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

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Abstract The present study firstly investigates the variations of microalgae in the constructed wetland. Then one of the potential microalgal species was isolated and cultured in autotrophic and mixotrophic growth 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 one year of monitoring. Under both autotrophic and mixotrophic cultivation conditions, an appropriate composition of each source was beneficial for respective biomass production, 8% (v/v) CO_2, and 40% piggery wastewater content. The specific growth rate and lipid productivity obtained with autotrophic growth were slightly higher than those obtained by mixotrophic growth. Both cultivation conditions led to dissimilar fatty acid compositions. Comparing the autotrophic and mixotrophic growth, the mixotrophic cultivation not only produced biomass, but also could assimilate up to 81.5% total nitrogen, 64.6% total phosphorus, and 60.7% chemical oxygen demand (COD) from piggery wastewater, respectively. The high biomass productivity was observed at 16.9:1.1:1 of COD/TN/TP of piggery wastewater.

Keywords: Biomass, *Scenedesmus*, wastewater

1. Introduction

Microalgae are considered a potential source of 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 (Sander and Murthy, 2010). The integration of algal biomass production and wastewater treatment is considered to be one of the most viable strategies for the cost reduction in algae-based biodiesel industry. Biodiesel production from algae 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 glycerol resulted in higher fatty acid methyl esters (FAMEs) productivity, an increase of over 72% compared to photoautotrophic culture (Das *et al*., 2011). Bohutskyi *et al*. (2016) 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 d^{-1} to 0.7-0.9 d^{-1} and biomass productivity from 10 to 20 mg/L.d to 40-60 mg/L.d with greater improvements for secondary effluents. Sydney *et al*. (2011) 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%. The lipid accumulation of microalgae in wastewater depends on growth conditions, especially the nutrients content (Chisti 2007; Pittman *et al*. 2011). Li *et al*. (2010) 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 (Scragg *et al*. 2002). Hence, high lipid productivity associated with biodiesel production needs both 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 (Wilkie *et al*. 2011). The specific objectives of this study was to investigate firstly 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 cultured in autotrophic and mixotrophic growth to compare the performance on biomass production and to evaluate the ability of wastewater treatment and simultaneous biodiesel production.

2. Materials and Methods

a. Collection and isolation of microalgal samples

Water samples (10 L each) were taken at three depths (0.2 m, 0.5 m, 0.8 m below the surface) of the water column at Linluo constructed wetland in southern Taiwan (20°39’02’’N; 120°31’38’’E). A 1000-mL of each sample, 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. The water sample was stored in a transparent plastic bottle in a dark environment and sent to the laboratory within 24 hours for algal cell identification and isolation. The strains were identified for taxonomy using Standard Methods (APHA, 1995) and morphology (Chen and Lee, 2016).

b. Microalgal culture

One of the dominant green algal species, *Scenedesmus* sp., was isolated from the sample water and cultured in a medium according to a method given by Norris *et al*. (1955). The microalgae that reached enough seed culture was inoculated in batch mode in a 1-L modified serum bottle containing 600 mL of pretreated piggery wastewater and Norris medium, respectively. The bottles were placed in the incubator, which continuously provided 5 kLux of illumination, 25 °C of temperature, and 0.8 L/min of air aeration. 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 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 days for experiments.

c. 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 hours. 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 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_0 are the dry weights of the microalgal biomass at times T_1 and T_0, respectively. The dry weight of cells was A stock culture of *Scenedesmus* sp. cells was collected by centrifugation at 2000 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) in a Soxhlet extractor and quantified gravimetrically. The lipid content (g g^{-1}) is expressed as the dry weight of the microalgal biomass. The biomass productivity was calculated from cultivation time (d) and biomass concentration (mg/L) (Chen and Lee, 2016).

d. Nutrients analysis

The samples were first filtered through a 0.45-μm membrane and then the filtrate was properly diluted and analyzed for chemical oxygen demand (COD), TN, and total phosphorus (TP). COD, TN, and TP concentrations were determined according to the Methods for Examination of Water and Wastewater (APHA, 1995). The characteristics of piggery wastewater used in this experiment were COD in the range of 98.2-492 mg/L, TN of 8.8-44.1 mg/L, and TP of 0.9-4.7 mg/L.

e. 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) was followed given by Chen and Lee (2016). The temperature program 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

a. Phytoplankton community composition of the Wetland

The whole Linluo constructed wetland mainly receives domestic and piggery wastewater, covering overall surface water area of 3.49 ha with a hydraulic retention time of 4.3 days and average water depth of less than 1.5 m. Fig. 1 shows a total of 4 phyla (22 total species), namely Bacillariophyta (8 species), Chlorophyta (8 species), Euglenophyta (2 species), and Cyanophyta (4 species) in the local wetland sample. Bacillariophyta and Chlorophyta made up the most significant proportion of the community composition. Chlorophyta accounted for the most species. One species of the Chlorophyta, *Scenedesmus* sp., was the second most abundant strain (4.43×10^4 cells/mL) for one year monitoring.

b. Optimization of photoautotrophic and mixotrophic growth

Photoautotrophic cultivation occurs when the microalgae use 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 undergo photosynthesis and use both organic compounds and inorganic carbon (CO_2) as a carbon source for growth. The cell growth, specific growth rate, and biomass productivity for various CO_2 is shown in Fig. 2. It can be seen that *Scenedesmus* sp. 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, and 0.136 d^{-1}, respectively. 8% CO_2 content resulted in the highest cell gworth, higher CO_2 concentration did not further improve cell growth. Similar results were reported for *Scenedesmus obliquus* by Kaewkannetra *et al*. (2012). 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 cultured at ranged from 40% to 80% piggery wastewater content (Fig. 3). High biomass productivity (48 mg/L.d) was found at 40% piggery wastewater content. This 40% piggery wastewater content culture reached 48 mg/L·d lipid productivity, compared to that obtained with the culture under 8% CO_2 content (46 mg/L.d).

c. Effect on lipid productivity and biodiesel composition

Fig. 4 shows the effects of CO_2 and piggery wastewater cultivation 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.d) 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 better performance of lipid productivity (8.3 mg/L.d) was obtained with 40% piggery wastewater content, similar to that of biomass productivity. 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 CO_2 cultivation, while that of C12:0 and C16:0 for piggery wastewater cultivation. 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.

d. Integration biomass productivity and nutrient removal

Fig. 5 shows the changes in TN, TP, and cell lipid content with time under 40% piggery wastewater cultivation. TN decreased from 44.3 mg/L to 8.2 mg/L and TP dropped from 20.9 mg/L to 7.4 mg/L after 8 days of cultivation (Fig. 5A, 5B). The COD drastically decreased from 319.4 mg/L to 125.4 mg/L in the same period (Fig. 5C). 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. 5D). Specific nutrient removal rates of 4.51 mg N/L·d, 1.69 mg P/L·d, 24.25 mg COD/L.d were obtained. These results are consistent with the nitrogen and phosphorous removal rates reported by Ruiz *et al*. (2013) for a culture of freshwater *Scenedesmus obliquus* with removal rates of 13.5-4.2 mg N/L·d and 1.49-0.32 mg P/L·d in a synthetic medium. The effect of COD/TP and TN/TP on biomass productivity of *Scenedesmus* sp. is shown in Fig. 6. The better performance of biomass producutivity was found when supplied with a high COD/TP and low TN/TP in piggery wastewater content. The high biomass productivity was observed at 16.9:1.1:1 of COD/TN/TP of piggery wastewater, while that of 15:2.1:1 for low biomass productivity.

4. Conclusion

The study investigated the variations of microalgae in the constructed wetland for one year monitoring to bioprostect the potential microalgal species on biomass production and simultaneous biodiesel production. It was concluded that (1) *Scenedesmus* sp. was the abundant strain in the constructed wetland; (2) the optimal CO_2 and piggery wastewater content for cell growth wer 8% and 40%, respectively; and (3) *Scenedesmus* sp. is a potential strain for integrating piggery wastewater treatment with algae biomass production, produced biodiesel practically and economically under mixotrophic growth.

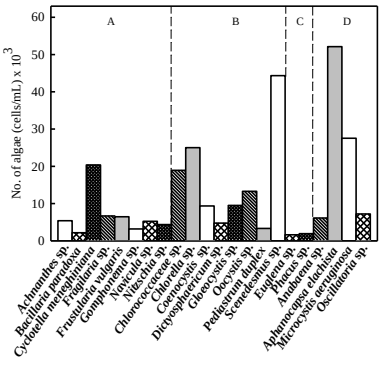

Fig. 1 Concentrations of various algal species in Linluo constructed wetland (A: Bacillariophyta; B: Chlorophyta; C: Euglenophyta; D: Cyanophyta).

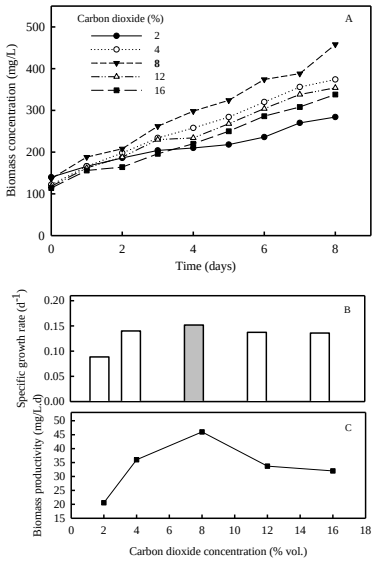

Fig. 2. Cell growth (A), specific growth rate (B), and biomass productivity (C) in various CO_2 content.

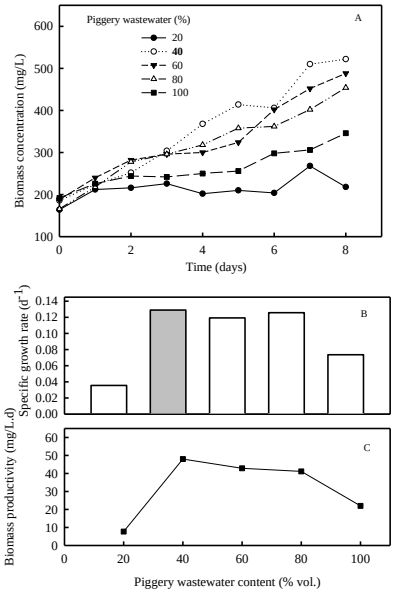

Fig. 3. Cell growth (A), specific growth rate (B), and biomass productivity (C) in various piggery wastewater content.

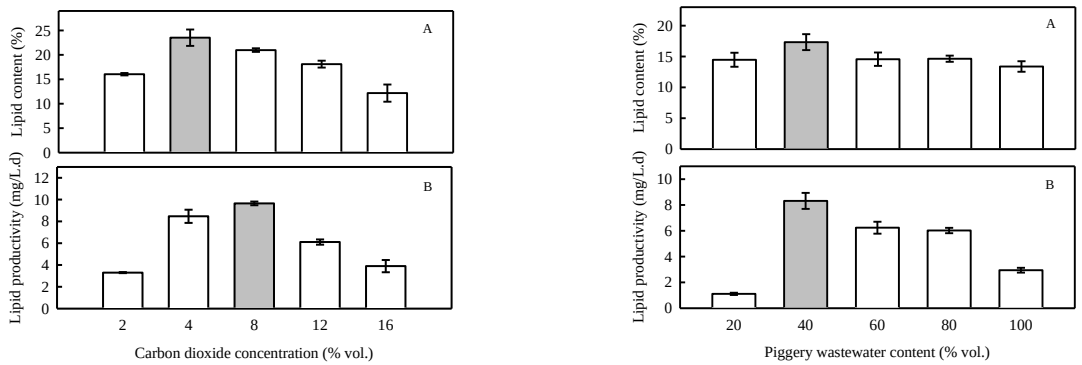

Fig.4. Lipid content and lipid productivity in various content of CO_2 (A, B) and piggery wastewater cultivation (C,D).

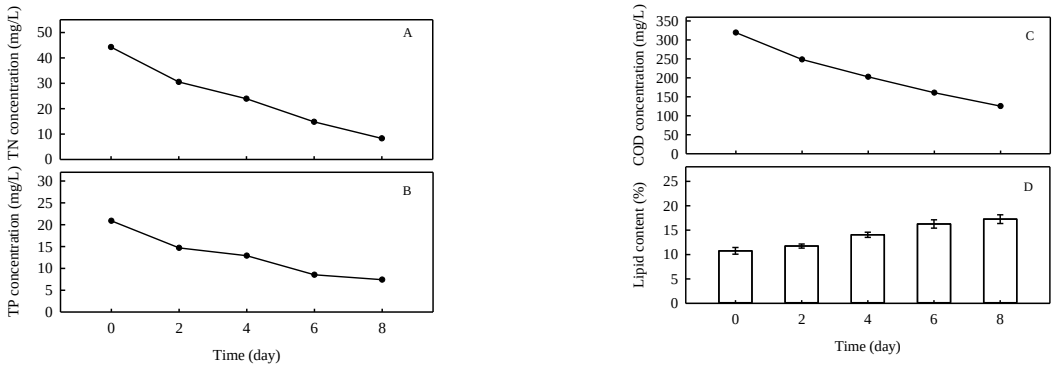

Fig.5. Total Nitrogen concentration (A), total phosphorus concentration (B), COD concentration (C), and cell lipid content (D) during batch culture on 40% piggery wastewater.

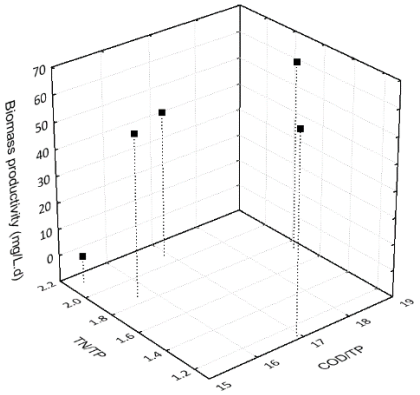

Fig. 6. The effect of ratio of TN, TP, and COD on biomass productivity of *Scenedesmus* sp.

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

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

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