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Microalgal Cultivation and Nutrient Removal from Digested Piggery Wastewater in a Thin-film Flat Plate Photobioreactor

Zhong-liang Sun[1] · Li-qin Sun[1] · Guo-zhong Chen[2]

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Abstract

This work investigated the cultivation of *Chlorella vulgaris* in a thin-film flat plate photobioreactor under outdoor conditions and using digested piggery wastewater as the culture medium. The algal cells were able to adapt quickly to the wastewater and outdoor conditions. A specific growth rate of 0.12 day^{-1} was obtained in the exponential growth phase, which was slightly higher than that during indoor cultivation using artificial culture medium. Results showed that *Chlorella vulgaris* effectively removed TN, TP, and COD by 72.48%, 86.93%, and 85.94%. Due to the difference in culture conditions and phosphorus availability, the biomass from outdoor cultivation contained higher lipid content and more unsaturated fatty acids compared to indoor cultures, while the amino acid composition was unaffected. Results of metallic element assay indicated that the biomass cultured with wastewater conformed to the standards required for animal feed additive production. The overall cost of the biomass production in the thin-film flat plate photobioreactor (32.94 US$/kg) was estimated to be 4.67 times lower than that of indoor cultivation (154.04 US$/kg). Together, these results provide a basis for large-scale outdoor production of microalgae and wastewater bioremediation.

Keywords *Chlorella vulgaris* · Digested piggery wastewater · Outdoor cultivation · Thin-film flat plate photobioreactor

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✉ Li-qin Sun
sliqin2005@ytu.edu.cn

[1] College of Life Sciences, Yantai University, Yantai 264005, People's Republic of China

[2] School of Life Sciences, Ludong University, Yantai 264025, People's Republic of China

Introduction

Microalgae technology is attracting considerable attention nowadays as a result of its potential as high-quality feedstock for production of renewable fuel, high-value pigments, biofertiliser, and animal feed supplements [1]. However, the algal-based commercial application is impeded due to the high cost of microalgae cultivation [2]. The use of chemical nutrients and fresh water in the cultivation are important cost factors, accounting for 23 to 30% of the total production cost [3]. There is no doubt that these barriers decrease the sustainability and competitiveness of microalgae-based technologies. In fact, the development of the livestock and poultry industry has led to an excessive disposal of waste into water bodies, thus reducing water quality and damaging aquatic ecosystems; many animal wastewaters have nutrient compositions similar to classic microalgae culture medium [4]; therefore, a coupled process combining wastewater treatment with microalgal biomass production could be an economically feasible option. Indeed, there is an increasing interest in using wastewater to grow microalgae because it provides an environmentally friendly method of bioremediation while reducing the costs of algal feedstock production [5, 6].

Numerous studies have demonstrated the feasibility of growing microalgae on digested piggery wastewater, which is one of the richest primary nutrient streams of nitrogen and phosphorus as well as micronutrients [3, 7]. Meanwhile, the genera *Chlorella* is one of the microalgal strains most frequently used because of its high adaptability in wastewater, high biomass productivity, and efficient nutrient removal ability [8, 9]. Besides, high-rate ponds are an efficient bioreactor used to produce microalgae and treat wastewater, which offer various advantages such as flexibility, low power consumption, and simple construction [10]. Nevertheless, there are some problematic issues accompanying the use of livestock wastewater for microalgae cultivation: for instance, the high turbidity in digested piggery wastewater because of the presence of solid particles that might influence light transmission [5]; the high nitrogen concentration might be toxic to microalgae, particularly when in the form of ammonium [11]. Some of these issues could be addressed through diluting the wastewater 20–100 times with fresh water [12]; however, the method of dilution with fresh water typically increases treatment costs and has relatively low techno-economic feasibility. In addition, open raceway ponds require a large land area to produce sufficient amounts of algal biomass, since the volume specific surface area of the bioreactor and the algal cell concentrations are typically lower than in a closed photobioreactor [13]. It is well known that the shortage of available land area is the weakest point of many pig-raising enterprises and wastewater treatment stations. Thus, the use of low-cost closed photobioreactors is a potential solution to reduce the required land area and improve nutrient removal.

In light of the above discussion, the objective of this work is to assess the production of *Chlorella vulgaris* in a thin-film flat plate photobioreactor using digested piggery wastewater, and also contributing to the recovery of nutrients from these effluents. As a comparison, indoor cultures of *Chlorella vulgaris* using artificial medium were conducted in controlled conditions. Fatty acid content and composition, protein content and amino acid composition, the metallic elements in the biomass harvested from wastewater, and artificial medium were investigated and compared. The cost of each cultivation system was calculated and assessed. It is hoped that the results of this study could provide a scientific basis, and support, for large-scale outdoor commercial production of microalgae and wastewater bioremediation.

Materials and Methods

Characteristics of Wastewater

The wastewater used in this study was collected from a pig farm located in Laiyang City, Shandong Province, China. The piggery wastewater was firstly digested in an anaerobic reactor for producing biogas and the effluent after separation of biogas residue was used as wastewater for algal cultivation; because of the high population of anaerobic bacteria originating from the biogas unit, which competed with microalgae cells for available nutrient, the addition of NaClO was used to sterilise the raw wastewater. Before each experiment, the precipitate resulting from oxidation was passed through a three-layer gauze filter. The characteristics of the sterilised wastewater are summarised in Table 1.

Microalgae, Inoculum Preparation, and Culture Conditions

A freshwater green algae *Chlorella vulgaris* was selected as the inoculation candidate due to its good performance in wastewater treatment. The microalgal strain was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, and the cell line number was FACHB-24. Inoculation was performed under sterile conditions, and it was cultivated in 100 mL of autoclaved BG11 medium in 250 mL conical flasks and expanded into a column photobioreactor with working volume of 1.0 L. The seed broth for outdoor batch experiments was prepared using 50% (*v*/*v*) wastewater as a culture medium. Microalgal cells were cultured in the column photobioreactor using white fluorescent tubes at a light intensity of 100 μmol/(photons m^2 s), and the culture photobioreactor was maintained at room temperature. For all batch experiments, the inoculum showed the chlorophyll concentration of 3 to 5 mg L^{-1}, which corresponded to a biomass of 0.05 to 0.10 g L^{-1}.

The experiments were performed in two groups: in the first group, experiments were run indoors using two rectangular glass tanks. The tank had a total volume of 37.5 L (a working volume of 25 L, 50 cm long × 15 cm wide × 50 cm height) and an illuminated area of 0.25 m^2 (supplementary material-Figure 1). To ensure a well-mixed medium and stabilised pH environment, one micro-bubble air diffuser (45 cm long × 1 cm diameter) was installed on the bottom of the glass tank to supply a gas mixture at 0.2 vvm (containing 2.0% CO_2). The culture temperature was maintained at room temperature. The average light intensity was kept

Table 1 Characteristics of digested piggery wastewater and BG11 medium for cultivating *Chlorella vulgaris*

Parameter	Wastewater	BG11
pH	8.0 ± 0.3	7.6 ± 0.2
Chemical oxygen demand (COD) (mg L^{-1})	813.12 ± 39.83	71.22 ± 5.36
Total nitrogen (mg L^{-1})	421.50 ± 9.19	120.00 ± 4.11
Total phosphorus (mg L^{-1})	5.00 ± 0.28	10.00 ± 0.2
Cu (mg L^{-1})	0.26 ± 0.01	0.02 ± 0.00
Fe (mg L^{-1})	1.29 ± 0.07	1.28 ± 0.04
Zn (mg L^{-1})	1.06 ± 0.03	0.05 ± 0.01
Mn (mg L^{-1})	0.12 ± 0.01	0.51 ± 0.07
As (mg L^{-1})	< 0.0006	–
Pb (mg L^{-1})	< 0.02	–

at 150 μmol/(photons m^2 s), and the light-dark schedule was 12 h on and 12 h off. One culture cycle lasted for 25 days.

The outdoor experiments of the second group were conducted under natural temperature and light conditions in the wastewater treatment station of a pig farm located in Laiyang City, Shandong Province, China (latitude 36° 34′ N, longitude 120° 31′ E), in summer (2 July to 28 July, 2017). Several thin-film flat plate photobioreactors were used, which were constructed from transparent polyethylene sheet with a thickness of 0.2 mm supported by stainless steel. The flat plate photobioreactors measured 80 cm long, 10 cm wide, and 120 cm in height and maintained a working volume of 70 L (supplementary material-Figure 2). Air was supplied using an air-sparger with the flow rate of 0.2 vvm at the bottom of each bioreactor. For the 25 L indoor culture, the modified BG11 medium containing 120 mg L^{-1} N-NO_3^- and 10 mg L^{-1} P-$H_2PO_4^-$ was used and the wastewater from anaerobic digestion unit was used for the 75-L outdoor culture.

For *Chlorella vulgaris* growth, the modified BG11 medium and wastewater were first sterilised by adding NaClO with the concentration of 1.0 mL L^{-1}, which contained 130 mg available chlorine per litre. After 12 h of disinfection, it was neutralised with $Na_2S_2O_3$ until the potassium iodide-starch test paper did not change colour. Before each experiment, the wastewater was filtered by a three-layer gauze to remove some precipitate formed by oxidation.

Determination of Microalgae Growth

The growth of algal cultures is expressed usually as the increment of biomass number of cells, amount of protein, pigments, etc., over a given period of time [14]. In this study, microalgae growth was evaluated via the chlorophyll concentration and the biomass dry weight. The chlorophyll concentration was determined as follows [7]: we centrifuged 10 mL of microalgal suspension at 9000 rpm for 5 min and discarded the supernatant, suspended the cells in 3 mL of the 90% methanol, then heated the suspension for about 5 min in a water bath at 80 °C, made the volume up to 5 mL, and if the pigment extract was too concentrated, further dilution was necessary until the absorbance could be read. The chlorophyll concentration in the extract was calculated by reading the absorption (A) of the pigment extract in a spectrophotometer at a given wavelength against a solvent blank by using the following equation:

$$\text{Chlorophyll}_{a+b}\left(\text{mg } L^{-1}\right) = (4.0 \times A_{665}) + (25.5 \times A_{650}) \tag{1}$$

Algal growth was measured every 5 days by measuring the concentration of the chlorophyll using a UV-2100 spectrophotometer (Unico, Shanghai, China), and the maximum specific growth rate (μ_{max}, day^{-1}) in the exponential stage was calculated as follows:

$$\mu_{\max}\left(d^{-1}\right) = \frac{\ln Chl_2 - \ln Chl_1}{t_2 - t_1} \tag{2}$$

where Chl_1 and Chl_2 were the chlorophyll$_{a+b}$ concentrations (mg L^{-1}) at times t_1 and t_2, respectively.

At the end of the batch experiments, the algal culture broth was harvested by centrifugation for 5 min at 9000 rpm. The algal pellets were then washed three times with distilled water and dried at 105 °C to a constant weight. The biomass dry weight (g L^{-1}) of the algal cells was determined gravimetrically.

Water Sampling and Chemical Analysis

The nutrient concentrations during the batch experiment were evaluated for the samples collected every 5 days. The samples collected from different culture condition groups were centrifuged at 9000 rpm, and the upper aqueous layer was appropriately diluted and analysed for COD, TN, and TP. The standard protocols of Water and Wastewater Analysis Methods were used to characterise the different nutrient concentrations in wastewater [15]. The removal efficiency of different nutrients was calculated according to the following equation:

$$E(\%) = \frac{C_t \times V_t - C_0 \times V_0}{C_0 \times V_0} \tag{3}$$

where, t corresponds to time, $E(\%)$ is the removal efficiency of different nutrients (e.g. COD, TN, or TP), C_0 is the initial concentration of nutrient, V_0 is the initial volume of the cultivation system, C_t is the concentration of nutrient at cultivation time t, and V_0 is the volume of the cultivation system at time t.

Trace elements and heavy metals in the wastewater, the normal culture medium, and the algal cells were measured by ICP-OES (Optima 2100DV, UK) after digestion using a microwave digestion instrument [16]. All assays were performed in triplicate, and the results of the study were expressed as the mean ± standard deviation of the replicates.

Algal Biomass Analysis

Lipid and Fatty Acids Analysis

Initially, the microalgal cell was harvested by centrifugation and then lyophilized. The modified method reported by Bligh and Dyer was used for extraction of total lipid from dry biomass, and the lipid content was quantified gravimetrically. The fatty acids methyl esters (FAMEs) were analysed according to a previously described method [17].

Protein and Amino Acid Analysis

The total protein content was determined using the Lowry method as described by Safi and Charton [18]. For the amino acid composition analysis, the protein was hydrolysed using 4.2 mol L^{-1} NaOH for tryptophan, performic acid for cysteine, and 6 mol L^{-1} HCl for the other amino acids. The hydrolysed amino acid samples were separated using ion exchange chromatography and quantified using an automatic amino acid analyser (Hitachi L8800, Japan).

Economic Feasibility Assessment

The total cost of the cultivation process contains both a fixed capital investment and operational costs [13]. In this study, the fixed investments included the construction of the photobioreactor, the air-supply system, and the illumination unit. It is worth noting that the costs should be calculated based on the batch experiments and the estimated lifetime of the equipment (Supplementary material-Table 1, Table 2). The process electricity consumption was calculated based on the instruments and their operating time: the unit electricity price was calculated on the basis of the local price ($ (USD equivalent) 0.078/kW h). The cost of

medium was estimated based on the market price listed on the Aladdin Chemical Website (http://www.aladdin-e.com/). In this study, the investment capital and process power consumption of indoor and outdoor cultivation are as listed in Table 6.

Results and Discussion

Physico-chemical Analysis of the Culture Medium

The physico-chemical characteristics of the digested piggery wastewater and artificial culture medium are listed in Table 1. After being pre-treated as described above, concentrations of COD, TN, and TP were 813.12 mg L^{-1}, 421.50 mg L^{-1}, and 5.00 mg L^{-1} in the digested piggery wastewater, while in artificial BG11 culture medium, they were 71.22 mg L^{-1}, 120.00 mg L^{-1}, and 10.00 mg L^{-1}, respectively. The wastewater used in other reports of different authors is usually diluted before inoculating the microalgal cells due to the high concentration of COD [3, 12]; however, based on the indoor acclimation results of microorganism used in this study, it was observed that algae grew normally in raw digested wastewater without dilution. Besides, the COD mainly consisted of acetic acid, propionic acid, and butyric acid and surplus sugar remaining after the anaerobic process, which was a readily available carbon source for microalgae growth [19]. Adding components such as citric acid, EDTA, and vitamins in artificial BG11 medium meant that the COD content was 71.22 mg L^{-1}: $NaNO_3$ and K_2HPO_4 were used as nitrogen, and phosphorus, sources for indoor cultivation. $N\text{-}NH_4^+$ was the main species of total nitrogen found in the wastewater and was the preferred source of nitrogen for microalgal growth; however, higher levels of ammonia may inhibit cell growth [20]; therefore, a high rate of flux of air was used to aerate the medium after sterilisation in this study, which not only helped to remove the surplus active chlorine but also helped in the volatilisation of NH_3. The concentration of TP was governed by environmental conditions, such as pH and oxidising environment, for pH values above 8.0 and high concentration of active chlorine, phosphorus precipitation may occur [21], hence the lower content of TP in wastewater compared to that in artificial medium. Besides the macronutrients, some metallic elements (e.g. Cu, Fe, Zn, Mn, As, and Pb) present in the wastewater were also assayed and compared with the normal medium used in large-scale microalgal cultivation. Except for the concentrations of Cu and Zn, the amounts of Fe and Mn in wastewater were consistent with those in BG11 medium. The toxic metal content in the wastewater was very low. According to the results of a comparison between the digested piggery wastewater and BG11 medium, the wastewater was suitable for microalgal cultivation without dilution, but, it is necessary to

Table 2 Growth kinetic parameters of the algal cells under different culture conditions and the removal efficiency of different nutrients

Culture condition	Maximum specific growth rate (day^{-1})	Maximum biomass concentration (g/L)	COD removal efficiency (%)	TN removal efficiency (%)	TP removal efficiency (%)
25 L indoors	0.07 ± 0.01 a	0.63 ± 0.05 a	–	32.44 ± 7.59	100.00 ± 0.00
70 L outdoors	0.12 ± 0.03 b	0.61 ± 0.04 b	85.94 ± 1.70	72.48 ± 10.50	86.93 ± 2.49

Values are means ± SD, $n = 3$ or 4. Values in a column with different letters are significantly different ($P < 0.05$) according to a t test

evaluate the safety thereof if the harvested biomass were to be developed for animal foodstuff or fish bait.

Algal Growth and Nutrient Removal Efficiency

In this study, the growth of algal cultures was expressed as the increment of chlorophyll concentration, and the result is shown in Fig. 1. After a 5-day lag period, the algal cells were able to adapt to the 2.0% CO_2 gas mixture and BG11 as growth substrates in the two 25-L bioreactors. The cell growth curves of two glass flat plate photobioreactors were similar because the same indoor culture conditions were used in each. Following the lag period, the cells entered the exponential phase, and continued until the end of the cultivation, indicating the suitability of indoor controlled conditions and a sufficiency of nutrients in the medium for microalgal cultivation. As shown in Table 2, the maximum specific growth rate of the indoor cultures was 0.07 day^{-1}. This resulted in a maximum of chlorophyll concentration as high as 18.34 mg L^{-1}, and 0.63 g L^{-1} biomass dry mass. As illustrated in Fig. 1 and Table 2, it is estimated that a higher biomass concentration would be reached if the cultivation time was prolonged because sufficient of the nitrogen source had not yet been utilised (the removal efficiency was only 32.44%); however, we failed to detect elemental phosphorus in the upper liquor after removing the microalgal cells. Similar results have been reported by others [22, 23]. This phenomenon in the culture process is due to the fact that orthophosphate is the best nutrient source for cell growth and it is usually absorbed excessively under optimal culture conditions.

For the outdoor culture, the algal cells were able to adapt quickly to outdoor conditions in the 75 L thin-film flat plate photobioreactors. After inoculation, the cells entered the exponential phase, and they reached the stationary phase at 10 days (Fig. 1). As shown in Table 2, the maximum specific growth rate in the exponential phase (before day 10) was

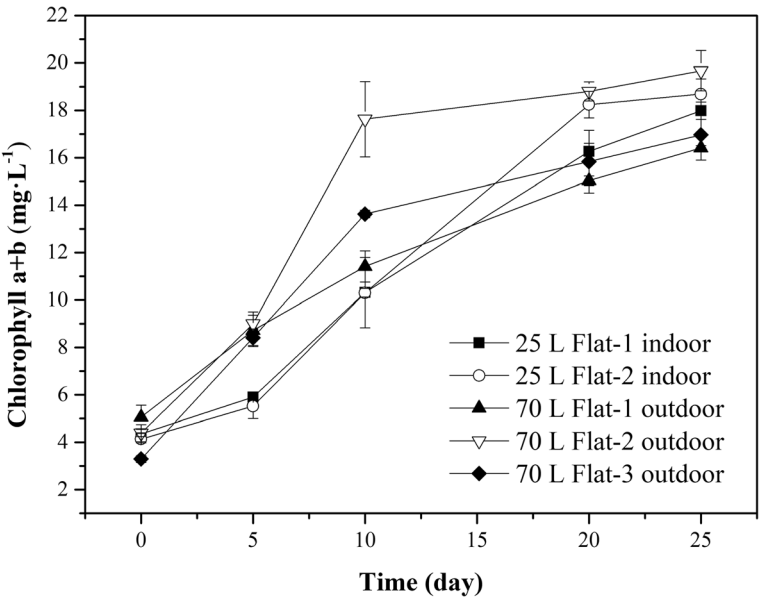

Fig. 1 The growth curve of *Chlorella vulgaris* under different cultivation conditions

0.12 day^{-1}, which was significantly higher ($P < 0.05$) than that under indoor cultivation (0.07 day^{-1}) according to a *t* test. This difference in growth rate is due to the fact that the algal cells are cultured under a higher light intensity (approximately 2000 μmol/(photons m^2 s) at midday) and over a longer day length (approximately 12 to 14 h day^{-1}) (Supplementary material-Figure 3), also indicating that digested piggery wastewater is suitable for microalgae cultivation. Nevertheless, the microalgal cells grew slowly from day 10 to the end of cultivation; this was accompanied by a lower average specific growth rate (0.015 day^{-1}), and 0.61 g L^{-1} biomass dry mass. As shown in Fig. 2, it can be seen that the nutrient source of phosphorus in wastewater was almost exhausted in the exponential growth phase. Besides, organophosphorus is the main species of TP in wastewater, and it should be digested to orthophosphate by bacteria before being assimilated by microalgal cells [24].

The removal capacity of various parameters such as TN, TP, and COD by *Chlorella vulgaris* was determined in the digested piggery wastewater during the incubation time (Fig. 2 and Table 2). Similar to the cell growth data, the maximum nutrient removal rates of microalgae were also observed during the exponential growth phase and strongly correlated with the microalgal growth. The nutrient removal percentages of TN, TP, and COD were found to be 72.48%, 86.93%, and 85.94%, respectively, as observed in the 75-L outdoor cultivation group, while nutrient removal percentages of 32.44% and 100.00% for TN and TP were found in the 25-L indoor cultivation group (Table 2). It was observed that the removal efficiency was found to be higher with orthophosphate in artificial culture medium compared to that in wastewater. In the literature, it was also reported that orthophosphate is the best source of phosphorus as it can be excessively absorbed by most microalgal species [25]. In outdoor culture, *Chlorella vulgaris* growth achieved high COD removal capacity. As shown in Fig. 2, the final concentration of COD reached 114.63 mg L^{-1}. Its value met the standards for effluent discharge from livestock and poultry (GB18596–2001, China). *Chlorella* strains generally have the ability to utilise organic substrates as carbon source for heterotrophic or mixotrophic growth [3]. Thus, their growth can effectively remove COD from various wastewater streams. Tan et al. (2018) reported that more than 90% of COD was removed from anaerobically digested starch wastewater by culturing *Chlorella*

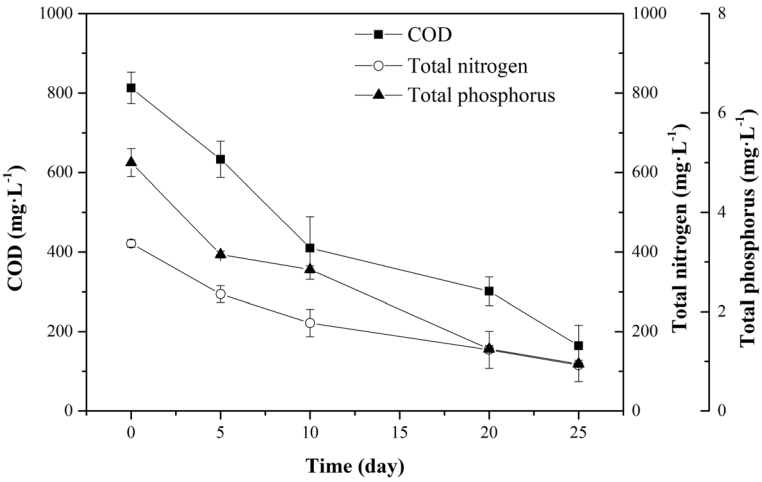

Fig. 2 Changes in TN, TP, and COD concentrations under outdoor cultivation

Table 3 The fatty acid composition of biomass cultured in different conditions

FA composition	FA content (% of total FA)	
	25 L indoors	70 L outdoors
12:0	0.94 ± 0.11	–
14:0	0.96 ± 0.14	0.58 ± 0.06
16:3	1.37 ± 0.01	3.37 ± 0.04
16:2	2.51 ± 0.08	6.52 ± 0.48
16:1	0.61 ± 0.86	–
16:0	19.51 ± 1.27	17.68 ± 0.68
18:3	17.29 ± 3.41	12.59 ± 0.39
18:2	9.63 ± 0.49	17.38 ± 0.92
18:1	12.35 ± 0.64	20.95 ± 1.26
18:0	–	2.35 ± 0.10
Others	34.82 ± 6.63 a	18.58 ± 2.87 b
C16	24.00 ± 2.04 a	27.57 ± 0.17 a
C18	39.28 ± 3.27 a	53.27 ± 2.67 b
SFA	21.41 ± 1.52 a	20.61 ± 0.37 a
MUFA	12.96 ± 1.50 a	20.95 ± 1.26 b
PUFA	30.81 ± 4.00 a	39.87 ± 0.79 b
Lipid content (% of DW)	22.81 ± 3.83 a	26.32 ± 4.41 b

Values are means ± SD, $n = 3$ or 4. Values in a row with different letters are significantly different ($P < 0.05$) according to a *t* test. *SFA*, saturated fatty acid; *MUFA*, monounsaturated fatty acid; *PUFA*, polyunsaturated fatty acid

pyrenoidosa therein [16]. Ebrahimian et al. (2014) used a mixture of primary and secondary municipal wastewater to culture *Chlorella vulgaris* and also obtained 100% COD removal [10]. Conversely, the concentrations of COD always increase when the artificial culture medium was used indoors. This may have been due to the exopolysaccharide secreted by the cells and decomposition of microalgal cells [26, 27]. At the end of outdoor batch culture, a total of 72.4% of all nitrogen had been removed from this wastewater. This exceeded the amount of nitrogen assimilated by microalgal cells according to the N-balance in an algal system (Table 2). Ammonium was the main species of TN in the digested piggery wastewater, and most microalgae prefer ammonium as the nitrogen source due to other nitrogen sources having to be converted to ammonium before being assimilated into biomass [28]; however, ammonium transformations in outdoor cultivation are complicated and include direct assimilation by microalgal cells or bacteria, ammonia evaporation, and nitrification [29]. Therefore, it can be suggested that nitrogen stripping played an important role in the outdoor system.

Chemical Composition of Algal Biomass

Table 3 summarises the composition of fatty acid and lipid content at the end of batch cultivation, under different conditions. There were eight to nine acids, and the fatty acids with 16 and 18 carbon atoms accounted for most of the total fatty acids (63.28% for biomass cultured indoors and 80.84% for biomass cultured outdoors), indicating that *Chlorella vulgaris* cultured with wastewater was suitable for high-quality biodiesel production [30]. Except for these detectable fatty acids, there also others present, such as fatty acids with 10, 20, and 22 carbon atoms. The other total fatty acid content extracted from the cells cultured indoors was higher than that outdoors. The difference between the indoor and outdoor cultivated cells was

Table 4 Amino acid and protein contents of biomass cultured in different conditions

Amino acid		Content (% DW)	
		25 L indoors	70 L outdoors
Essential amino acids	Phe	1.50 ± 0.04	1.08 ± 0.01
	Thr	1.52 ± 0.11	1.20 ± 0.04
	Met	0.52 ± 0.23	0.54 ± 0.32
	Val	1.66 ± 0.40	1.25 ± 0.05
	Ile	1.06 ± 0.01	0.86 ± 0.00
	Leu	2.36 ± 0.03	1.68 ± 0.03
	Trp	0.22 ± 0.10	0.23 ± 0.07
	Lys	1.48 ± 0.26	1.05 ± 0.20
	Total	10.32 ± 0.79	7.89 ± 0.67
Non-essential amino acids	Ala	3.45 ± 0.83	1.98 ± 0.11
	Gly	1.74 ± 0.64	1.34 ± 0.15
	Tyr	0.81 ± 0.26	0.64 ± 0.06
	Ser	1.28 ± 0.37	0.99 ± 0.10
	His	0.43 ± 0.06	0.30 ± 0.01
	Arg	1.56 ± 0.07	1.28 ± 0.14
	Glu	3.64 ± 0.55	2.44 ± 0.81
	Asp	2.85 ± 0.11	2.30 ± 0.26
	Pro	2.62 ± 0.60	2.02 ± 0.46
	Cys	0.86 ± 0.15	0.77 ± 0.06
	Total	19.24 ± 3.22	14.06 ± 2.05
Essential amino acid percentage (% total amino acids)		34.91 ± 1.55 a	35.95 ± 1.71 a
Total amino acids (% DW)		29.56 ± 1.36 a	21.95 ± 1.05 b
Total protein content (% DW)		36.87 ± 1.69 a	29.22 ± 2.38 b

Values are means ± SD, $n = 3$ or 4. Values in a row with different letters are significantly different ($P < 0.05$) according to a t test

likely due to the differences in cultivation conditions. The algal cells cultured indoors are cultured in optimal controlled conditions rather than the uncontrolled outdoor natural environment. Therefore, algae cultivated outdoors are more likely to synthesise some fatty acids, which play an important role in enhancing membrane fluidity and maintaining high photosynthetic activity [31, 32]. As indicated in Table 3, the higher lipid content of 26.32% was observed in culture supplemented with wastewater and this might be mainly due to the deprivation of available phosphorus in the culture medium. Previously reported studies have also indicated that the depletion of phosphorus resulted in enhanced lipid content in microalgae [23]. In addition, the proportion of unsaturated fatty acids (including monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)) in the algal cells cultivated outdoors

Table 5 Metallic element analysis of the algal cells cultured in different conditions

Parameter	25 L indoors	70 L outdoors
Cu (mg kg^{-1})	15.71 ± 0.41 a	178.51 ± 2.33 b
Fe (mg kg^{-1})	560.73 ± 0.27 a	610.30 ± 0.17 a
Zn (mg kg^{-1})	21.05 ± 0.71 a	401.06 ± 3.53 b
Mn (mg kg^{-1})	328.51 ± 6.77 a	90.12 ± 2.41 b
As (mg kg^{-1})	–	0.47 ± 0.02
Pb (mg kg^{-1})	–	0.14 ± 0.04

Values are means ± SD, $n = 3$ or 4. Values in a row with different letters are significantly different ($P < 0.05$) according to a t test

was higher than that from indoor cultures, especially in the case of PUFA. A similar result was reported elsewhere [33]. Some unsaturated fatty acids are beneficial to treatment of cardiovascular diseases, and they are often used to enhance animal growth because of their antioxidant properties [34].

As with other *Chlorella sp.*, the microorganism used in this study is rich in high-quality proteins. And it is used to enhance animal growth when it is applied as aquaculture or animal feed [35, 36]. As listed in Table 4, the protein contents and amino acid compositions of *Chlorella vulgaris* were compared between indoor and outdoor cultivation. The cells cultured indoor had total protein contents as high as 36.87%, which was higher than that of the cells from outdoor culture, at 29.22% of dry mass. Several studies have previously demonstrated that a sufficient nitrogen source in the medium helps to increase the protein content in *Chlorella* and other algal species. What was inconsistent with this study is that $N\text{-}NH_4^+$ in the wastewater was more readily assimilated into protein than $N\text{-}NO_3^-$ used in artificial medium in other studies [37]. This reason for this difference was likely to have been due to deprivation of available phosphorus in the final growth stage that resulted in the higher lipid content and the lower protein content of the outdoor culture. However, the amino acid composition and the total essential amino acid percentage concentration showed few differences in response to different cultivation conditions. Some abundant amino acids, such as proline, aspartic acid, glutamic acid, alanine, and leucine, were consistent with reports by others [38]. Combined with this study, it can be found that the percentages of amino acids in the algal cells are similar and they are also stable under different cultivation conditions. In addition, the biomass harvested from digested piggery wastewater was found that contained similar protein content and amino acid compositions when compared with other works [39, 40], and was also suitable for development into feed additives.

Except for the biochemical components, some metallic elements were assayed (Table 5). Metallic pollution is the main reason behind restrictions against the recycling of wastewater from the livestock and poultry industries. The sources of metallic pollution were the metal additives in fodder and the sterilisation of breeding environments, including added Cu, Zn, Fe, and so on. In a piggery, bluestone is usually used to expel tapeworms, and elements such as zinc and iron are important promoters of animal growth; therefore, an abundance of metallic elements has been detected in animal excrement; however, they tend to be deposited in solid sediment during anaerobic processes (70% of total metal). As illustrated in Table 1, the digested piggery wastewater composition with respect to metallic elements such as Cu, Fe, Zn, and Mn was 0.26 mg L^{-1}, 1.29 mg L^{-1}, 1.06 mg L^{-1}, and 0.12 mg L^{-1}, respectively. After 25 days, the results of metal absorption assay are as listed in Table 5. It was observed that the Cu and Zn content of dry biomass was greater in outdoor cultivation using wastewater in comparison to indoor cultivation using BG11 medium, while the percentage of Mn was lower.

Table 6 The cost of the biomass cultured in different conditions ($ (USD equivalent))

	25 L × 2 indoors	70 L × 3 outdoors
Capital investment	0.83	1.79
Power	3.17	2.30
Medium	0.40	0.13
CO_2	0.46	–
Total biomass (g)	31.55	128.10
Cost ($ (USD equivalent)/kg DW)	154.04	32.94

As previously discussed, the additional amount of metal elements was likely to change during an animal's growth, if the algal biomass harvested from wastewater were to be developed into feed additives. Besides, the heavy metals detected in algal biomass, such as As and Pb, conformed to the national standards of feed grade *Spirulina* powder (GB/T 17243-1998, China) and the provincial standard of feed grade *Chlorella* powder (DB32/T 564-2010, Jiangsu, China).

Economic Analysis of the Microalgae Production

The costs of the biomass production in different conditions are listed in Table 6, which mainly included the capital investment, electricity power, medium, and CO_2 costs. Compared with indoor cultivation, the cost of producing algal biomass using wastewater under an outdoor environment was far less expensive with an estimated cost some 4.67 times lower than the cost of the 25-L indoor cultures. In the 70-L outdoor culture, the low cost of thin-film flat plate photobioreactor significantly decreased the capital investment burden. Besides, the chemical reagent and CO_2 were not needed in outdoor experiments due to the fact that the TN, TP, organic matter, and trace elements in wastewater supplied the nutrients for algal cell growth; however, the cost of power is the main investment, which ranged from 56.23 to 65.63% of the total cost, in outdoor and indoor cultivation, which was consistent with previous studies using different algal species. Despite this, the cost of biomass production was still high, which mainly resulted from the small scale of the production system and the long incubation time.

As discussed earlier, the algal cells achieved a higher growth rate in the exponential phase. In this sense, a semi-continuous or continuous cultivation mode has the greater potential for decreasing the production cost than batch cultivation; therefore, the electricity consumption may be decreased by 50% if the algal cell growth rate remained high. According to the test results from the outdoor culture system in this study, it has the potential for industrial production on a large scale. The investment and process operation cost may be decreased by a third if the culture volume were doubled. On the other hand, after removing the algal cells and disinfecting by use of a simple technique, the wastewater could be reused to wash the piggery. Compared with the indoor culture, the algal cells cultured using wastewater had a similar biochemical composition and could be developed into feed additives or fish bait. Thus, the cost of wastewater treatment has the potential to be decreased for full-scale breeding enterprises.

Conclusion

This study demonstrated *Chlorella vulgaris* growth and nutrient removal from digested piggery wastewater in a thin-film flat plate photobioreactor. The microalgae could adapt to the wastewater under outdoor conditions with a higher growth rate in their exponential phase compared to indoor cultivation. Fatty acid composition and lipid/protein contents were distinct between indoor and outdoor cultures, while the amino acid composition was stable. Metallic elements absorbed by cells conformed to current standards for feed additives. The cost of outdoor biomass production was lower compared to indoor cultures. Results indicated that outdoor cultivation with a low-cost bioreactor has the potential for biomass production and wastewater treatment.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

References

1. Markou, G., & Nerantzis, E. (2013). Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. *Biotechnology Advances, 31*(8), 1532–1542.
2. Abodeely, J. M., Coleman, A. M., Stevens, D. M., Ray, A. E., Cafferty, K. G., & Newby, D. T. (2014). Assessment of algal farm designs using a dynamic modular approach. *Algal Research-Biomass Biofuels and Bioproducts, 5*, 264–273.
3. Zhou, W. G., Chen, P., Min, M., Ma, X. C., Wang, J. H., Griffith, R., Hussain, F., Peng, P., Xie, Q. L., Li, Y., Shi, J., Meng, J. Z., & Ruan, R. (2014). Environment-enhancing algal biofuel production using wastewaters. *Renewable & Sustainable Energy Reviews, 36*, 256–269.
4. Cai, T., Park, S. Y., & Li, Y. B. (2013). Nutrient recovery from wastewater streams by microalgae: status and prospects. *Renewable & Sustainable Energy Reviews, 19*, 360–369.
5. Deng, X. Y., Gao, K., Zhang, R. C., Addy, M., Lu, Q., Ren, H. Y., Chen, P., Liu, Y. H., & Ruan, R. (2017). Growing *Chlorella vulgaris* on thermophilic anaerobic digestion swine manure for nutrient removal and biomass production. *Bioresource Technology, 243*, 417–425.
6. Jebali, A., Acien, F. G., Sayadi, S., & Molina-Grima, E. (2018). Utilization of centrate from urban wastewater plants for the production of *Scenedesmus sp.* in a raceway-simulating reactor. *Journal of Environmental Management, 211*, 112–124.
7. Fernandez-Linares, L. C., Barajas, C. G., Paramo, E. D., & Corona, J. A. B. (2017). Assessment of *Chlorella vulgaris* and indigenous microalgae biomass with treated wastewater as growth culture medium. *Bioresource Technology, 244*(Pt 1), 400–406.
8. Avagyan, A. B. (2011). Water global recourse management through the use of microalgae addressed to sustainable development. *Clean Technologies and Environmental Policy, 13*(3), 431–445.
9. Ebrahimian, A., Kariminia, H. R., & Vosoughi, M. (2014). Lipid production in mixotrophic cultivation of *Chlorella vulgaris* in a mixture of primary and secondary municipal wastewater. *Renewable Energy, 71*, 502–508.
10. Mehrabadi, A., Craggs, R., & Farid, M. M. (2015). Wastewater treatment high rate algal ponds (WWT-HRAP) for low-cost biofuel production. *Bioresource Technology, 184*, 202–214.
11. Morales-Amaral, M. D., Gomez-Serrano, C., Acien, F. G., Fernandez-Sevilla, J. M., & Molina-Grima, E. (2015). Outdoor production of *Scenedesmus sp.* in thin-layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source. *Algal Research-Biomass Biofuels and Bioproducts, 12*, 99–108.
12. Kim, H. C., Choi, W. J., Ryu, J. H., Maeng, S. K., Kim, H. S., Lee, B. C., & Song, K. G. (2014). Optimizing cultivation strategies for robust algal growth and consequent removal of inorganic nutrients in pretreated livestock effluent. *Applied Biochemistry and Biotechnology, 174*(4), 1668–1682.
13. Norsker, N. H., Barbosa, M. J., Vermue, M. H., & Wijffels, R. H. (2011). Microalgal production - a close look at the economics. *Biotechnology Advances, 29*(1), 24–27.
14. Becker, E. W. (1994). Microalgae: biotechnology and microbiology. *Quarterly Review of Biology*, p56–p61.
15. SEPE. (2002). *Water and wastewater analyzing methods*. Beijing: China Environmental Science Press.
16. Tan, X. B., Zhao, X. C., Zhang, Y. L., Zhou, Y. Y., Yang, L. B., & Zhang, W. W. (2018). Enhanced lipid and biomass production using alcohol wastewater as carbon source for *Chlorella pyrenoidosa* cultivation in anaerobically digested starch wastewater in outdoors. *Bioresource Technology, 247*, 784–793.
17. Sun, Z., Xue, S., Yan, C., Cong, W., & Kong, D. (2016). Utilisation of tris(hydroxymethyl)aminomethane as a gas carrier in microalgal cultivation to enhance CO_2 utilisation and biomass production. *RSC Advances, 6*(4), 2703–2711.
18. Safi, Charton, Pignolet, Silvestre and Vaca-Garcia. (2013) Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. Journal of Applied Phycology, 25, 523–529, 2.

19. Perez-Garcia, O., Escalante, F. M. E., de-Bashan, L. E., & Bashan, Y. (2011). Heterotrophic cultures of microalgae: metabolism and potential products. *Water Research, 45*(1), 11–36.
20. Collos, Y., & Harrison, P. J. (2014). Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Marine Pollution Bulletin, 80*(1-2), 8–23.
21. Shih, Y. J., Abarca, R. R. M., de Luna, M. D. G., Huang, Y. H., & Lu, M. C. (2017). Recovery of phosphorus from synthetic wastewaters by struvite crystallization in a fluidized-bed reactor: effects of pH, phosphate concentration and coexisting ions. *Chemosphere, 173*, 466–473.
22. Kim, T. H., Lee, Y., Han, S. H., & Hwang, S. J. (2013). The effects of wavelength and wavelength mixing ratios on microalgae growth and nitrogen, phosphorus removal using *Scenedesmus sp.* for wastewater treatment. *Bioresource Technology, 130*, 75–80.
23. Michelon, W., Da Silva, M. L. B., Mezzari, M. P., Pirolli, M., Prandini, J. M., & Soares, H. M. (2016). Effects of nitrogen and phosphorus on biochemical composition of microalgae polyculture harvested from phycoremediation of piggery wastewater digestate. *Applied Biochemistry and Biotechnology, 178*(7), 1407–1419.
24. Kurade, M. B., Kim, J. R., Govindwar, S. P., & Jeon, B. H. (2016). Insights into microalgae mediated biodegradation of diazinon by *Chlorella vulgaris*: microalgal tolerance to xenobiotic pollutants and metabolism. *Algal Research-Biomass Biofuels and Bioproducts, 20*, 126–134.
25. Chu, F. F., Chu, P. N., Shen, X. F., Lam, P. K. S., & Zeng, R. J. (2014). Effect of phosphorus on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. *Bioresource Technology, 152*, 241–246.
26. Li, Y. C., Chen, Y. F., Chen, P., Min, M., Zhou, W. G., Martinez, B., Zhu, J., & Ruan, R. (2011). Characterization of a microalga *Chlorella sp.* well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. *Bioresource Technology, 102*(8), 5138–5144.
27. Wang, R. M., Tian, Y., Xue, S. Z., Zhang, D. M., Zhang, Q. H., Wu, X., Kong, D. Z., & Cong, W. (2016). Enhanced microalgal biomass and lipid production via co-culture of *Scenedesmus obliquus* and *Candida tropicalis* in an autotrophic system. *Journal of Chemical Technology and Biotechnology, 91*(5), 1387–1396.
28. Salama, E. S., Kurade, M. B., Abou-Shanab, R. A. I., El-Dalatony, M. M., Yang, I. S., Min, B., & Jeon, B. H. (2017). Recent progress in microalgal biomass production coupled with wastewater treatment for biofuel generation. *Renewable & Sustainable Energy Reviews, 79*, 1189–1211.
29. Gonzalez-Fernandez, C., Molinuevo-Salces, B., & Garcia-Gonzalez, M. C. (2011). Nitrogen transformations under different conditions in open ponds by means of microalgae-bacteria consortium treating pig slurry. *Bioresource Technology, 102*(2), 960–966.
30. Demirbas, M. F. (2011). Biofuels from algae for sustainable development. *Applied Energy, 88*(10), 3473–3480.
31. Klyachko-Gurvich, G., Doucha, J., Kopetskii, J., Semenenko, V., & Tsoglin, L. (2010). Desaturation of fatty acids as an adaptive response to shifts in light intensity. *Physiologia Plantarum, 107*, 240–249.
32. Zili, F., Mezhoud, N., Trabelsi, L., Chreif, I., & Ben Ouada, H. (2015). Fatty acid composition of the thermophilic *Gloeocapsa gelatinosa* under different combinations of temperature, light intensity, and $NaNO_3$ concentration. *Journal of Applied Phycology, 27*(1), 97–107.
33. Zhu, L. D., Wang, Z. M., Takala, J., Hiltunen, E., Qin, L., Xu, Z. B., Qin, X. X., & Yuan, Z. H. (2013). Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production. *Bioresource Technology, 137*, 318–325.
34. Zielinska, A., Chojnacka, K., & Labuda, M. (2011). Technology for production of mineral feed additives based on microalgal biomass. *Przemysl Chemiczny, 90*, 1092–1095.
35. Li, Y., Xiao, G. Q., Mangott, A., Kent, M., & Pirozzi, I. (2016). Nutrient efficacy of microalgae as aquafeed additives for the adult black tiger prawn, *Penaeus monodon*. *Aquaculture Research, 47*(11), 3625–3635.
36. Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Chowdhury, M. A. K., Parsaeimehr, A., Liang, Y. M., & Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology, 30*(1), 197–213.
37. Xia, L., Song, S. X., & Hu, C. X. (2016). High temperature enhances lipid accumulation in nitrogen-deprived *Scenedesmus obtusus* XJ-15. *Journal of Applied Phycology, 28*(2), 831–837.
38. Wang, S. K., Hu, Y. R., Wang, F., Stiles, A. R., & Liu, C. Z. (2014). Scale-up cultivation of *Chlorella ellipsoidea* from indoor to outdoor in bubble column bioreactors. *Bioresource Technology, 156*, 117–122.
39. Duong, V. T., Ahmed, F., Thomas-Hall, S. R., Quigley, S., Nowak, E., & Schenk, P. M. (2015). High protein- and high lipid-producing microalgae from northern Australia as potential feedstock for animal feed and biodiesel. *Frontiers in Bioengineering and Biotechnology, 3*, 53–60.
40. Ogbonna, J. C., Masui, H., & Tanaka, H. (1997). Sequential heterotrophic/autotrophic cultivation – an efficient method of producing *Chlorella* biomass for health food and animal feed. *Journal of Applied Phycology, 9*(4), 359–366.