Water-saving analysis on an effective water reuse system in biodiesel feedstock production based on *Chlorella zofingiensis* fed-batch cultivation

Kang Yang, Lei Qin, Zhongming Wang, Wei Feng, Pingzhong Feng, Shunni Zhu, Jingliang Xu and Zhenhong Yuan

ABSTRACT

The micralgae-based biofuel obtained from dairy wastewater (DWW) is considered a promising source of energy. However, this process consumes water due to the concentration of wastewater being normally too high for some micoralgae cultivation, and dilution is always needed. In this work, the cultivation of microalgae has been examined in non-recirculated water (NR) and recirculated water systems (R). The growth of *Chlorella zofingiensis* and the nutrient removal of DWW have been recorded. The comparison indicates the R had a little more advantage in biomass and lipid output (1.55, 0.22 g, respectively) than the NR (1.51, 0.20 g, respectively). However, the total chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), and total phosphorus (TP) removals of the R were lower than those of the NR system during the culture. The highest removal of total COD, TKN, and TP were 85.05%, 93.64%, and 98.45%, respectively. Furthermore, no significant difference has been observed in the higher heating value and lipid content of the biomass of the R and NR. The results show the R can save 30% of the total water input during the culture. All above results indicate the R system has great potential in industry.

Key words | *Chlorella zofingiensis*, dairy wastewater, fed-batch, water-saving potential

Kang Yang†
Lei Qin†
Zhongming Wang
Pingzhong Feng
Shunni Zhu
Jingliang Xu
Zhenhong Yuan (corresponding author)
Key Laboratory of Renewable Energy, Chinese Academy of Sciences,
Guangzhou Institute of Energy Conversion,
Guangzhou 510640,
China
E-mail: *yuanzh@ms.giec.ac.cn*

Wei Feng
Beijing Key Laboratory of Bioprocess, Department of Biochemical Engineering,
Beijing University of Chemical Technology,
Beijing 100029,
China

Kang Yang
University of Chinese Academy of Sciences,
Beijing 100049,
China

†These two authors contributed equally to this work.

INTRODUCTION

Biodiesel, as a form of green and alternative renewable energy, attracts ever-increasing attention from researchers, policy makers, and traders (Lam & Lee 2012). Unlike grain and cellulose, which are the main feedstock of first and second generation biofuel, microalgae is considered to be third generation feedstock as it can be cultivated in marine water, fresh water, and even wastewater. Furthermore, microalgae can grow quickly with a high lipid content, which can be used as source of biofuel (Markou *et al.* 2012).

The microalgae biomass is not very economically feasible because water and fertilizer are needed in the process and costs can be very high (4.2 and 14.8% of raw materials and utilities costs, respectively; Acien *et al.* 2012). Such costs can be reduced by using wastewater that is rich in nitrogen and phosphorus (e.g., piggery wastewater, dairy wastewater (DWW), and urban wastewater) to cultivate microalgae (Christenson & Sims 2011; Wang *et al.* 2012; Qin *et al.* 2014). However, in the wastewater, the concentration of urea, ammonium, organic acids, pesticides, and other compounds are normally high enough to suppress the growth of microalgae (Hodaifa *et al.* 2008). Ji *et al.* (2013) reported that dilution of the wastewater can improve the *Chlorella vulgaris* YSW-04 growth, lipid productivity, and nutrient removal of the piggery wastewater. Wang *et al.* (2010) reported that the average specific growth rates of *Chlorella* sp. in the first 7 days increased (0.282, 0.350, 0.407 and 0.409 d^{-1}) with the dilution ratio (10×, 15×, 20× and 25×, respectively) of digested dairy manure. Our latest research also showed that appropriate dilution of high concentration piggery wastewater can increase the growth of *Chlorella zofingiensis* (Zhu *et al.* 2013). Hence, dilution of high concentration wastewater is needed to improve the growth of microalgae. Based on this concept, a huge amount of water is needed when using wastewater with a high concentration for microalgae cultivation. Although water contributes just 4.2% to the raw materials and utilities cost of microalgae biomass production (Acien *et al.* 2012), clean water is essential

doi: 10.2166/wst.2015.139

and indispensable to human and other life. Hence, we should take water-saving into account in the microalgae cultivation process. Nevertheless, to date, related literature was rare.

This work aims to evaluate the quantity of water saved during microalgae fed-batch cultivation in a recirculated water system. The biomass of *C. zofingiensis*, the lipid output, and the nutrient removal of wastewater during the different culture stages, and the amount of water saved in the whole culture period were investigated, followed by assessing the influence of the recirculation of water.

MATERIAL AND METHODS

Algal strain and cultivation condition

The *C. zofingiensis* was inoculated into 250 ml Erlenmeyer flasks containing 100 ml BG-11 medium. The inoculum was incubated under illumination at ~200 μmol photon m^{-2} s^{-1} at 25 °C, and was aerated with air (supplemented with 5% CO_2) at a rate of 1 vvm (volume gas per volume media per minute) for 4 days.

Dairy wastewater

The DWW was collected from the Yili Dairy Co., Ltd in Foshan, Guangdong, China. Large solid particles were removed from the sample by sedimentation, and suspended solids were removed by centrifuge (5,000 rpm, 10 minutes). The supernatant was dealt with by autoclaving and stored at 4 °C for the following experiments. The characteristics and concentration of the autoclaved DWW were then determined (Table 1).

Experiment design

All cultivations of *C. zofingiensis* were performed in 1 l glass photobioreactors (PBRs) ($\Phi = 5.5$ cm × 70 cm) containing 500 ml of mixed liquor (250 ml autoclaved DWW and 250 ml sterile water) in the beginning. *C. zofingiensis* was harvested by centrifugation (3,000 rpm, 15 minutes, 4 °C) from a 4-day culture in BG11, washed with 15 mg L^{-1} $NaHCO_3$ solution and inoculated into each PBR. Each PBR was aerated with air supplemented with 5% CO_2 at 1 vvm. The whole cultivation period (288 hours) was artificially divided into four stages (each of 72 hours), based on the cultivation time of commercialized *Chlorella* and the time for nutrient removal from wastewater by microalgae, for fed-batch cultivation. At the end of the first, second and third stage, half the cultured liquid (250 ml) was taken out from the PBRs, followed by centrifuging (5,000 rpm, 5 minutes). Then, 125 ml sterile water plus 125 ml autoclaved DWW (NR) or 125 ml recirculated supernatant (RS) (produced by centrifugation) plus 125 ml autoclaved DWW (R) was added into the corresponding PBR. The collected biomass for each stage was washed twice with distilled water and dried by lyophilization for subsequent analysis. All treatment groups were carried out in duplicate.

Table 1 | Characteristics of autoclaved DWW used in this work

Parameter	Concentration
pH	9.11 ± 0.01
COD (mg L^{-1})	1,858 ± 17
TN (mg L^{-1})	136.5 ± 2.2
TP (mg L^{-1})	85.0 ± 1.9
NH_3-N (mg L^{-1})	90.4 ± 1.6
Suspended solids (mg L^{-1})	395 ± 12

Algal growth determination and nutrient removal analysis

The pH of the culture broth was measured every 24 hours. Microalgae suspension (5 ml) was collected every 24 hours from each PBR for growth and nutrient removal determination. The samples were filtered using a syringe with 0.22 μm filter paper (Whatman GF/C, GE Healthcare UK Limited, Buckinghamshire, UK). The filtrates were appropriately diluted for chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), total phosphorus (TP) and ammonium (NH_3-N) analysis following the Hach DR2700 spectrophotometer manufacturer's manual (Hach Co., Loveland, CO, USA). The filter papers were rinsed twice with ultrapure water and then incubated at 105 °C until they reached a constant weight. The biomass concentration during the cultivation period was calculated through the following equation:

$$\text{Biomass concentration (g L}^{-1}) = P_1 - P_0/0.005$$

where P_0 was the weight of the filter paper; P_1 was the weight of the filter paper after filtration.

The total removal rate was calculated through the following equation:

$$\text{Total removal rate (\%)} = \frac{(\sum A_i - R_i)}{\sum A_i \times 100\%}$$

where A_i was defined as the added amount of COD, total nitrogen (TN), and TP at different stages; R_i was the residual at the end of different stages (i = 1,2,3,4).

Lipid content analysis and elemental analysis

Total lipid was extracted following the method of Bigogno *et al.* (2002). The lipid content was calculated as follows:

$$\text{Lipid content } (\%) = \frac{W_l}{W_a} \times 100\%$$

where W_l was the weight of the extracted lipid; W_a was the weight of the corresponding dry biomass.

The lyophilized algal powder for each stage was analyzed for nitrogen, carbon, hydrogen and sulfur contents determination using an elemental analyzer (Elemental AnalyserVario-EL, Elementar Analysensysteme GmbH, Hanau, Germany). To quantify the suitability of biomass as a potential biofuel, the higher heating value (HHV) was calculated according to the report by Maddi *et al.* (2011).

RESULTS AND DISCUSSION

The growth condition and characteristics of *C. zofingiensis*

Irradiance and broth temperature of the whole cultivation period were measured daily at 8:00, 10:00, 12:00, 14:00, 16:00 and 18:00 hours (Supplementary data, available online at http://www.iwaponline.com/wst/071/139.pdf). The biomass and pH profiles of the cultivation are shown in Figure 1. The biomass concentrations of the R were a little higher than those of the NR system in stages two to four except at 288 hours. In stage one, the biomass concentrations of the NR and R systems were nearly the same because of the same culture conditions. In stage two, the biomass concentrations of the NR and R system rapidly reached 1.49 and 1.62 g L^{-1}, respectively. Although the biomass concentrations of the NR and R systems can also increase to 1.58 and 1.69 g L^{-1}, respectively, in stage three, the initial concentrations are significantly higher than those from stage two. The biomass concentrations of the NR and R systems were 1.25 and 1.21 g L^{-1}, respectively, at the end of stage four. Many factors, including nutrients, light, temperature, salinity, etc., can affect algae growth (von Alvensleben *et al.* 2013). During the whole culture, the same irradiance and temperature applied for the NR and R systems. Hence, it could be concluded that the residual nutrition in the RS promotes the growth of *C. zofingiensis*.

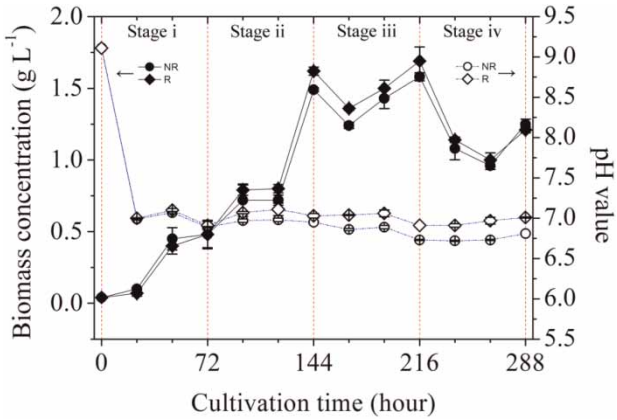

Figure 1 | The growth curves and pH profiles of *C. zofingiensis* of the NR and R systems during the whole cultivation period (means ± sd).

The pH declined sharply from 9.11 and fell between 6.7 and 7.1. The CO_2 was consumed due to the photosynthesis of microalgae. If CO_2 in the culture system is insufficient enough, the pH of the culture system would increase. In this work, 5% CO_2 is supplemented continuously throughout the whole cultivation period; CO_2 dissolved in the water in HCO_3^- form ensures the pH of the culture system maintained at a relatively stable range (Zhou *et al.* 2012a). The pH in the R was higher than those of the NR system in stages two to four. Fuggi *et al.* (1988) reported that acidic materials will be secreted to block cell division when cell concentration keeps increasing. Hence, we supposed that more acidic materials were accumulated in the R system as the import of RS, in turn increased the difference between the R and NR systems.

Nutrient removal efficiencies

The nutrient input in different cultivation stages, and the total removal rates of COD, TP and TKN at the end of each stage are shown in Figure 2. Just like many other studies (Zhou *et al.* 2012b; Franchino *et al.* 2013), NH_3-N at each stage was removed almost completely in 48 hours (data not shown). Unlike the biomass concentration profile, the total removal rates, including COD, TKN and TP, of the NR were higher than those of the R system. The R had a higher nutrient load than the NR system because the additional nutrient in the RS was brought into the R system during stages two to four (Figure 2). The total removal rates in stages two to four, regardless of COD, TKN and TP, for NR and R showed no significant change

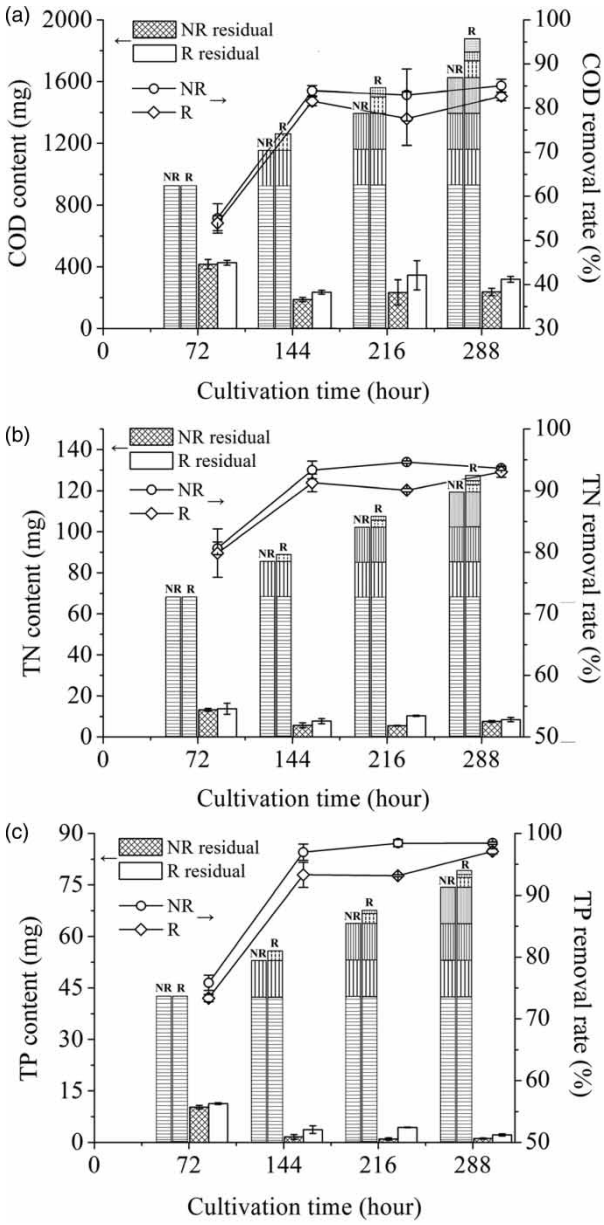

Figure 2 | Nutrient removal rates of (a) COD, (b) TN and (c) TP by *C. zofingiensis* at different stages (mean ± sd). Stack columns stand for the total amount of nutrients for each stage. ▭: Added in the beginning. ▥: Added at the end of stage i (autoclaved DWW). ▥: Added at the end of stage ii (autoclaved DWW). ▥: Added at the end of stage iii (autoclaved DWW). ▥: Added at the end of stage i (RS for stage i). ▥: Added at the end of stage ii (recirculated supernatant for stage ii). ▥: Added at the end of stage iii (recirculated supernatant for stage iii).

(77.61–85.05%, 90.08–93.64%, and 93.16–98.45%, respectively) which are higher than the corresponding values for stage one. The microalgae cells assimilated carbon, nitrogen and phosphorus, etc. from the wastewater for the nucleic acid, phospholipid and protein syntheses (Yan *et al.* 2013). During stages two to four, the biomass was taken out and lower concentration nutrient compared with stage one, was added into the cultivation system. The removal of COD, TKN and TP in this fed-batch cultivation were better than our previous research, which used DWW and piggery wastewater for batch *C. zofingiensis* cultivation (Zhu *et al.* 2013; Huo *et al.* 2012).

Lipid content and elemental analysis

The lipid content and elemental analysis for each stage are shown in Table 2. The lipid content reduced from 15.8 to 11.5% via 14.9 and 14.0% (NR) and from 15.7 to 12.7% via 15.2 and 14.3% (R) as the cultivation proceeded. This may be caused for the following reasons. (1) Growth stage limited. When nutrients are sufficient, the cells are highly productive. Under a nutrient-depletion situation, cell division and productivity are greatly reduced and lipid can be accumulated if the depletion is sustained for a certain period (Nigam *et al.* 2011). According to the experimental results, the algae rapidly grew during almost the whole cultivation period. Hence, the cells were not really in the lipid accumulation stage because of the addition of nutrient at the beginning of each stage. (2) Phosphorus limited. According to the high uptake rate of phosphorus and very low residual content in this study, insufficient phosphorus may be a suppression factor under the nitrogen starvation stage for lipid increase (Chu *et al.* 2013).

Diversified biofuel production from microalgae is necessary to improve the overall energy balance (Lam & Lee 2012). In this work, elemental analysis was performed to evaluate the biofuel potential of the algae biomass. The HHV is based on the elemental composition of the biomass and is a measure of the amount of energy stored within it. According to Table 2, the HHV values of *C. zofingiensis* biomass in this work were consistent with the HHV of energy grass *Miscanthus* (19.1 MJ kg^{-1}), wood material (19.6 MJ kg^{-1}) and sunflower (20.3 MJ kg^{-1}) (Friedl *et al.* 2005). The HHV values of different stages did not display significant differences. Hence, we could make a decision to complete the cultivation according only to the biomass or the lipid production and the nutrient removal efficiency.

Economic assessment and future prospects

There are many challenges to combine wastewater treatment with biofuel feedstock production through microalgae cultivation. Those challenges include nutrient supply and recycling, gas transfer and exchange, environment control to prevent contamination explosion, land and water

Table 2 | Elemental analysis and lipid contents of *C. zofingiensis* cultivated in NR and R systems at different stages

	72 h		144 h		216 h		288 h	
	NR	R	NR	R	NR	R	NR	R
Carbon	48.00	48.48	47.19	46.60	46.36	46.08	46.39	46.52
Hydrogen	7.13	7.14	7.15	7.10	7.12	7.07	7.15	7.08
Nitrogen	9.28	9.41	6.10	6.25	5.10	5.40	5.91	7.17
Sulfur	0.76	0.77	0.61	0.73	0.53	0.66	0.64	0.74
Oxygen*	34.83	34.20	38.96	39.32	40.90	40.79	39.91	38.49
C/N	5.17	5.15	7.73	7.45	9.08	8.53	7.85	6.49
C/H ratio	6.73	6.79	6.60	6.56	6.51	6.52	6.49	6.57
HHV(MJ/kg)	20.52	20.77	19.73	19.46	19.20	19.11	19.33	19.54
Lipid content (%)	15.8 ± 1.1	15.7 ± 0.6	14.9 ± 1.1	15.2 ± 0.9	14.0 ± 0.1	14.3 ± 1.2	11.5 ± 0.1	12.7 ± 0.3

Note: Oxygen* = 100(%)-C (%)-H (%)-N (%)-S (%).

availability (Christenson & Sims 2011). The cultivation of microalgae is water intensive and large-scale production of microalgae with wastewater requires plenty of clean water to dilute high concentration wastewater for better growth of microalgae. Water saving should be an important consideration to realize the real industrialization of microalgae energy. According to this work, the R not only has higher total biomass or lipid yield (1.55, 0.22 g, respectively) than the NR system (1.51, 0.20 g, respectively), but also saves 375 ml fresh water, about 30% of the total water input, in the 500 ml cultivation system, according to Table 3.

Water requirements will differ drastically for open raceway ponds (ORP) compared to a tubular or flat plate PBR. Furthermore, water requirements for cultivation are shown to be geographically dependent (Quinn & Davis 2014). It should be noted that this study has examined only one dilution condition, which was only conducted in tubular PBR. There are some limitations to revealing a general view of the water saving. However, this work offers a potential water-saving idea in wastewater treatment combined with microalgae production. Several further studies are needed to implement this technology. First, PBRs used in this study are easy to operate, to control and prevent contamination, but they are expensive. Cheaper culture systems, such as ORP, should be investigated. Second, the DWW suffered from sedimentation, followed by centrifugation and autoclaving previously used in this study. Hence, methods that are easy, low-cost, suitable for large-scale sterilization and contamination control should be researched further. Furthermore, CO_2 supplementary methods, which can increase the utilization efficiency of CO_2, should also be developed. In summary, there are still lots of research interests that should be introduced to the commercial utilization of microalgae feedstock produced by wastewater.

CONCLUSION

The quantity of water used in the wastewater diluting process saved in *C. zofingiensis* fed-batch cultivation by using

Table 3 | The input and output of the R and NR culture systems and the water-saving evaluation

	Input of water (ml)						Output of biomass or lipid (g, means ± sd)			
	NR			R			NR		R	
	DWW	SW	RS	DWW	SW	RS	Biomass	Lipid	Biomass	Lipid
0–72 h	250	250	0	250	250	0	0.12 ± 0.023	0.02 ± 0.002	0.12 ± 0.025	0.02 ± 0.003
72–144 h	125	125	0	125	0	125	0.37 ± 0.004	0.06 ± 0.004	0.41 ± 0.007	0.06 ± 0.003
144–216 h	125	125	0	125	0	125	0.40 ± 0.007	0.06 ± 0.001	0.42 ± 0.025	0.06 ± 0.002
216–288 h	125	125	0	125	0	125	0.63 ± 0.018	0.07 ± 0.003	0.60 ± 0.011	0.08 ± 0.003
Total	625	625	0	625	250	375*	1.51 ± 0.016	0.20 ± 0.004	1.55 ± 0.004	0.22 ± 0.010

the recirculated water system has been analyzed. The R achieves higher biomass or lipid output than the NR, and also saves 375 ml fresh water, which is about 30% of the total water input. This amount of saved water will be considerable if applied to the large-scale production of microalgae. In stages two to four, the removal of COD, TKN and TP for NR and R were 77.61–85.05%, 90.08–93.64% and 93.16–98.45%, respectively. Furthermore, there is no significant difference in HHV and the lipid content between the R and NR. Such results provide fundamental support for further research on water saving issues in microalgae biofuel feedstock production with DWW.

ACKNOWLEDGEMENTS

The authors are grateful for the support of the National Program on Key Basic Research Project of China (grant no. 2011CB200905), the National High Technology Research and Development Program of China (grant no. 2013AA065803), the National Natural Science Foundation of China (grant no. 31100189).

REFERENCES

Acien, F. G., Fernandez, J. M., Magan, J. J. & Molina, E. 2012 Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnol. Adv.* **30** (6), 1344–1353.

Bigogno, C., Khozin-Goldberg, I., Boussiba, S., Vonshak, A. & Cohen, Z. 2002 Lipid and fatty acid composition of the green oleaginous alga Parietochloris incisa, the richest plant source of arachidonic acid. *Phytochemistry* **60** (5), 497–503.

Christenson, L. & Sims, R. 2011 Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnol. Adv.* **29** (6), 686–702.

Chu, F. F., Chu, P. N., Cai, P. J., Li, W. W., Lam, P. K. & Zeng, R. J. 2013 Phosphorus plays an important role in enhancing biodiesel productivity of Chlorella vulgaris under nitrogen deficiency. *Bioresour. Technol.* **134**, 341–346.

Franchino, M., Comino, E., Bona, F. & Riggio, V. A. 2013 Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. *Chemosphere* **92** (6), 738–744.

Friedl, A., Padouvas, E., Rotter, H. & Varmuza, K. 2005 Prediction of heating values of biomass fuel from elemental composition. *Anal. Chim. Acta* **544**(1–2), 191–198.

Fuggi, A., Pinto, G., Pollio, A. & Taddei, R. 1988 Effects of NaCI, Na_2SO_4, H_2SO_4, and glucose on growth, photosynthesis, and respiration in the acidophilic alga *Dunaliella acidophila* (Volvocales, Chlorophyta). *Phycologia* **27** (3), 334–339.

Hodaifa, G., Martínez, M. A. & Sánchez, S. 2008 Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*. *Bioresour. Technol.* **99** (5), 1111–1117.

Huo, S., Wang, Z., Zhu, S., Zhou, W., Dong, R. & Yuan, Z. 2012 Cultivation of *Chlorella zofingiensis* in bench-scale outdoor ponds by regulation of pH using dairy wastewater in winter, South China. *Bioresour. Technol.* **121**, 76–82.

Ji, M. K., Kim, H. C., Sapireddy, V. R., Yun, H. S., Abou-Shanab, R. A., Choi, J., Lee, W., Timmes, T. C., Inamuddin & Jeon, B. H. 2013 Simultaneous nutrient removal and lipid production from pretreated piggery wastewater by Chlorella vulgaris YSW-04. *Appl. Microbiol. Biotechnol.* **97** (6), 2701–2710.

Lam, M. K. & Lee, K. T. 2012 Microalgae biofuels: A critical review of issues, problems and the way forward. *Biotechnol. Adv.* **30** (3), 673–690.

Maddi, B., Viamajala, S. & Varanasi, S. 2011 Comparative study of pyrolysis of algal biomass from natural lake blooms with lignocellulosic biomass. *Bioresour. Technol.* **102** (23), 11018–11026.

Markou, G., Angelidaki, I. & Georgakakis, D. 2012 Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. *Appl. Microbiol. Biotechnol.* **96** (3), 631–645.

Nigam, S., Rai, M. P. & Sharma, R. 2011 Effect of nitrogen on growth and lipid content of *Chlorella pyrenoidosa*. *Am. J. Biochem. Biotechnol.* **7** (3), 124–129.

Qin, L., Shu, Q., Wang, Z., Shang, C., Zhu, S., Xu, J., Li, R., Zhu, L. & Yuan, Z. 2014 Cultivation of *Chlorella vulgaris* in dairy wastewater pretreated by UV irradiation and sodium hypochlorite. *Appl. Biochem. Biotechnol.* **172** (2), 1121–1130.

Quinn, J. C. & Davis, R. 2014 The potentials and challenges of algae based biofuels: a review of the techno-economic, life cycle, and resource assessment modeling. *Bioresour. Technol.* **184**, 444–452.

von Alvensleben, N., Stookey, K., Magnusson, M. & Heimann, K. 2013 Salinity tolerance of *Picochlorum atomus* and the use of salinity for contamination control by the freshwater Cyanobacterium *Pseudanabaena limnetica*. *PLoS One* **8** (5), e63569.

Wang, H., Xiong, H., Hui, Z. & Zeng, X. 2012 Mixotrophic cultivation of Chlorella pyrenoidosa with diluted primary piggery wastewater to produce lipids. *Bioresour. Technol.* **104**, 215–220.

Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J. & Ruan, R. R. 2010 Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae Chlorella sp. *Bioresour. Technol.* **101** (8), 2623–2628.

Yan, C., Zhao, Y., Zheng, Z. & Luo, X. 2013 Effects of various LED light wavelengths and light intensity supply strategies on synthetic high-strength wastewater purification by *Chlorella vulgaris*. *Biodegradation* **24** (5), 721–732.

Zhou, W., Hu, B., Li, Y., Min, M., Mohr, M., Du, Z., Chen, P. & Ruan, R. 2012a Mass cultivation of microalgae on animal

wastewater: a sequential two-stage cultivation process for energy crop and omega-3-rich animal feed production. *Appl. Biochem. Biotechnol.* **168** (2), 348–363.

Zhou, W., Min, M., Li, Y., Hu, B., Ma, X., Cheng, Y., Liu, Y., Chen, P. & Ruan, R. 2012b A hetero-photoautotrophic two-stage cultivation process to improve wastewater nutrient removal and enhance algal lipid accumulation. *Bioresour. Technol.* **110**, 448–455.

Zhu, L., Wang, Z., Shu, Q., Takala, J., Hiltunen, E., Feng, P. & Yuan, Z. 2013 Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment. *Water Res.* **47** (13), 4294–4302.

First received 19 November 2014; accepted in revised form 12 March 2015. Available online 27 March 2015