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Culturing *Chlorella* in Anaerobically Digested Piggery Wastewater for Biodiesel Feedstock and Nutrient Removal

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Abstract

Anaerobic digestion of piggery waste generates effluents which containing high concentration of inorganic nitrogen and phosphorus can result in eutrophication of natural water bodies when discharged without adequate treatment. Microalgae can be cultured in effluent derived from the anaerobic digestion of piggery waste for nutrient removal and biodiesel feedstock production. In this study, the piggery wastewater was used as a nutrient source for culturing the green microalgae, Chlorella sp B5. The effect of dilution of the effluent on the nutrient removal efficiency of Chlorella sp.B5 was determined. During an eight day batch culture, Chlorella sp.B5 grew fastest in an diluted effluent with a initial COD concentration of about 300-400 mg/L. The maximum specific growth rate and biomass concentration of Chlorella sp. B5 were 0.306 day^{-1} and 0.19 g/L, respectively. The culturing system of Chlorella sp. B5 removed ammonia, total phosphorus and COD of piggery wastewater by 60 – 95.8%, 22 – 68% and 34 – 73.8%, respectively. The lipid content of algal biomass after 8 days were 28-30% of dry weight.

Keywords: piggery wastewater, Chlorella, Biodiesel feedstock, Nutrient removal, Lipid.

1. Introduction

The potential of microalgae as a source of biofuel feedstock has been widely recognised since research efforts began in the 1980's and early 1990's [1]. Currently, the rationale for extensive development of renewable fuel resources is seen as key to meeting world demand in a sustainable manner . Microalgae have the capability to produce biomass with a high lipid/oil content for use as a precursor for liquid biofuels [2]. To date, research efforts have been largely focused on reduction of costs in producing biodiesel feedstock from microalgae. One key challenge is coupling microalgae biofuel feedstock production with the treatment of organic waste effluents such as anaerobically digested piggery wastewater [3]. This effluent can serve as an alternative nutrient source to support microalgal production, as it is rich in inorganic nitrogen and phosphorous (NH_4^+, NO_3^-, PO_4^{3-}). Additionally, pathogens that impair microalgae cultures can be eliminated in two-stage (thermophilic and mesophilic) anaerobic digestion process [4]. Use of such effluents as a surrogate for costly mineral medium sources with associated reductions in demand for fresh water is therefore appealing from a techno-economic perspective.

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A strain of *Chlorella sp.* B5 is used in the present study for treatment of piggery waste effluent, and a source of biodiesel feedstock. *Chlorella sp.* B5 was grown mixotrophically in diluted piggery wastewater from Co Dong pig farm, Son Tay, Hanoi, Vietnam. The capacity of this farm is 1000-2000 pigs per litter in average. The duration of a litter usually is from four to five months to breed from baby pigs (around 7-10 kg) to sold-out pigs (about 80-100 kg). The wastes collected to a pit included manure, urine, feed residues, wastewater from pig washing and pigsty flushing, etc. The waste mix was pumped to a upflow anaerobic sludge blanket (UASB) system for anaerobic digestion (Fig.1). The relationship between the initial COD values and microalgae growth rate, elimination of nutrients (NH_4^+-N, TKN, PO_4^{3-}-P, total P and COD) and cellular lipid content were investigated.

2. Materials and methods

2.1. Microalgal strain and culture conditions

Strain B5 was locally isolated from a pond in Co Dong pig farm, Son Tay, Hanoi, and screened as described in [5]. The strain was morphologically identified as *Chlorella* sp. and cultured in Bold Basal Medium in the presence of vitamins (BBM+V). Culture flasks were maintained at room temperature (25–32 ^{0}C) under a static condition. Light irradiation of the culture was 1500 lux, and was achieved using two cool-white fluorescence tubes, with a 12:12 h light:dark cycle. Light intensity was measured at the

surface of the culture broth using a Testo 545 lux meter (Testo GmbH & Co, Germany).

2.2. Sampling site of piggery wastewater

The piggery wastes were digested in a UASB system (see Fig. 1). Then, the effluent stream was used for culturing *Chlorella sp.* B5 to remove nitrogen and phosphate, as well as generate biomass for biodiesel feedstock in the form of intracellular microalgal lipids. Four sampling trips were taken for characterizing the effluent from the UASB. The first and forth samplings were collected in the period of mature pigs (80-100 kg). The second was at the beginning of a litter, which pig's weightline was 7-10 kg and the feed has special high protein for weaned pigs. The third one was at the period of growing pigs (30-50 kg). Prior to microalgae culture, the effluent was filtered over a glass cotton layer in the laboratory to remove crude solids, and then diluted with distilled water to obtain a range of COD concentrations.

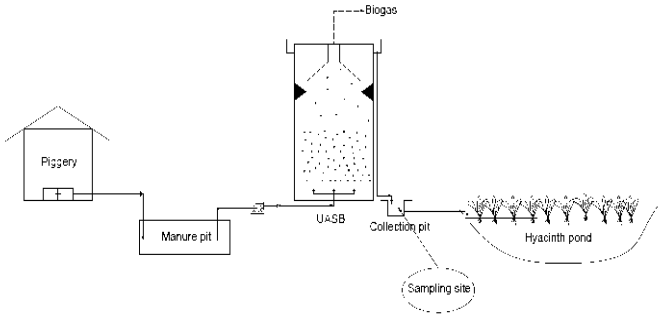


Fig.1. Schematic diagram of manure and effluent treatment at the Co Dong pig farm, Hanoi.

2.3. Diluted wastewater to various COD concentrations

Digested wastewater was diluted to a range of COD concentrations as follows: 250, 300, 400, 530, 600, and 740 mg COD/L. *Chlorella sp.* B5 in exponential phase was transferred to 250mL conical flasks containing 100 mL of diluted piggery wastewater (triplicate cultures for each COD concentration). The optical density (OD) of all flasks at the starting experiment were 0.12 equivalent to 13.2 mg biomass/L. The flasks were manually agitated thrice daily to maintain cells in suspension. All experiments were terminated after 8 days.

2.4. Determination of cell growth rate

The growth rate of each strain was characterized based on measurement of cell density every day using a UV/VIS Lambda EZ210 spectrophotometer (PerkinElmer, USA), at λ = 685 nm. The specific growth rate of each microalgae strain was calculated from the slope of linear regression of time (days) and cell density (optical density OD_{685nm}) [6], $\mu = (\ln OD_t - \ln OD_o)/(t - t_o)$; where μ (day^{-1}) is the specific growth rate, OD_t is algal cell density at time (t), and OD_o is algal cell density at the start of the exponential phase (t_o).

2.5. Biomass dry weight (DW) and total lipid content

Biomass dry weight was determined as follows: on the terminal day of culture, 50 mL of microalgae suspension was sampled and filtered on a pre-weighed Whatman GF/F glass fiber filter (0.7 µm pore size and 47 mm diameter; Whatman, USA) using a Kontec Ultraware microfiltration apparatus. The algal pellets were dried overnight at 80^{o}C and weighed.

The total lipid content of microalgae dry biomass was measured via the use of a modified Folch et al method, and then quantified gravimetrically as described in our published article [7]. The dry biomass was transferred into a centrifuge tube containing a mixture of chloroform:methanol (2:1, v/v), vigorously shaken and then placed into an ultrasonic water bath (Telsonic Ultrasonic Inc., USA) for 2 hours. The supernatant was then collected via pipette and transferred into another centrifuge tube. The extraction procedure was repeated twice to ensure adequate lipid extraction. NaCl at 0.9% solution was added to the supernatant to make the ratio of lipid solution: solvent to 1:5, v/v. The centrifuge tube was then vortex-mixed for 5 mins, and then allowed to undergo phase separation for 15 minutes. The lower phase in the centrifuge tube was removed and deposited into a pre-weighed aluminum container to evaporate the solvent, then dried overnight at 80 ^{0}C and re-weighed to determine dry weight.

2.6. Nutrient removal efficiency

The removal efficiencies of organic polutants and nutrients after 8-day batch, i.e. chemical oxygen demand (COD), ammonium (NH_4^+-N), total Kjeldahl nitrogen (TKN), phosphate (PO_4^{3-}P) and total phosphorus (total P), were examined. The analysis of COD, NH_4^+-N, TKN, PO_4^{3-}P and total P was conducted according to methods in the Standard Methods for the Examination of Water and Wastewater [8].

Table 1. Characteristics of piggery wastewater at Co dong pig farm, Son Tay, Hanoi.

Parameters	Sampling				QCVN 40:2011/ BTNMT
	1st	2nd	3rd	4th	
pH	7.68	7.89	7.82	8.45	5.5-9
TS (mg/L)	3470	3243	3012	3323	-
TSS (mg/L)	134	256	273	242	100
COD (mg/L)	1995	1879	1632	1768	150
BOD_5(mg/L)	390	240	310	–	50
PO_4^{3-}-P (mg/L)	188	-	-	246	-
Total P (mg/L)	635	297	374	541	6
NH_4^+ -N (mg/L)	863	-	-	756	10
TKN (mg/L)	966	872	834	762	40
*N:P **	*3.4:1*	*6.5:1*	*4.9:1*	*3.1:1*	

(*): N = TKN/14, P = Total P/31

3. Results and discussion

3.1. Characteristics of piggery wastewater

Piggery wastewater used for microalgae culturing was characterized and the results were shown in the Table 1.

Concentrations of pollutants such as BOD,COD, nitrogen (i.e. NH_4^+-N and TKN) and phosphorus (i.e. PO_4^{3-}P and total P) after UASB treatment still exceeded the Vietnamese Environmental Standards for discharge of wastewater, i.e. QCVN 40: 2011/ BTNMT (column B). The values of COD and BOD were 10-13 times and 5-8 times greater than the respective standard. Digested wastewater still contained high concentrations of nitrogen and phosphorus, i.e. 22-24 times greater than the Standard for TKN, and 49.5-105 times for total P. The ratio of N:P fluctuated from 3:1 to 6.5:1 during four sampling periods. The varied concentrations of the pollutants were related to pig olds, which resulted from different diets. The grower diet comprised of more protein and minerals than for mature pigs. As shown in Table 1, the N:P ratio of 6.5:1 was for young pigs which were provided with feed rich in protein. The ratio then decreased for growing pigs (i.e. 4.9:1) and mature pigs (i.e. about 3: 1). Table 1 presents that most of samples contained an N:P ratio of lesser than the Redfield values, which indicate that favour ratios of N:P for phytoplankton as well as for microalgae is from 16:1 to 25:1. Therefore the result in this study shows that nitrogen was in low level to phosphorus. It also means that nitrogen was the limited factor in this type of piggery wastewater.

3.2. Culturing Chlorella B5 in piggery wastewater

The piggery wastewater samples were diluted to six levels of COD concentration, ranging from 250 mg/L to 740 mg/L. In preliminary experiments, with undiluted wastewater, high concentrations of NH_4^+-N and COD (see Table 1), the color of wastewater was dark which inhibited the growth of *Chlorella* sp B5. Thus, wastewater samples were diluted by at least two times to achieve a COD concentration less than 800 mg/L. The dilution ratios ensured the maximum ammonium concentration was less than 150 mg/L, to prevent inhibition of microalgae growth. The initial density of *Chlorella* sp. B5 inoculum in the culture medium was equivalent to 13 mg/mL for all cultures. The growth curves of the microalga are presented in Fig. 2.

As can be seen in Fig. 2, *Chlorella* sp. B5 grew relatively well in the diluted wastewater at an initial COD concentrations in the range of 250-400 mg/L. At higher COD solutions (i.e. 530-740 mg/L) and elevated TKN concentrations, cell growth rate declined, which due to dark colour of wastewater hindered light penetration to microalgae culture. *Chlorella sp.* growth increased more rapidly in diluted wastewater at 300-400 mgCOD/L compared to a control (BBM media only), which implies that *Chlorella* sp.B5 could grow mixotrophically. Specific growth rates of *Chlorella* sp. B5 in diluted wastewaters were highest at initial COD concentrations of 300 – 400 mg/L i.e. $0.26d^{-1}$, resulting in the highest biomass concentration after 8 days at ~0.2 g/L. The lag phase of growth was about two days for COD of 600 and 740 mg/l, but only one day for the other cultures (see Fig. 2).

Optimum COD concentrations for the growth of *Chlorella* sp. B5 in this study (COD: 300-400 mg/L) were lower than those quoted in Wang's report [9] i.e. COD:0.7-1 g/L. It is possible that *Chlorella pyrenoidosa* in Wang's study may have a higher tolerance to piggery wastewater than the *Chlorella* sp B5 learnt in this study.

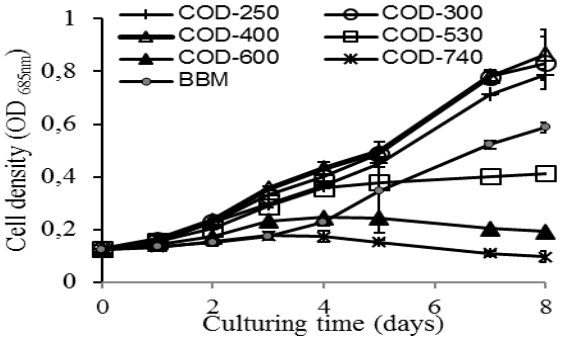
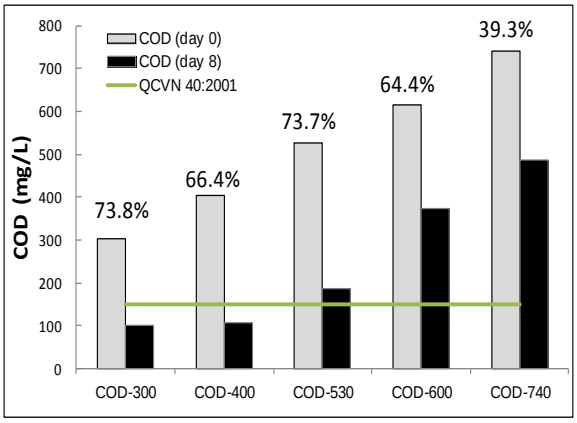
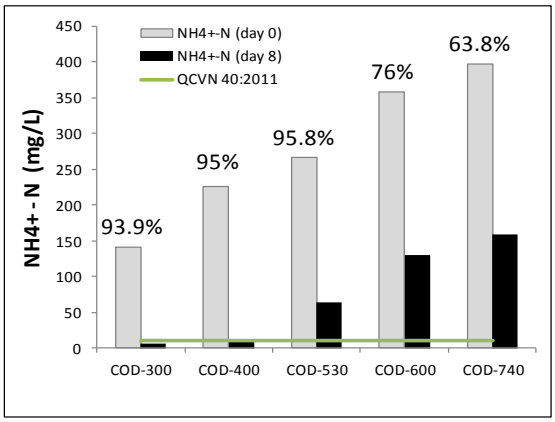


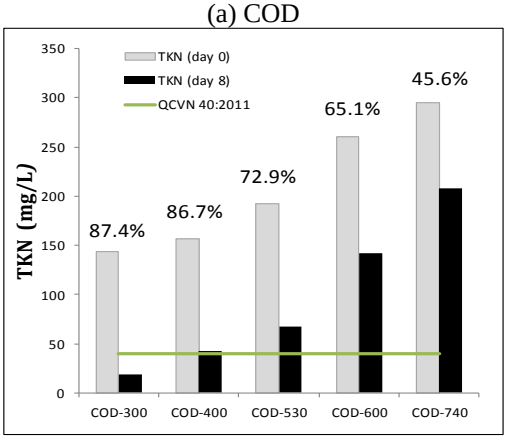
Fig. 2. Growth curves of *Chlorella* sp. B5 in diluted piggery wastewater medium at different initial COD values



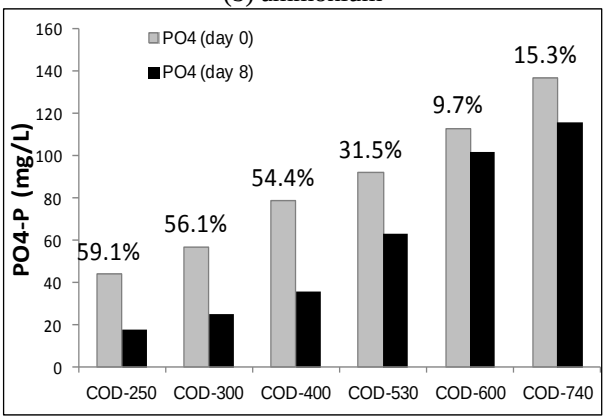
(a) COD



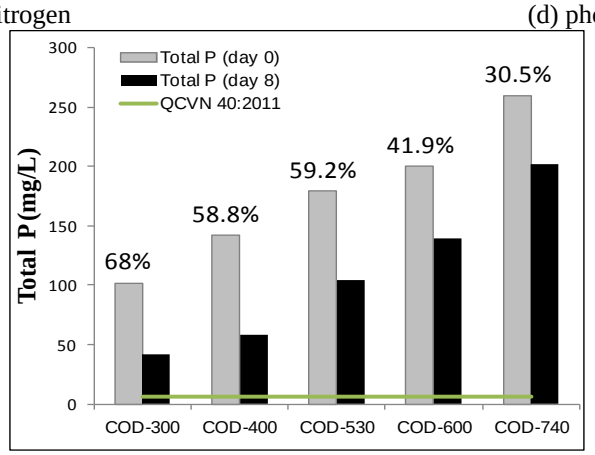
(b) ammonium



(c) total nitrogen



(d) phosphate



(e) total phosphorus (percentages indicate removal efficiency of certain pollutants)

Fig. 3. Removal rates

3.3. Nutrient removal efficiencies

In addition to differences in microalgae growth rates, the efficiency of nutrient removal also varied according to intial COD. The removal rates of COD ranged from 34 to 73.8% after 8 days of culture (see Fig. 3a), with the greatest reduction of 66 to 74% achieved with an initial COD concentrations ranging between 250 and 400 mg/L. Reduction in COD levels shows that *Chlorella* B5 is capable of growing mixotrophically, using the fixed carbon in wastewater as a source of energy and a substrate for cell growth. The most effective removal i.e.73.7% of COD on day 8 was observed for the culture with initial COD of 400 mg/L, where the COD level declined to 106 mg/L i.e. lower than specified in the QCVN 40:2011/ BTNMT (i.e.150 mg/L) (Fig. 3a).

Nitrogen was the limiting growth factor, where it was exhausted after 5 days of culturing (data not show). Removal efficiencies of NH_4^+-N

were as high as 95.8% at an initial COD level of 400 mg/L (see Fig. 3b), and 60% for an initial COD level of 740 mg/L. The higher the initial COD concentrations, the lesser the efficiency of NH_4^+-N removal. It related to the decline of cell growth rate in the COD removal above mentioned. In this study, the efficiency of NH_4^+-N removal was greater than reported in Park's study (i.e. 95.8% vs. 47%, respectively), but less for PO_4^{3-}-P removal (i.e. 56% vs. 68%, respectively) [10]. Strong reductions in total nitrogen (TKN) and total phosphorus were found for all four diluted wastewater samples (Fig. 3c and 3d). It is clear from experimental data that the removal rate of both total nitrogen and total phosphorus in wastewater increased with a COD values of less than 530 mg/L.

3.4. Total lipid content

The lipid contents produced in the control of BBM's solution and a diluted piggery wastewater of 400 mg COD/L were at almost equal levels i.e. 28.3 and 28.8% dry weight, respectively on day 8 of cultures (see Table 2). Cellular lipid contents for biomass grown in the cultures of 250 and 300 mg COD/L were slightly higher, i.e. 30% dry weight. This is likely due to a limited availability of nitrogen in these cultures.

Results show that *Chlorella* sp. B5 accumulated intracellular lipid during the cultivation period in wastewater. Other cultures in diluted wastewater contained a low lipid content, i.e. <14% dry wt, due to poor growth of *Chlorella* sp. B5 in these solutions. According to Mata *et al*. [2], a lipid content in range of greater than 30% could adopt a requirement for biodiesel processes. Thus, the biomass of *Chlorella* sp. B5 is deemed suitable as a biodiesel feedstock.

Conclusions

Coupling *Chlorella sp.* B5 cultivation with treatment of diluted anaerobic digested piggery wastewater can effectively eliminate organic pollutants and nutrients. The highest growth rate of *Chlorella sp.* B5 occurred in diluted wastewater at an initial COD concentration of 300-400 mg/L. These cultures also had the highest nutrient removal rates i.e. up to 73.7% for COD, up to 95.8% for NH_4^+-N, up to 86.7% for TKN, up to 56.1% for PO_4^{3-}-P and up to 59.2 for total P. During cultivation in diluted wastewater the lipid content of *Chlorella* sp. B5 was 28-30%, and is potential source of biodiesel feedstock.

Acknowledgements

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Table 2. Total lipid content of biomass cultivated in BBM solution and diluted piggery wastewater after 8 days. Data expressed in mean values ±SD (n=3)

	BBM	Diluted wastewater with various initial COD concentration					
Lipid content (% DW)		COD-250	COD-300	COD-400	COD-530	COD-600	COD-740
	28.3±0.5	30.0±0.4	30.1±0.5	28.8±0.8	14.0±0.7	13.8±0.5	13.2±0.7

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