastewater [14]. The characteristics of piggery wastewater used in this experiment were COD in the range of 98.2–492 mg L^{-1}, TN of 8.8–44.1 mg N L^{-1}, and TP of 0.9–4.7 mg P L^{-1}.

2.5. Extraction method and analysis of FAMEs

Freeze-dried biomass (0.1 ± 0.002 g) was placed in 50-mL Teflon-capped Pyrex tubes and mixed with a premixed homogeneous solution of NaOH catalyst (2.5 wt.%) and methanol. 8.0 mL of alkali catalyst was added to the tubes. The transesterification reaction and FAME analysis (Agilent 7820A, USA, flame ionization detector, and a DB-23 Agilent column) were followed given by Chen and Lee [18]. The temperature program was as follows: the injector was held at 250°C while the detector was kept at 300°C. The oven was at 50°C initially, ramped to 175°C at 25°C min^{-1}, ramped again to 230°C at 4°C min^{-1} for 4 min, and finally held at 230°C, giving a total heating time of 23.75 min. FAMEs were identified by comparing their retention times with those of a 37-component FAME mix (Supelco, USA) and quantified by comparison with the prepared calibration curves.

3. Results and discussion

3.1. Phytoplankton community composition of the Wetland

The Dashu constructed wetland mainly receives domestic and manure wastewater, covering overall nine sequential components with average depth of from 0.24 to 1.1 m. Fig. 2 shows a total of four phyla (22 total species), namely Bacillariophyta (eight species), Chlorophyta (eight species), Euglenophyta (two species), and Cyanophyta (four species) in the local wetland sample. Chlorophyta and Cyanophyta made up the most significant proportion of the community composition. One species of the Chlorophyta, *Scenedesmus* sp., was the second most abundant strain (4.43×10^4 cells mL^{-1}) for 1 year monitoring.

3.2. Comparison of photoautotrophic and mixotrophic growth

The microalgae under photoautotrophic cultivation which uses light as the energy source, and inorganic carbon (e.g., CO_2) as the carbon source to form chemical energy through photosynthesis. While mixotrophic cultivation undergoes photosynthesis and uses both organic compounds and inorganic carbon (CO_2) as a carbon source for growth. The cell growth, specific growth rate, and BP of *Scenedesmus* sp. grown under different CO_2 contents are shown in Fig. 3. It can be seen that the microalgae survived in all of the cultures and no obvious lag phases were observed. The average specific growth rates under cultures with CO_2 content levels of 2%, 4%, 8%, 12%, and 16% were 0.09, 0.14, 0.152, 0.137,

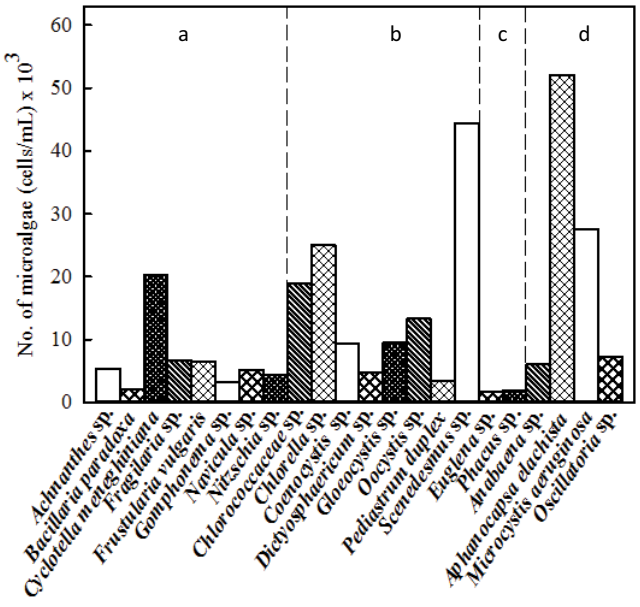

Fig. 2. Concentrations of various algal species in Dashu constructed wetland (a: Bacillariophyta; b: Chlorophyta; c: Euglenophyta; and d: Cyanophyta).

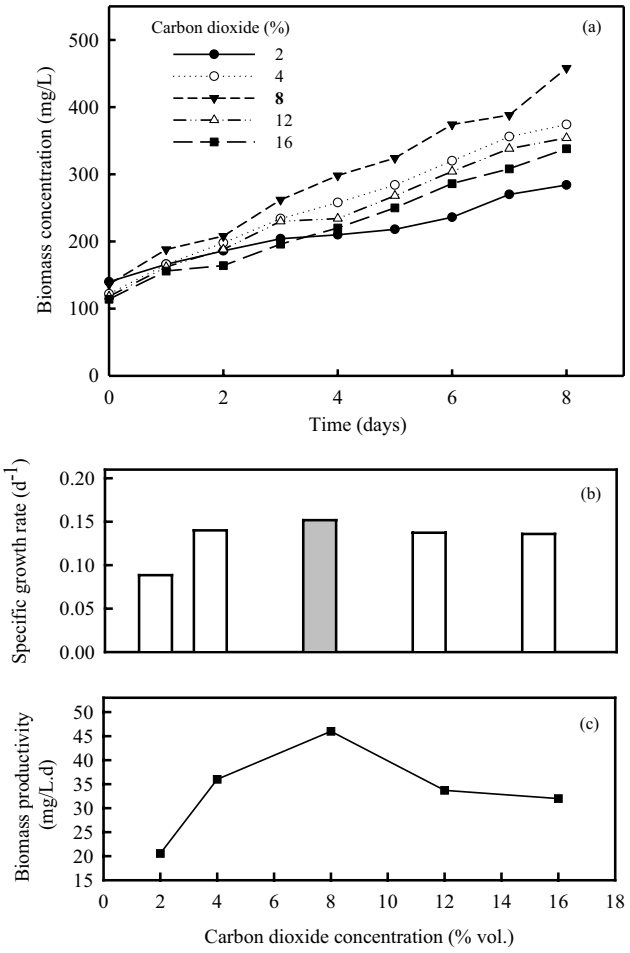

Fig. 3. Cell growth (a), specific growth rate (b), and biomass productivity (c) of *Scenedesmus* sp. under different CO_2 contents.

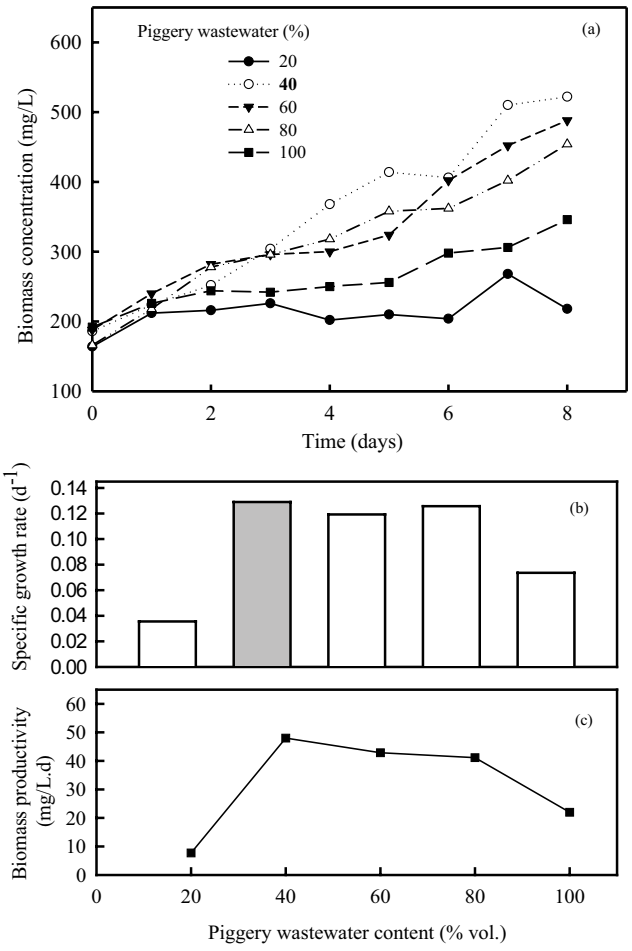

Fig. 4. Cell growth (a), specific growth rate (b), and biomass productivity (c) of *Scenedesmus* sp. under different piggery wastewater contents.

and 0.136 d^{-1}, respectively. Eight percentage of CO_2 content resulted in the highest cell growth, higher CO_2 concentration did not further improve cell growth. Similar results were reported for *Scenedesmus obliquus* by Kaewkannetra et al. [19]. This is probably due to excess CO_2 being converted to H_2CO_3, resulting in a reduction of pH in the culture, thereby affecting cell growth.

Under piggery wastewater cultivation, the better cell growth of *Scenedesmus* sp. was cultivated at ranged from 40% to 80% piggery wastewater content (Fig. 4). The highest BP of 48 mg L^{-1} d^{-1} was observed that cultivated under 40% piggery wastewater content. Under mixotrophic condition, the cellular photosynthetic components concentration particularly depended on nutrients content. Previous studies reported that optimal growth of the microalgae at the suitable dilution of piggery wastewater effluent, due to the nutrient uptake by microalga increased [20–22]. This finding is similar to those of the *C. zofingiensis* whose nutrient removal and highest productivities of biomass and lipid were primarily affected by suitable concentration of piggery wastewater [10]. The 40% piggery wastewater content culture reached 48 mg L^{-1} d BP, compared with that obtained with the culture under 8% CO_2 content (46 mg L^{-1} d^{-1}).

3.3. Lipid productivity and FAME composition

Fig. 5 shows the effects of the respective CO_2 and piggery wastewater contents on lipid content and lipid productivity. Although 4% CO_2 resulted in the highest lipid content (23.5%), the highest lipid productivity (9.6 mg L^{-1} d^{-1}) was achieved with 8% CO_2 content. The results show that high CO_2 content not only decreased cell growth but also significantly hindered lipid accumulation. Lipid productivity is the mass of lipids produced per unit volume of the culture per unit time; it depends on the algal growth rate and the lipid content of the biomass. Under piggery wastewater cultivation, the best performance of lipid productivity (8.3 mg L^{-1} d^{-1}) was obtained with 40% piggery wastewater content, similar to that of BP.

Table 1 compares the FAME composition of *Scenedesmus* sp. grown in CO_2 and piggery wastewater. The microalgae produced fatty acids, comprising mainly lauric acid (C12:0), tridecanoic acid (C13:0), palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). C16:0 and C18:3 were abundant in *Scenedesmus* sp. cells under photoautotrophic cultivation with CO_2, while that of C12:0 and C16:0 for mixotrophic cultivation with piggery wastewater.

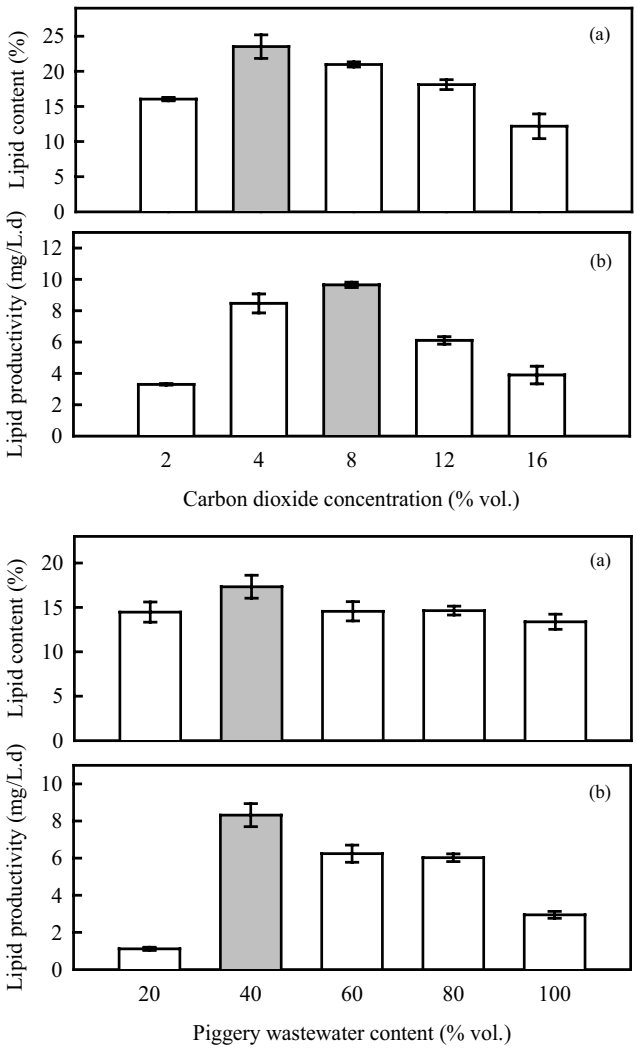

Fig. 5. Lipid content (a) and lipid productivity (b) of *Scenedesmus* sp. under different contents of CO_2 and piggery wastewater.

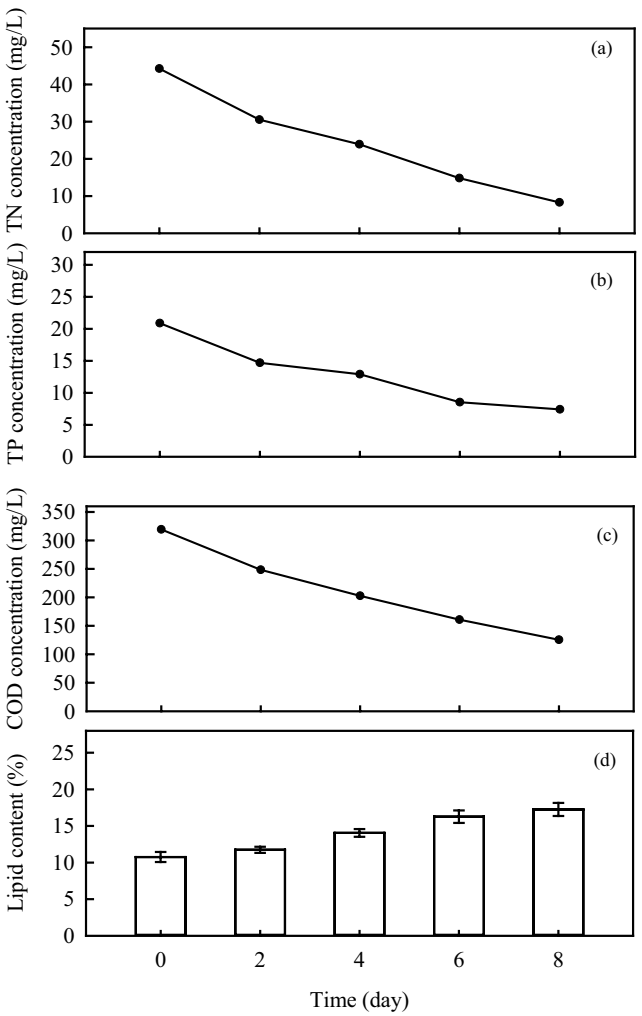

Fig. 6. Total nitrogen concentration (a), total phosphorus concentration (b), COD concentration (c), and lipid content of *Scenedesmus* sp. (d) under 40% piggery wastewater content.

Table 1
The FAME composition of *Scenedesmus* sp. grown in CO_2 and piggery wastewater, respectively

CO_2 conc. (vol.%)	C13:0	C16:0	C16:1	C18:2	C18:3
2	11.04 ± 0.25	28.24 ± 0.84	10.32 ± 0.41	14.64 ± 0.20	35.75 ± 0.48
4	9.11 ± 0.22	30.12 ± 2.49	10.05 ± 0.04	15.11 ± 0.75	35.61 ± 2.00
8	9.10 ± 0.43	33.70 ± 0.00	10.00 ± 0.11	17.28 ± 0.13	29.92 ± 0.40
12	10.76 ± 1.19	28.91 ± 1.67	11.05 ± 1.24	18.35 ± 0.55	30.92 ± 1.07
16	13.00 ± 0.12	32.41 ± 0.37	9.75 ± 0.17	15.68 ± 0.04	29.16 ± 0.38

Piggery wastewater content (vol.%)	C12:0	C13:0	C16:0	C16:1	C18:2	C18:3
20	19.27 ± 0.02	11.71 ± 0.08	24.96 ± 0.17	9.52 ± 0.09	7.22 ± 0.26	27.31 ± 0.11
40	45.06 ± 0.52	11.49 ± 0.15	16.34 ± 0.08	9.08 ± 0.08	9.13 ± 0.23	8.90 ± 0.30
60	45.67 ± 0.28	11.11 ± 0.95	16.08 ± 0.23	8.74 ± 0.03	8.62 ± 0.48	9.79 ± 0.49
80	43.85 ± 1.00	10.49 ± 0.60	17.38 ± 0.03	9.36 ± 0.14	7.56 ± 0.19	11.36 ± 0.10
100	18.11 ± 5.90	12.96 ± 0.20	24.66 ± 2.25	12.36 ± 1.00	11.59 ± 1.02	20.32 ± 1.82

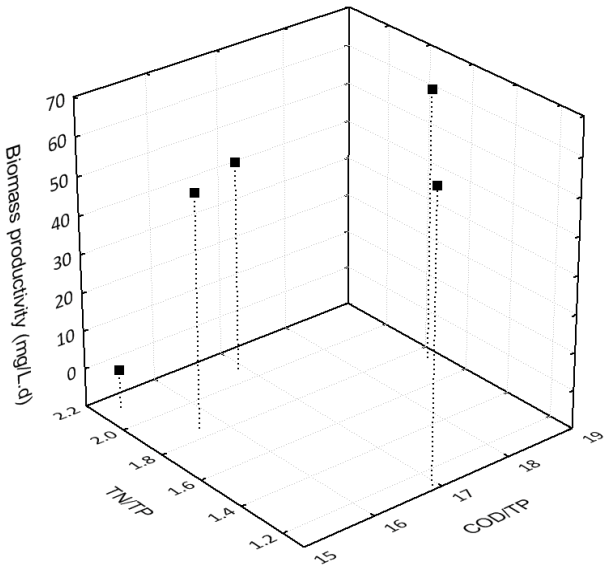

Fig. 7. The effect of ratio of TN, TP, and COD on biomass productivity of *Scenedesmus* sp.

The combined percentages of each these two fatty acids were 63.6% and 61.4% of the total fatty acids for 8% CO_2 and 40% piggery wastewater content, respectively, thereby being suitable for applications in the biodiesel industry [23].

3.4. Integration biomass productivity and nutrient removal

Fig. 6 shows the changes in TN, TP, COD, and cell lipid content with time under 40% piggery wastewater cultivation. TN decreased from 44.3 to 8.2 mg L^{-1} and TP dropped from 20.9 to 7.4 mg L^{-1} after 8 d of cultivation (Figs. 6(a) and (b)). The COD drastically decreased from 319.4 to 125.4 mg L^{-1} in the same period (Fig. 6(c)). The removal efficiencies of TN, TP, and COD are 81.5%, 64.6%, and 60.7%, respectively. The decrease in nutrients resulted in an increase in cell lipid content (Fig. 6(d)). Specific nutrient removal rates of 4.51 mg N L^{-1} d^{-1}, 1.69 mg P L^{-1} d^{-1}, and 24.25 mg COD L^{-1} d^{-1} were obtained. These results are consistent with the nitrogen and phosphorous removal rates reported by Ruiz et al. [24] for a culture of freshwater *S. obliquus* with removal rates of 13.5–4.2 mg N L^{-1} d^{-1} and 1.49–0.32 mg P L^{-1} d^{-1} in a synthetic medium.

The effect of COD/TP and TN/TP on BP of *Scenedesmus* sp. is shown in Fig. 7. The better performance of BP was found when supplied with a high COD/TP and low TN/TP in piggery wastewater content. The high BP was observed at 16.9:1.1:1 of COD/TN/TP of piggery wastewater. Accordingly, nutrient removal and simultaneous biomass production by *Scenedesmus* sp. grown in piggery wastewater as >15:1 of COD/TP and <2:1 of TN/TP could be concluded in the study.

4. Conclusion

The study investigated the variations of microalgae in the constructed wetland during 1 year monitoring to bioprospect the potential microalgal species on biomass production and simultaneous biodiesel production. *Scenedesmus* sp. was the abundant strain in the constructed wetland. The optimal CO_2 and piggery wastewater content for the microalgal growth were 8% and 40%, respectively. The BP of *Scenedesmus* sp. was high in >15:1 of COD/TP and <2:1 of TN/TP of piggery wastewater, which also showed high nutrient removal efficiency, thereby integrating piggery wastewater treatment with algae biomass production, producing biodiesel practically and economically under mixotrophic growth.

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