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Fed-batch cultivation of *Desmodesmus* sp. in anaerobic digestion wastewater for improved nutrient removal and biodiesel production

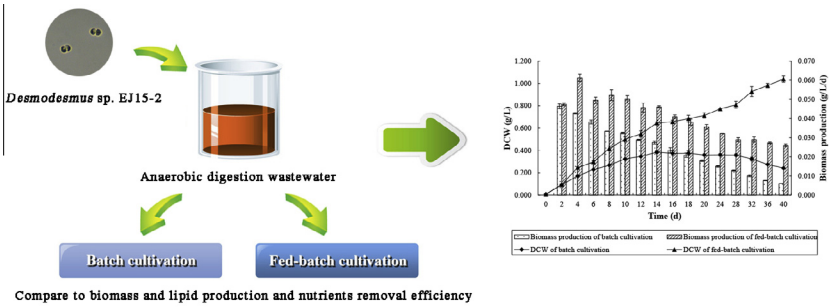
Fang Ji [a,c], Yuguang Zhou [a,c,*], Aiping Pang [a,c], Li Ning [a,c], Kibet Rodgers [a,c], Ying Liu [b,c], Renjie Dong [a,c]

[a] College of Engineering/Biomass Engineering Center, China Agricultural University, Beijing 100083, China
[b] College of Agriculture and Biotechnology, China Agricultural University, Beijing 100193, China
[c] Key Laboratory of Clean Production and Utilization of Renewable Energy, Ministry of Agriculture, P. R. China, Beijing 100083, China

HIGHLIGHTS

- *Desmodesmus* sp. can grow culture in diluted anaerobic digestion wastewater (ADW).
- Highest biomass production of fed-batch cultivation was 1.039 g/L.
- Highest lipid production of fed-batch cultivation was 261.8 mg/L.
- Fed-batch observed more than 300% higher biomass production than batch.
- Maximum removal of TN, NH_4-N and PO_4-P were 236.143, 268.238 and 6.427 mg/L.

GRAPHICAL ABSTRACT



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ABSTRACT

Desmodesmus sp. was used in anaerobically digested wastewater (ADW) for nutrients removal and the biodiesel production was measured and compared using fed-batch cultivation was investigated and compared with batch cultivation. The *Desmodesmus* sp. was able to remove 236.143, 268.238 and 6.427 mg/L of TN, NH_4-N and PO_4-P respectively after 40 d of fed-batch cultivation, while in batch cultivation the quantities of TN, NH_4-N and PO_4-P removed were 33.331, 37.227 and 1.323 mg/L. Biomass production of *Desmodesmus* sp. was also enhanced in fed-batch cultivation, when ADW loading was carried out every 2 days; the biomass concentration peaked at 1.039 g/L, which was three times higher than that obtained in batch cultivation (0.385 g/L). The highest lipid production (261.8 mg/L) was also recorded in fed-batch cultivation as compared to batch cultivation (83.3 mg/L). Fed-batch cultivation of *Desmodesmus* sp. could provide effective control of nutrients limitation and/or ammonia inhibition on microalgae cultivation.

1. Introduction

As one of the most potential energy sources, microalgae could be used as a biofuel to replace part, if not all, of fossil fuels in the near future (Wijffels and Barbosa, 2010). As compared to higher plants, microalgae have relatively high growth rates and tolerance to varying environmental conditions (Pandey et al., 2013), and also have the ability to remove nitrogen and phosphorus while accumulating lipids when cultured in wastewater (Zhang et al., 2012; Zhu et al., 2013). However, algae cultivation and lipid extraction is not economically feasible due to higher average production costs associated with these processes. Thus, improving biomass and lipid production, and reducing freshwater consumption during algae cultivation, are two important concerns that needs to be addressed in order to reduce the unit cost of production (Samorì et al., 2012;

* Corresponding author at: P.O. Box 50, No. 17 Qinghua Donglu, Haidian District, Beijing 100083, China. Tel.: +86 10 62737858; fax: +86 10 62737885.
E-mail address: zhouyg@cau.edu.cn (Y. Zhou).

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Wu et al., 2013). Furthermore, the simultaneous treatment of wastewater and algae growth could offer economical benefits.

Anaerobic digestion is commonly used to decompose organic waste and produce biogas (ca. 60% CH_4 and 40% CO_2). However, anaerobic digestion could reduce 30–40% of nutrients from manure used as feedstock (Wang et al., 2010a), the anaerobic digestion wastewater (ADW) could incur eutrophication of nearby waterways and volatilization of ammonia (Levine et al., 2011); and available evidence shows that excessive application of ADW to farmlands poses highly potential risks on environment (Singh et al., 2011). Hence, significant amount of nutrients essential for algae growth are normally retained in ADW, making it a good medium for algae cultivation as well as cultivation of other plants (Wang et al., 2010a). Microalgae cultivation in ADW effluent is a potential option for environmental sustainability and carbon neutrality. Although the productivity was lower than that obtained through cultivation with some commercial standard media due to relatively low carbon levels and high ammonium concentration (about 65–82% of total nitrogen in ADW is ammonium) (Cai et al., 2013b; Kebede-Westhead et al., 2006), it was actually reasonable considering the lower production costs derived from the use of ADW as a cheaper culture medium (Bhatnagar et al., 2011). This approach could not only protect the environment, but also produce biofuel directly. Improving biomass and lipid production makes the extraction of the target fuel more economical feasible and therefore, it is necessary to improve the process of microalgae cultivation in ADW in order to benefit from a cost-reduced production. Thus, fed-batch process is a potential means of achieving this objective and making it the subject of this study. Although some researchers described the advantages of fed-batch process over batch and continuous processes in terms of better control of nutrients concentration and higher level of biomass and lipid productivity (Minihane and Brown, 1986), the batch process is still the method that most widely applied for microalgae cultivation incorporated with wastewater treatment. The fed-batch process could control nutrient levels adequately due to its flexibility in supplying the nutrients (Rodrigues et al., 2011). Therefore, fed-batch addition of nutrients is an effective way of removing much more nutrients from ADW and protecting algae from high turbidity and ammonium concentration during cultivation (Hongyang et al., 2011; Yuan et al., 2011; Wang et al., 2010c). Fed-batch cultivation was investigated with the aim of increasing the microalgae biomass production as well as treating wastewater that associated with the use of ADW as a nutrient source for algae cultivation. The present study has been focused on culturing *Desmodesmus* sp. in diluted ADW using fed-batch cultivation process in order to improve biomass and lipid production, and also increase the amount of nutrients removed therefrom.

2. Methods

2.1. Microalgae strain and inoculum production

Desmodesmus sp. EJ15-2 was obtained from the BioEnergy Engineering and Low Carbon Technology Laboratory of China Agricultural University, Beijing, China (Ji et al., 2013). The medium used in this study was BG-11 medium which contained 1,500 mg of $NaNO_3$, 40 mg of K_2HPO_4, 75 mg of $MgSO_4{\cdot}7H_2O$, 36 mg of $CaCl_2{\cdot}2H_2O$, 6 mg of citric acid, 6 mg of ferric ammonium citrate, 1 mg of $EDTANa_2$, 20 mg of Na_2CO_3, 2.86 mg of H_3BO_3, 1.86 mg of $MnCl_2{\cdot}4H_2O$, 0.22 mg of $ZnSO_4{\cdot}7H_2O$, 0.39 mg of $Na_2MoO_4{\cdot}2H_2O$, 0.08 mg of $CuSO_4{\cdot}5H_2O$, and 0.05 mg of $Co(NO_3)_2{\cdot}6H_2O$ per liter. The initial pH value of medium was adjusted to 7.0 with 1 mol/L HCl.

Microalgae were inoculated in autoclaved BG-11 medium (121 °C, 20 min) in 100 mL Erlenmeyer flasks. At the beginning of each experiment, 60 mL of medium was put into flasks with precultured microalgae. The culture flasks were incubated in a growth chamber under the optimized conditions at 30 ± 1 °C with a continuous photon flux density of 98 ± 2 μmol/m^2/s by cool-white fluorescent light illumination and light/dark cycles (L:D) of 14:10 h (Ji et al., 2013). Periodic agitations were performed for three times each day.

2.2. ADW collection and pretreatment

ADW was collected from the Bei Langzhong Piggery Farm Biogas Plant (Beijing, China) in April 2013. In order to avoid variation in its composition, ADW was stored at 4 °C immediately after removing large particles and microorganisms through 1.2 μm glass microfiber filters (Whatman Inc., USA). The ADW (pH = 9.0) was a complex mixture with varying chemical properties, illustrated as follows: 4,050 ± 319 mg/L chemical oxygen demand (COD), 774.47 ± 32.99 mg/L total nitrogen (TN), 708.78 ± 17.17 mg/L ammonia nitrogen (NH_4-N), 70.12 ± 2.76 mg/L nitrate nitrogen (NO_3-N) and 31.24 ± 0.56 mg/L phosphate (PO_4-P).

2.3. Experimental design

Microalgae strain had been cultivated in 5.0% ADW for three generations to obtain stable characteristics. To determine the optimal ADW concentration for the growth of *Desmodesmus* sp. EJ15-2, batch experiments were performed for 10 days with three initial ADW dilutions of 2.5%, 5.0% and 10.0%. Light and temperature conditions were set as mentioned in Section 2.1 and the cultures were inoculated to achieve an initial optical density (OD) of 0.1 for all experimental units. The ODs of the cultures and concentration of nutrients (TN, NH_4-N, and PO_4-P) in the media were measured daily. The optimal ADW concentration was then determined based on biomass production and nutrient removal ratio.

Fed-batch process was conducted in 1 L reactors with 800 mL medium on a magnetic stir running at 200 rpm for 2 min every half an hour. Cultivation methods were the same as in batch operation. An optimal ADW concentration obtained from batch experiments was always used at the beginning of fed-batch cultivation. In order to avoid any inhibition of ammonia by maintaining ammonia at the optimal concentration, and to achieve high biomass production, ADW was loaded every 2 days during a total operation of 40 days. After sampling, the reactors were replenished with ADW and deionized water to optimal NH_4-N concentration based on the affect of samples and ADW at the same time. All experiments were carried out in at least triplicates and average values were statistically analyzed.

2.4. Analytical methods

2.4.1. Nutrients analysis

Samples for physicochemical analysis of ADW were first centrifuged at 10,000 rpm for 10 min and the supernatants were then filtered using 0.45 μm glass microfiber filters (Whatman Inc., USA). The filtrates were properly diluted and analyzed for NH_4-N, PO_4-P and COD concentration according to the spectrophotometric method cited in Hach DR 2700 Spectrophotometer Manual (Hach Company, USA).

The amount of NO_3-N was measured with a flow injection analyzer (AA3, Seal Analytical Inc., UK). TN was determined colorimetrically as nitrate after the samples had been oxidized. The pH of the solution was determined with a pH meter (Orion-3 STAR, Thermo Fisher Scientific Inc., USA).

Nutrient removal efficiencies were obtained according to Eq. (1):

Table 1
Comparison of biomass production, lipid content and nutrients removal performance in different ADW loading ratios with 10-day cultivation.

ADW loading ratio (%)	Initial concentration (mg/L)			Final concentration (mg/L)			Biomass production (g/L)	Lipid content
	TN	NH_4-N	PO_4-P	TN	NH_4-N	PO_4-P		
2.5	24.978 ± 1.743	22.441 ± 0.362	0.806 ± 0.044	4.360 ± 0.202	0	0	0.269 ± 0.000	29.3 ± 1.1
5.0	44.080 ± 1.611	40.149 ± 1.205	1.323 ± 0.023	10.749 ± 0.727	2.922 ± 0.148	0	0.324 ± 0.003	25.7 ± 0.5
10.0	77.447 ± 3.299	70.818 ± 1.717	3.124 ± 0.056	13.540 ± 0.479	2.401 ± 0.000	0	0.290 ± 0.009	24.2 ± 0.7

$$R_i = (S_{i0} - S_{it})/S_{i0} \times 100\% \quad (1)$$

where: R_i represents the removal efficiency of substrate i (TN, NH_4-N, or PO_4-P); S_{i0} and S_{it} are defined as the mean values of substrate i concentration at initial time t_0 and time t_i, respectively.

The rate of nutrient removal was calculated as given by Eq. (2):

$$r_i = (S_{i0} - S_{it})/(t_i - t_0) \quad (2)$$

where: r_i (g/L/d) is the rate of nutrient removal; S_{i0} is the initial substrate concentration (TN, NH_4-N or PO_4-P) and S_{it} is the corresponding substrate concentration at time t_i.

2.4.2. Determination of biomass concentration

The biomass concentration was evaluated using dry cell weight (DCW) method. Optical density of samples at 680 nm (OD_{680}) was employed to determine the cell density by a spectrophotometer (UV-7504PC, Xinmao Instrument, Shanghai, China).

A linear correlation was obtained and shown in Eq. (3):

$$y = 0.3021x - 0.0221 (R^2 = 0.998) \quad (3)$$

with: y, DCW in g/L, and 680 nm absorbance x.

The biomass productivity was calculated according to Eq. (4):

$$P = (y_i - y_0)/(t_i - t_0) \quad (4)$$

where: P is the biomass productivity (g/L/d); y_i and y_0 are DCW (g/L) at time t_i and t_0 (initial time), respectively.

2.4.3. Lipid extraction and fatty acid methyl ester (FAME) contents

Samples were harvested and freeze-dried at −80 °C for 48 h using a vacuum freeze dryer (FD-1B-05, Boyikang Instrument, Beijing, China) prior to lipid analysis. Total lipid extractions were performed as described by Bligh and Dyer (1959). FAME contents in the lyophilized cells were analyzed using one-step extraction–transesterification method as described by Indarti et al. (2005). The composition of fatty acid was analyzed by a gas chromatography spectrometer (GC-2010 Plus, Shimadzu, Japan) with a HP-Wax capillary column (30 m × 0.32 mm, Agilent Technologies, USA). Operating conditions were set as follows: injector temperature 220 °C, oven temperature ramped from standby to 100 °C and kept constant for 3 min; ramped to 200 °C at 4 °C/min, held at 200 °C for 5 min; then raised to 250 °C at 3 °C/min and held for 10 min. The flow rate of carrier gas (99.999% pure nitrogen, Beijing AP BAIF Gases Industry, Co., Ltd., Beijing, China) was maintained at 2.0 mL/min. The FAME contents can be calculated by comparing the peak areas of tested samples and the control sample (the standard FAME sample with HPLC-grade purity) at the same retention time points, which are corresponding to different detected peak signals.

3. Results and discussion

3.1. Batch cultivations

The initial concentrations of nitrogen and phosphorus in ADW were significantly higher than the recommended concentration (N: 20–250 mg/L; P: 3–10 mg/L) for microalgae cultivation (Singh et al., 2011) Therefore, ADW must be diluted prior to loading. The removal of nitrogen and phosphorus was as the results of nutrients uptaken by microalgae during growth. The removal amounts of TN, NH_4-N and PO_4-P in different initial concentrations of ADW for 10-day batch cultivations are exhibited in Table 1. The TN removal efficiencies achieved were 82.5 ± 0.8%, 75.6 ± 1.6% and 82.5 ± 0.6% in 2.5%, 5.0% and 10.0% ADW, respectively. The TN could not be entirely removed since there were still some nitrogen origins which could not be completely assimilated by microalgae (Wang et al., 2010a). The amount of removed NH_4-N tended to increase with high initial ADW concentration. Approximately 22.441, 37.227 and 68.417 g/L were removed from the cultures containing 2.5%, 5.0% and 10.0% of ADW, respectively. The NH_4-N in all samples was almost completely removed within 10 days because ammonium is the preferred nitrogen source for microalgae growth (Peccia et al., 2013). The PO_4-P removal efficiency decreased with increasing initial ADW concentration at the first few days (100%, 55.3% and 7.6% of PO_4-P could be removed at the Day 3 from 2.5%, 5.0% and 10.0% ADW, respectively); and the maximum amount of PO_4-P removed was 3.124 mg/L in the cultures with an initial concentration of 10.0% ADW.

The performance of biomass production and lipid content using three different ADW concentration levels as nutrient sources in cultures of *Desmodesmus* sp. EJ15-2 is shown in Table 1. The DCW increased slightly when the ADW concentration increased from 2.5% to 5.0%, and then decreased when the concentration further increased. Similar results have been reported in other investigations about microalgae cultivation in wastewater (Cai et al., 2013a), where it was observed that relatively higher loading ratios could decrease growth rates due to the inhibition of ammonia in wastewater. Among different loading ratios of ADWs, relatively lower ADW concentrations recorded higher lipid contents, a characteristic which had been evidenced in other studies as well (Cai et al., 2013a). Therefore, it can be concluded that 5.0% ADW concentration is better for *Desmodesmus* sp. due to higher biomass and lipid productivity that observed.

3.2. Fed-batch cultivations

3.2.1. Nutrient removal

The profiles of TN, NH_4-N and PO_4-P concentrations and removal efficiencies with time in ADW for 40-day fed-batch cultivation are depicted in Fig. 1. The concentrations of TN, NH_4-N and PO_4-P increased after loading fresh ADW every 2 days, but decreased rapidly due to absorption and consumption of nutrients by microalgae. The removal efficiencies of TN and NH_4-N increased along with time and reached 94.2% and 91.1%, respectively after 40 days of cultivation, while the maximum PO_4-P removal efficiency reached a peak value of 95.6% on the Day 6 and then decreased. Fig. 1a and b illustrates that the nitrogen demand for microalgae growth was almost the same as that provided by ADW. However, Fig. 1c indicates that the surplus phosphorus from fresh ADW loading would not be used further by microalgae after 8 days. The rate of TN, NH_4-N and PO_4-P removal stayed in the ranges of 6.706–9.464, 5.904–8.920 and 0.161–0.390 mg/L/d, respectively during this period.

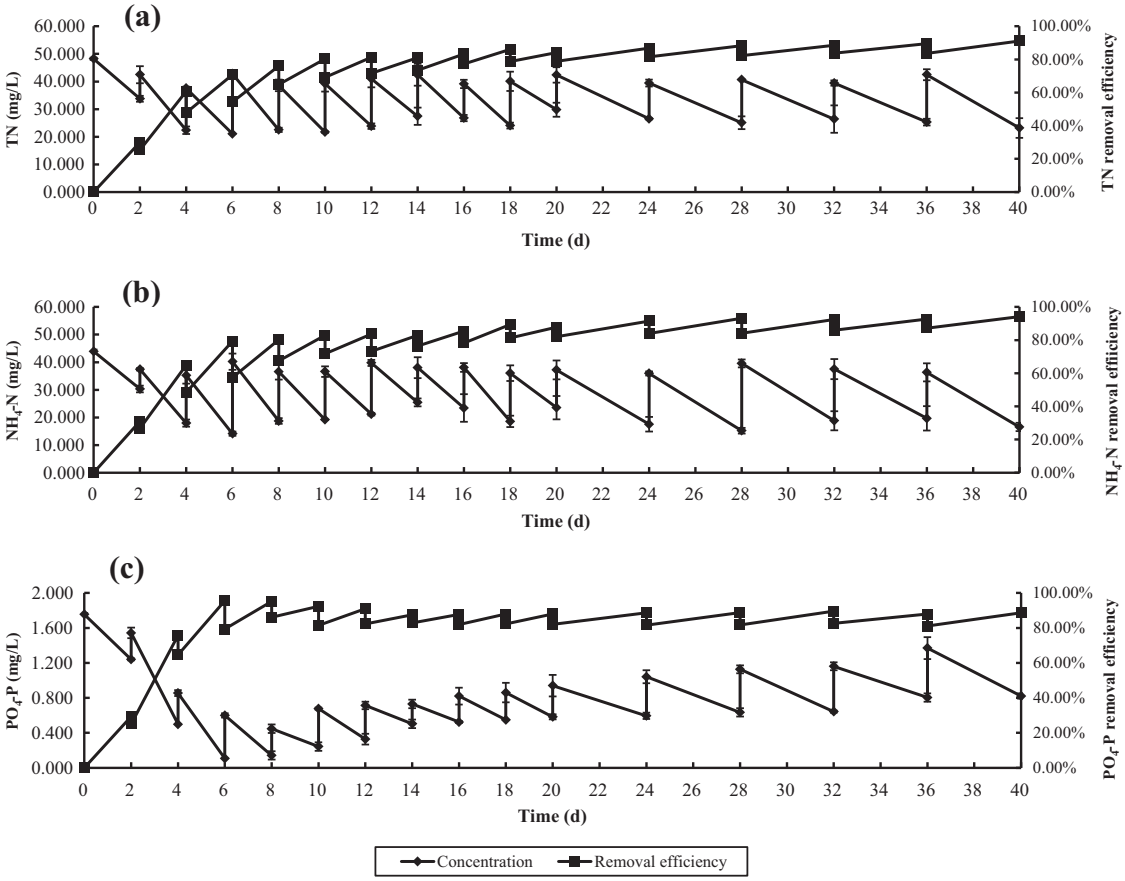

Fig. 1. Removal of (a) TN, (b) NH_4-N and (c) PO_4-P from ADW by *Desmodesmus* sp. EJ15-2 in fed-batch culture.

Table 2
Comparison of nutrient removal between batch and fed-batch experiments.

Nutrients	Batch experiments (5.0% ADW) [a]				Fed-batch experiments [b]			
	Removal efficiency (%)	Removal amount (mg/L)	Removal rate (mg/L/d)	Max. removal rate (mg/L/d)	Removal efficiency	Removal amount (mg/L)	Removal rate (mg/L/d)	Max. removal rate (mg/L/d)
TN	75.6	33.331	3.333	6.227	94.2%	236.143	5.904	9.464
NH_4-N	92.7	37.227	3.723	7.701	91.1%	268.238	6.706	8.920
PO_4-P	100	1.323	0.132	0.244	88.7%	6.427	0.161	0.390

[a] Batch experiments were conducted for 10 days.
[b] Fed-batch experiments were conducted for 40 days.

The performance of batch and fed-batch experiments in removing nutrients are compared in Table 2. In batch experiments, 75.6%, 92.7% and 100% of TN, NH_4-N and PO_4-P were removed while in fed-batch experiments; the removal efficiencies for TN, NH_4-N and PO_4-P were 94.2%, 91.1% and 88.7%, respectively. The amount of TN, NH_4-N and PO_4-P removed in the 10-day batch experiments were 33.331, 37.227 and 1.323 mg/L, respectively, while 236.143, 268.238 and 6.427 mg/L of TN, NH_4-N and PO_4-P, respectively, were removed in fed-batch experiments after 40 days of cultivation. The phenomenon observed in this study corresponds to the results obtained by Xie et al. (2013) and Min et al. (2011), who also reported that fed-batch cultivation had more capability to increase biomass production and nutrient reduction than that of in batch cultivation.

The detection of ammonia as a nitrogen source in the medium served the purpose of identifying inhibition (6 mM) or toxic concentrations (10 mM) (Carvalho et al., 2004). He et al. (2013) reported that the inhibitory ammonia level of *Chlorella vulgaris* was 17 mg/L, while the toxic level was 143 mg/L. The utilization of nitrogen from ADWs is subject to the lower initial concentration in batch systems, which lead to lower biomass yields. Therefore, supplying external nitrogen has been commonly suggested as a method to improve growth rate and biomass production of microalgae (Sturm and Lamer, 2011).

In general, the optimal N/P ratio was suggested as from 6.8:1 to 10:1 for freshwater algae growth (Wang et al., 2010b); and a proper N/P would be 16:1 for ammonia as nitrogen source (Kim et al., 2013). However, the ADWs used in this study and the final removal amount in fed-batch cultivation showed that N/P ratio was 25:1 and 36:1, respectively. It indicated that phosphorus is not an inhibiting element for this microalgae strain. Due to varying internal N/P ratio reported for different algae strains, and under

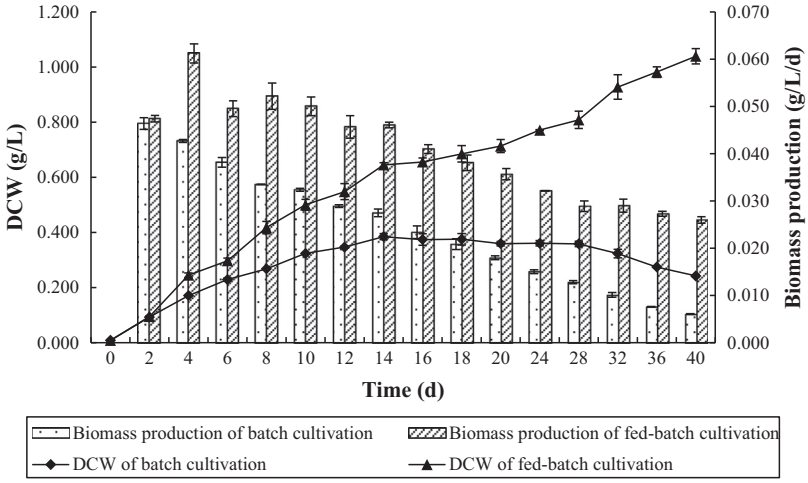

Fig. 2. Comparison of DCW and biomass production between batch and fed-batch cultivation in ADWs.

Table 3
Comparison of biomass and lipid production by microalgae in ADWs.

Microalgae species	ADW source	Loading ratio (%)	Biomass production (g/L)	Lipid production (mg/L)	Refs.
Chlorella minutissima	Poultry litter	4	0.340	40.1	Singh et al. (2011)
Chlorella sorokiniana	Poultry litter	4	0.366	45.0	Singh et al. (2011)
Scenedesmus bijuga	Poultry litter	4	0.352	41.2	Singh et al. (2011)
Neochloris oleoabundans	Cow manure	0.5	0.399	37.9	Levine et al. (2011)
Scenedesmus sp.	Pig manure	10	0.458–0.556	n.a.	Park et al. (2010)
Desmodesmus sp.	Pig manure	Fed-batch	1.039	261.8	This study

n.a.: not available.

different growth conditions (Ho et al., 2003), it might be possible that the ratio in the supplied wastewater was suboptimal and varying for microalgae growth (Boelee et al., 2011).

3.2.2. Biomass growth

On the basis of preliminary batch experiments results, high initial ADW concentration negatively affected the biomass production of microalgae. Fed-batch cultivation of *Desmodesmus* sp. was conducted at optimal ADW concentration (5.0% ADW which contained approximately 40 mg/L NH_4-N) determined during preliminary batch-cultivation experiments. Comparisons of algae growth and biomass production in batch and fed-batch cultivation are illustrated in Fig. 2. Microalgae in batch and fed-batch experiments did not show obvious lag phases, since this wild microalgae strain was pre-adapted to the diluted ADWs in preliminary experiments of this study. Comparing those two treatments, both biomass productivities increased significantly in the first 12 days with the microalgae in fed-batch cultivation growing faster than the cultures in batch cultivation. Afterwards, algae growth in batch experiments moved into stagnate phase where there was no further change in biomass concentration. Nevertheless, fed-batch experiment still witnessed a rapid growth with a specific growth rate and optimal culturing conditions without nutrients overload or limitation. As shown in Fig. 2, in fed-batch cultivation, the

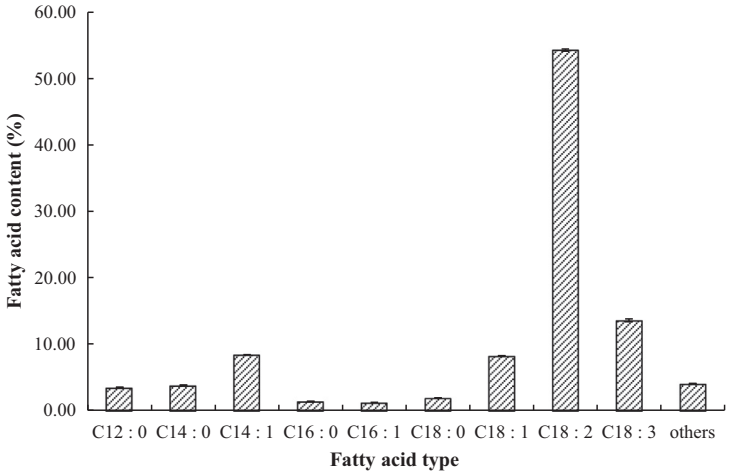

Fig. 3. Fatty acid profiles derived from triacylglycerol, phospholipid and free fatty acids in *Desmodesmus* sp.

biomass productivity was continuous and relatively stable during the first 10 days; and therefore the DCW increased steadily while in batch experiment biomass productivity decreased continuously. Moreover, nutrient reduction in fed-batch cultivation was stable as illustrated in Fig. 1. The highest DCW achieved in fed-batch cultivation after 40 days was 1.039 ± 0.028 g/L, which was more than 300% higher than that obtained from batch cultivation (0.324 g/L).

3.2.3. Lipid production and FAME composition

ADW has been documented as a potential source of nutrients for algae biomass production associated with wastewater treatment. However, previous studies have also indicated that the inhibition on microalgae growth was observed when it was cultivated in ADWs with high mixing ratios (Table 3). To achieve higher biomass and lipid production, the potential inhibition of high ammonium concentration and turbidity should be avoided. Thus, fed-batch cultivation could provide nutrients continuously and a means to avoid the undesirable effects of high initial concentrations and therefore achieve high biomass productivity (Zheng et al., 2013). Table 3 summarized the performance of different species of microalgae in terms of biomass and lipid production when different ADWs were used as substrate (culture media). The final values of 1.039 g/L (biomass concentration) and 261.8 mg/L (lipid concentration) were achieved by *Desmodesmus* sp. in this study.

Fatty acid composition of lipid from *Desmodesmus* sp. cultured in ADW using fed-batch process was determined on the last day and the results were shown in Fig. 3. The FAME content was 10.2% of DCW, while the percentage values of saturated, monounsaturated and polyunsaturated fatty acids in *Desmodesmus* sp. lipids were 10.6%, 20.1% and 69.3%, respectively. The linoleic acid (C18:2) presented a significant percentage (54.3%) among all FAMEs, which indicated that the lipid accumulation could be enhanced when cultures of *Desmodesmus* sp were subjected to low nutrient availability (Wang et al., 2010a).

4. Conclusions

Fed-batch cultivation of *Desmodesmus* sp. improved the amount of nitrogen and phosphorus that removed from ADWs. Moreover, this process could provide more effective control of nutrients limitation and/or ammonia inhibition on microalgae cultivation and growth. With the optimized culture and feeding conditions, the biomass and lipid contents reached 1.039 g/L and 261.8 mg/L, while the amount of TN, NH_4-N and PO_4-P removed were 236.143, 268.238 and 6.427 mg/L, respectively. Therefore, it can be concluded that *Desmodesmus* sp. under fed-batch cultivation mode performed better than the cultures of the same algae species under batch cultivation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.09.144.

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