J. Microbiol. Biotechnol. (2013), **23**(1), 92–98
http://dx.doi.org/10.4014/jmb.1210.10033

pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Increased Microalgae Growth and Nutrient Removal Using Balanced N:P Ratio in Wastewater

Lee, Seung-Hoon[1,2], Chi-Yong Ahn[1,2], Beom-Ho Jo[1], Sang-Ah Lee[3], Ji-Yeon Park[4], Kwang-Guk An[3], and Hee-Mock Oh[1,2]*

[1]*Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea*
[2]*University of Science and Technology (UST), Daejeon 305-350, Korea*
[3]*Department of Biological Science, School of Biological Sciences and Biotechnology, Chungnam National University, Daejeon 305-764, Korea*
[4]*Clean Fuel Department, Korea Institute of Energy Research, Daejeon 305-343, Korea*

Received: October 15, 2012 / Revised: October 30, 2012 / Accepted: November 3, 2012

Microalgal cultivation using wastewater is now regarded as essential for biodiesel production, as two goals can be achieved simultaneously; that is, nutrient removal efficiency and biomass production. Therefore, this study examined the effects of carbon sources, the N:P ratio, and the hydraulic retention time (HRT) to identify the optimal conditions for nutrient removal efficiency and biomass production. The effluent from a 2nd lagoon was used to cultivate microalgae. Whereas the algal species diversity and lipid content increased with a longer HRT, the algal biomass productivity decreased. Different carbon sources also affected the algal species composition. Diatoms were dominant with an increased pH when bicarbonate was supplied. However, 2% CO_2 gas led to a lower pH and the dominance of filamentous green algae with a much lower biomass productivity. Among the experiments, the highest chlorophyll-*a* concentration and lipid productivity were obtained with the addition of phosphate up to 0.5 mg/l P, since phosphorus was in short supply compared with nitrogen. The N and P removal efficiencies were also higher with a balanced N:P ratio, based on the addition of phosphate. Thus, optimizing the N:P ratio for the dominant algae could be critical in attaining higher algal growth, lipid productivity, and nutrient removal efficiency.

Key words: Biodiesel, microalgae, nitrogen, N:P ratio, phosphorus, wastewater

In the context of a shrinking availability of petroleum-based fuels and imminent energy crisis, microalgae have been suggested as a promising feedstock for biofuel production owing to a number of advantages, including a higher photosynthetic efficiency, higher biomass production, and higher growth rates when compared with other oil crops [4, 9, 23]. Microalgae have an approximately 50% carbon dry weight, meaning that about 1.8 kg of CO_2 is required to generate 1 kg of algal biomass [1]. Moreover, producing 1 kg of microalgae-based biodiesel requires about 3,726 kg of water, 0.33 kg of nitrogen, and 0.71 kg of phosphate, if freshwater is used without recycling [26]. Therefore, this vast consumption of water resources, inorganic nutrients (mainly nitrogen and phosphate), and CO_2 makes microalgal cultivation too expensive, which is a critical problem [15].

Population growth poses a serious threat to the environment owing to the release of vast amounts of domestic wastewater [14]. The major effect of releasing wastewater rich in organic compounds and inorganic chemicals, such as phosphates and nitrates, is the eutrophication of freshwater ecosystems [6, 17]. Therefore, additional physical and chemical tertiary wastewater treatments are needed before any freshwater release, yet this is often too costly to implement [8]. One possible solution to overcome the high cost of microalgal cultivation is to use wastewater treatment to obtain a microalgae biomass as a by-product, which is essential for biodiesel production [20, 21].

The use of algae for municipal wastewater treatment in ponds is already well established [20]. The carbon/nitrogen and carbon/phosphorus ratios in domestic sewage (C/N, 3.5; C/P, 20) and dairy lagoon water (C/N, 3; C/P, 10) are

---

*Corresponding author
Phone: +82-42-860-4321; Fax: +82-42-879-8103;
E-mail: heemock@kribb.re.kr

low compared with the typical ratios in a rapidly growing algae biomass (C/N, 6; C/P, 48) [19].

The 2nd effluents of domestic wastewater plants usually contain relatively low inorganic nutrients (around 10–15 mg/l total nitrogen and 0.5–1 mg/l total phosphorus), so an optimum N:P ratio and nutrient content are needed for good growth in 2nd effluents as well as the efficient removal of inorganic nutrients. The concentrations of nitrogen and phosphorus present in water have a fundamental and direct influence on algal growth kinetics, and are also closely related to nutrient removal efficiency and lipid accumulation [7].

Accordingly, this study examined the effects of the carbon sources, N:P ratio, and hydraulic retention time (HRT) to find the optimal conditions for biomass production and nutrient removal efficiency when using the effluent from a 2nd lagoon for microalgal cultivation.

MATERIALS AND METHODS

Wastewater Characterization

The effluent from a 2nd lagoon was collected at the Daejeon Metropolitan City Facilities Management Corporation. The average total phosphorus (TP) and total nitrogen (TN) during experiments were 0.13 ± 0.01 mg/l and 7.04 ± 1.03 mg/l, respectively.

Microalgae Culture Condition

The microalgae in the effluent from the 2nd lagoon, including *Scenedesmus*, *Chlorella*, *Nitzschia*, and other filamentous microalgae, were cultivated in five sets of treatments conducted in parallel (Table 1). The microalgae were cultured in plastic baskets (35 cm height, 30 cm diameter) with a working volume of 18 L under semi-continuous operation for 17 days. The temperature during experiments was 23.8–25.8°C. All the treatments, except for treatment 1 and 5, were supplied with ambient air at 0.3 v/v/m, whereas treatment 1 was supplied with 2% CO_2 gas at 0.3 v/v/m. Up to 0.5 mg/l of phosphorus in K_2HPO_4 was supplied as treatment 3, and 2.3 mg/l of carbon in Na_2CO_3 as the carbon source was supplied as treatment 5.

Algal Biomass Determination

An aliquot (10 ml) of the culture was filtered through a glass microfiber filter (GF/C, Whatmann). The retained cells were dried at 105°C for 24 h and cooled to room temperature, and the dry weight was measured.

Table 1. Culture conditions of phosphorus addition, carbon sources, and hydraulic retention time (HRT).

Treatment No.	P addition	Carbon source	HRT
Treatment 1	-	2% CO_2 gas	3 days
Treatment 2	-	Air	3 days
Treatment 3	0.5 mg/l P	Air	3 days
Treatment 4	-	Air	6 days
Treatment 5	-	2.3 mg/l C in Na_2CO_3	3 days

Chlorophyll-*a* Determination

A known volume of the microalgae suspension was centrifuged (4,000 rpm, 10 min, 4°C) and all the residue were extracted using a 90% (v/v) acetone solution. The chlorophyll-*a* concentration in the pooled extract was then spectrophotometrically measured at four wavelengths (630, 647, 664, and 730 nm) and calculated using Jeffrey's method [10].

Phosphorus and Nitrogen Analysis

The concentrations of phosphorus and nitrogen were determined after persulfate oxidation to orthophosphate and nitrate, respectively. The total phosphorus (TP), total dissolved phosphorus (TDP), and total particulate phosphorus (TPP) were analyzed using the ascorbic acid method [2]. The total nitrogen (TN), total dissolved nitrogen (TDN), and total particulate nitrogen (TPN) were analyzed using the ultraviolet spectrophotometric screening method [2]. TDP and TDN were determined after filtering the water samples through a 0.20 µm cellulose filter (Minisart, Sartorius Stedim). TPP and TPN were the difference of TP − TDP and TN − TDN. The phosphorus and nitrogen removal efficiencies were calculated as TPP divided by TP, and TPN divided by TN, respectively.

Fatty Acid Analysis

The fatty acids were analyzed using the modified method of Lepage and Roy [13]. The crude lipid (~10 mg) was dissolved using 2 ml of a freshly prepared chloroform–methanol mixture [2:1 (v/v)] and transferred into a capped test tube. One milliliter of chloroform containing nonadecanoic acid (500 mg/l) as the internal standard and 1 ml of methanol and 300 ml of sulfuric acid as transmethylation reagents were mixed for 5 min and then incubated at 100°C for 10 min. The fatty acid-containing phase was separated by adding 1 ml of distilled water and then recovered. The organic phase was filtered using a hypodermic 0.22 µm PVDF syringe filter (Millex-GV; Millipore, USA). The methyl esters of the fatty acids were analyzed using a gas chromatograph (GC-7890; Agilent, USA) equipped with a flame ionization detector and HP-INNO wax capillary column (Agilent Technologies, USA). The temperatures of the injector and detector were set at 250°C and 275°C, respectively. The oven temperature conditions were maintained at 50°C for 1 min, 200°C for 12 min, and 250°C for 2 min. Mix RM3, Mix RM5, GLC50, GLC70 (Supelco Co., USA), and α-linolenic acid (Sigma Chemical Co., USA) were used as the standard materials. All the reagents were of analytical grade. The components were then identified by comparing their retention times and fragmentation patterns with those of the standards [25].

RESULTS

Comparison of Microalgal Growth Under Different Conditions

The semi-continuous microalgae cultures were grown for 17 days under 5 different conditions, as described in Table 1. The highest chlorophyll-*a* concentration, 486.7 µg/l, was obtained with the addition of phosphate (Fig. 1). The chlorophyll-*a* concentration with a balanced N:P ratio when supplying additional phosphorus up to 0.5 mg/l was about 4.7–5.0 times higher than with the other treatments.

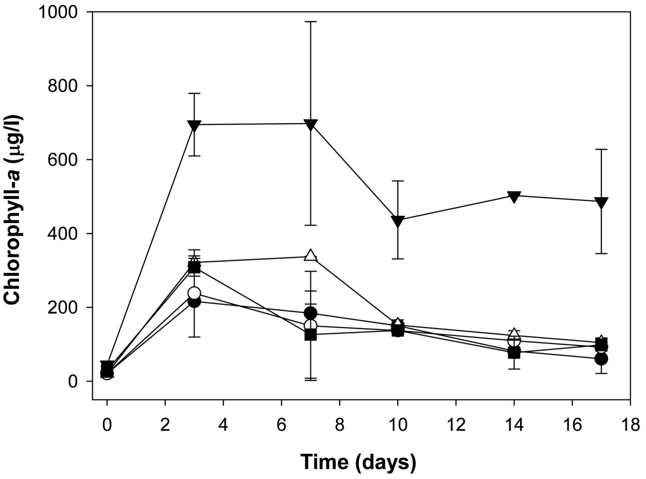

Fig. 1. Chlorophyll-*a* concentrations in semi-continuous culture using wastewater effluent under five different conditions (●, 2% CO_2; ○, ambient air; ▼, 0.5 mg/l P; △, 6-day HRT; ■, bicarbonate). HRT was 3 days for all treatments except "6-day HRT". Each value indicates the mean ± SD of duplicates.

With the phosphate supplement, the *Scenedesmus*, *Chlorella*, and *Coelastrum* genera and filamentous Chlorophyceae dominated. The second highest chlorophyll-*a* concentration, 104.5 μg/l, was obtained with the longer 6-day HRT, which increased the number of microalgal species and cultivated microalgal biomass when compared with the 3-day HRT. The supply of 2% CO_2 or bicarbonate did not contribute to the algal biomass productivity when compared with that with ambient air. The chlorophyll-*a* concentrations were 60.6 μg/l and 90.8 μg/l with the 2% CO_2 and bicarbonate, respectively. Diatoms were dominant with an increased pH when bicarbonate was supplied. However, the 2% CO_2 gas led to a lower pH and the dominance of filamentous green algae with a much lower biomass productivity.

Comparison of Nutrient Removal Efficiency

After 17 days, over 70% phosphorus removal efficiency was achieved by all five treatments (Fig. 2A). The TP concentrations in the 2nd lagoon effluent were below 0.15 mg/l, and the TDP concentrations were below 0.04 mg/l. The supply of 2% CO_2 gas only produced a 7% increase in phosphorus removal efficiency when compared with that with ambient air. However, the phosphorus removal efficiency decreased approximately 6% when bicarbonate was supplied. The longer 6-day HRT had an adverse effect on the phosphorus removal efficiency. The TP concentrations increased to 0.52–0.61 mg/l with the addition of phosphate, yet the TDP concentrations were below 0.04 mg/l, which was similar to the other treatments (Fig. 2B). The addition of phosphate resulted in the highest phosphorus removal efficiency of 92.4%, based on a balanced N:P ratio that was optimal for algal growth.

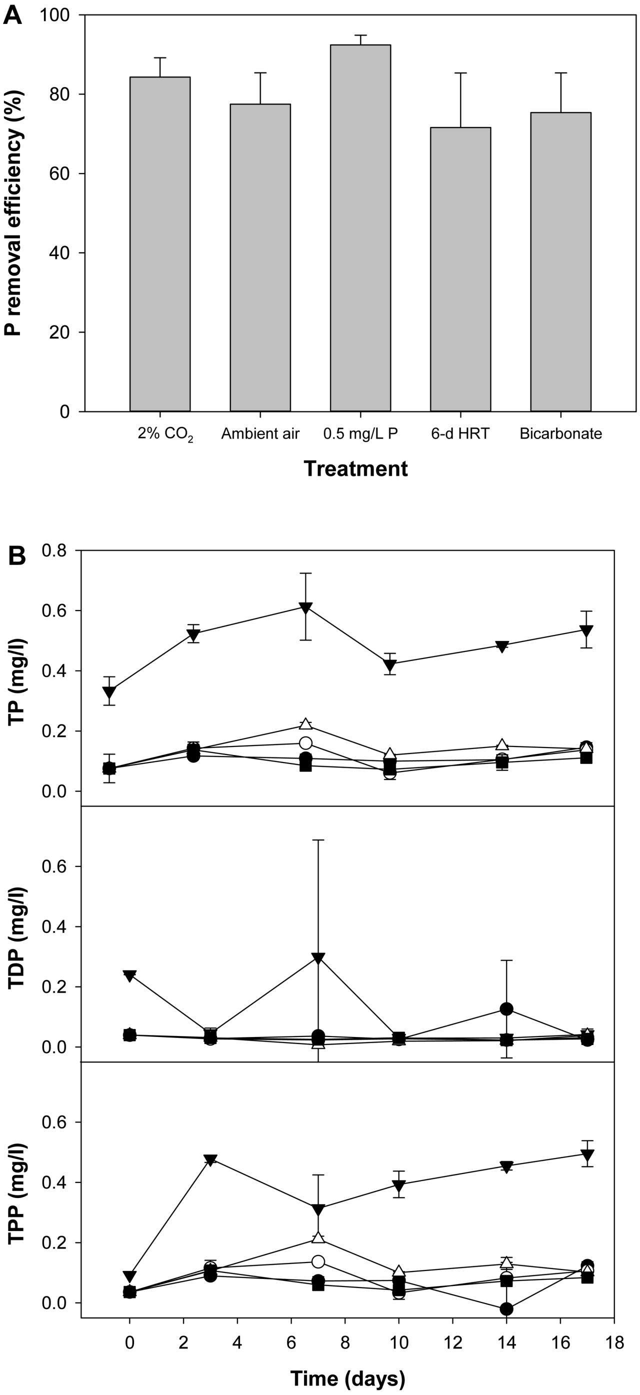

Fig. 2. Phosphorus removal efficiency (**A**) and phosphorus concentration (TP, TDP, and TPP) (**B**) in semi-continuous culture using wastewater effluent under five different conditions (●, 2% CO_2; ○, ambient air; ▼, 0.5 mg/l P; △, 6-day HRT; ■, bicarbonate).
Each value indicates the mean ± SD of duplicates.

The nitrogen removal efficiency for all the treatments, except for the addition of phosphate, was below 30% (Fig. 3A). The TN concentrations in the effluent were 8.1–

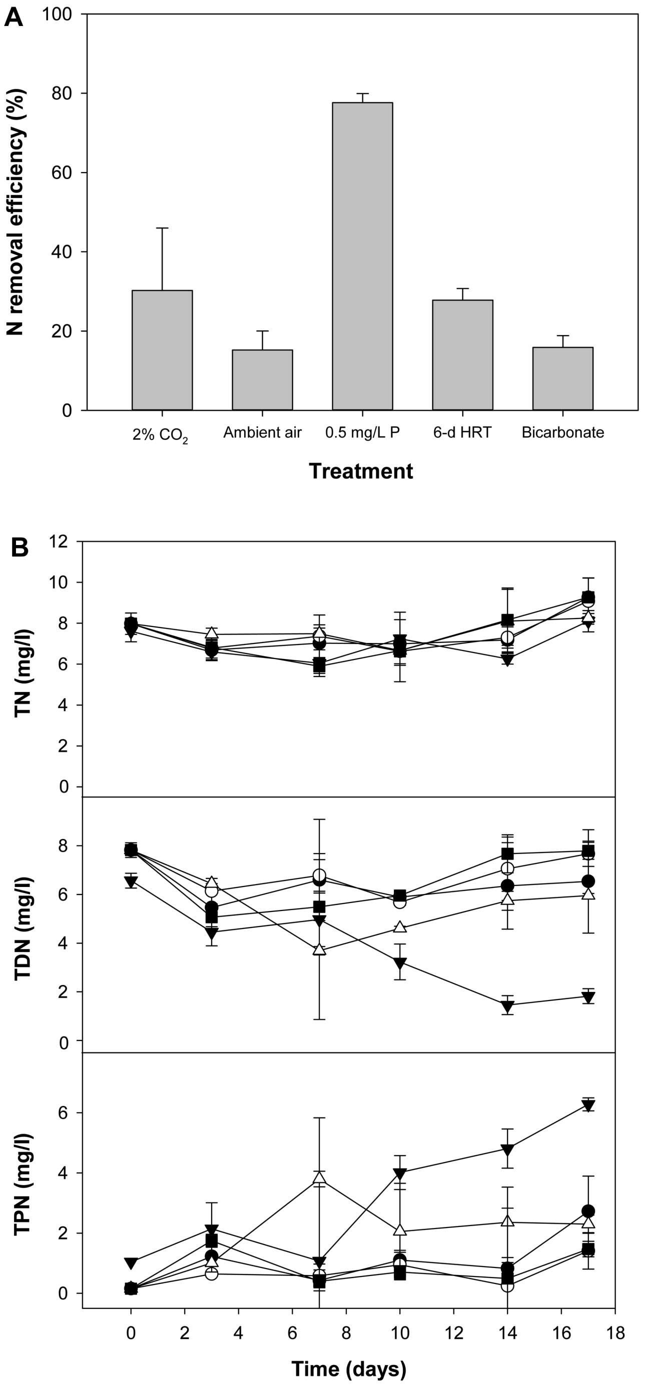

Fig. 3. Nitrogen removal efficiency (**A**) and nitrogen concentration (TN, TDN, and TPN) (**B**) in semi-continuous culture using wastewater effluent under five different conditions (●, 2% CO_2; ○, ambient air; ▼, 0.5 mg/l P; △, 6-day HRT; ■, bicarbonate). Each value indicates the mean ± SD of duplicates.

9.2 mg/l and the TDN concentrations were 5.0−7.9 mg/l. The TDN concentration decreased to 1.8 mg/l with the addition of phosphate (Fig. 3B). Supplying 2% CO_2 gas doubled the nitrogen removal efficiency compared with that with ambient air. However, bicarbonate did not increase the nitrogen removal efficiency. The highest nitrogen removal efficiency of over 77% was achieved with the addition of phosphorus up to 0.5 mg/l.

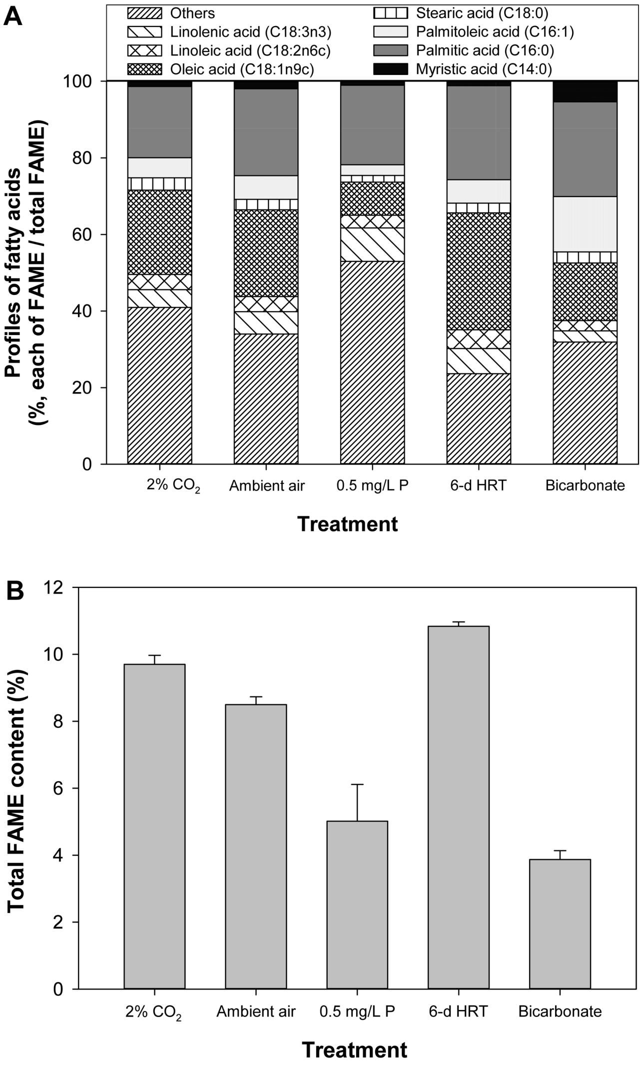

Fig. 4. Fatty acid methyl ester (FAME) profiles (**A**) and contents (**B**) after 17-day cultivation under five different conditions. Each value indicates the mean ± SD of duplicates.

Fatty Acid Composition and Productivity

The major fatty acid compositions were determined using a GC analysis on day 17 (Fig. 4A). Palmitic acid (C16:0) and oleic acid (C18:1n9c) were commonly dominant in all the treatments, ranging from 19% to 25% and 9% to 31%, respectively. Meanwhile, myristic acid (C14:0), palmitoleic acid (C16:1), stearic acid (C18:0), linoleic acid (C18:2n6c), and linolenic acid (C18:3n3) were the minor fatty acids. Bicarbonate addition led to the dominance of diatoms with the highest palmitic acid and palmitoleic acid contents when compared with the other treatments.

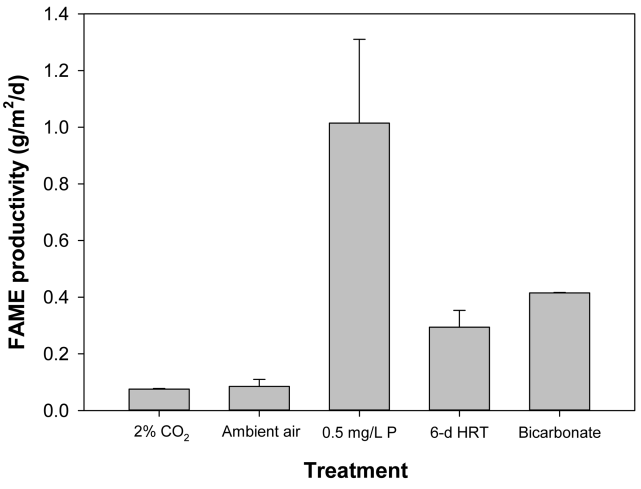

Fig. 5. Total fatty acid methyl ester (FAME) productivity for 17-day cultivation under five different conditions.
Each value indicates the mean ± SD of duplicates.

The total FAME contents of the five treatments ranged from 3.9% ± 0.3% to 10.8% ± 0.1% of the dry weight. The highest FAME content, 10.8% ± 0.1%, was obtained with the 6-day HRT. The total FAME content with the longer 6-day HRT was about 1.1−2.8 times higher than that with the other treatments. The supply of phosphate and bicarbonate did not contribute to the total FAME content when compared with that with air. Instead, the total FAME content decreased with the supply of phosphate and bicarbonate (Fig. 4B).

Although the FAME content was relatively lower with the addition of phosphate, the highest biomass productivity resulted in the highest FAME productivity of 1.0 ± 0.3 g/m^2/day (Fig. 5). The FAME productivity with the balanced N:P ratio based on supplying additional phosphate was about 2.4−13.4 times higher than that with the other treatments. The supply of bicarbonate and longer 6-day HRT contributed to the FAME productivity when compared with that with ambient air.

Discussion

This study attempted to find the optimal conditions for algae biomass production for making biodiesel and for nutrient removal efficiency using the effluent from a 2nd lagoon. Specifically, we examined the effects of such factors as the carbon sources, N:P ratio, and HRT.

For microalgal growth, the 2nd effluent of domestic wastewater usually contains relatively low inorganic nutrients (around 10−15 mg/l nitrogen and 0.5−1 mg/l phosphorus) [24]. The 2nd effluent in this study contained 7.04 ± 1.03 mg/l of TN and 0.13 ± 0.01 mg/l of TP. The nutrient concentrations influence algal growth, which in turn relates to nutrient removal efficiency and lipid accumulation [3, 7]. In this study, a balanced N:P ratio was identified as the key optimal condition for FAME productivity and nutrient removal efficiency. The highest chlorophyll-*a* concentration, N and P removal efficiency, and FAME productivity were obtained with a balanced N:P ratio based on supplying additional phosphorus up to 0.5 mg/l.

The composition of the microalgal community determines the optimal nutrient ratio in wastewater. However, the culture conditions also affect the microalgae composition. Kapdan and Aslan [12] reported that the N:P ratio has a substantial effect on nutrient removal efficiency, and found that the optimum ratio for *Chlorella vulgaris* is 8:1. This is similar to the optimum N:P ratio (14:1) in this study. A balanced N:P ratio based on the addition of phosphate produced the highest N and P removal efficiency.

All the treatments, except for the addition of phosphate, were under phosphorus limitation, since the TDP remained at the bottom level (0.13 ± 0.01 mg/l) in all the treatments and even dropped to the bottom within a day after the addition of phosphate.

The FAME, rather than the total extracted lipids, was measured as an indicator of the algal biodiesel productivity, as not all extracted lipids can be converted into biodiesel [22]. The FAME content of *Scenedesmus* sp. cultivated in the municipal wastewater effluent was from 11.32% to 12.08% [16]. Phosphate limitation increased the total cellular lipids, mainly due to the dramatic increase in the triacylglycerol (TAG) levels from 6.5% up to 39.3% of the total lipids [7]. Similarly, the FAME contents of the other treatments were 1.7−2.2 times higher than that of the phosphate supplement, (*i.e.*, unlimited phosphorus). The longer 6-day HRT, which increased the number of microalgal species compared with that with the 3-day HRT, resulted in the highest FAME content of 10.8% ± 0.1%. The oleic acid content was 8% higher with the 6-day HRT than with the 3-day HRT. However, when bicarbonate was supplied, the FAME content was lower than that with the phosphate supplement, and diatoms were dominant with an increased pH.

Some recent studies have reported a reasonable FAME or lipid productivity in wastewater-grown microalgae (Table 2). Johnson and Wen [11] assessed the growth of *Chlorella* in dairy manure wastewater, and compared the growth of the microalgae in a suspended culture and attached culture. The total fatty acid contents of the microalgae were similar from both growth systems (~9% DW), yet the total lipid productivity of the attached culture was 0.23 g/m^2/day. Christenson and Sims [5] assessed the growth of a mixed culture of microalgae in domestic wastewater with an attached rotating algal biofilm reactor (RABR). The RABR system showed a FAME productivity of 2.2 g/m^2/day. Moreover, Mulbry *et al.* [18] examined *Rhizoclonium hieroglyphicum* grown in raw swine effluent

Table 2. Comparison of FAME or lipid productivity in microalgae grown under various wastewater conditions.

Wastewater type	Microalgal species	FAME or lipid productivity (g/m^2/day)	References
Agricultural wastewater (dairy manure with polystyrene rocker system)	*Chlorella* sp.	0.23 (lipid)	[11]
Agricultural wastewater (dairy effluent + CO_2, maximum manure loading rate)	*Rhizoclonium hieroglyphicum*	0.21 (FAME)	[18]
Domestic wastewater (suspended culture)	Mixed culture	1.0 (FAME)	[5]
Domestic wastewater (rotating algal biofilm reactor)	Mixed culture	2.2 (FAME)	[5]
Domestic wastewater (2nd effluent, suspended culture)	Mixed culture	1.1 (FAME)	This study

with and without additional CO_2. In this study, although the FAME content was relatively lower with the addition of phosphate, the highest biomass productivity resulted in the highest FAME productivity of 1.0 ± 0.3 g/m^2/day. Therefore, optimizing the N:P ratio for the dominant algae could be a key factor for higher nutrient removal efficiency and FAME productivity.

Therefore, the current study identified a key element in wastewater treatment that combines nutrient removal efficiency with microalgae cultivation for biofuel production. Balancing the N:P ratio through the addition of phosphate increased the nutrient (N and P) removal efficiency and algal fatty acid production in a mixed culture. The phosphorus removal efficiency was nearly complete, regardless of the treatment. However, the nitrogen removal efficiency was much higher with the addition of phosphate. The highest chlorophyll-*a* concentration and fatty acid productivity were obtained with a balanced N:P ratio based on the addition of phosphorus up to 0.5 mg/l, since phosphorus was in short supply when compared with nitrogen. Therefore, optimizing the N:P ratio for the dominant algae would appear to be critical in attaining higher algal growth, lipid productivity, and nutrient removal efficiency.

Acknowledgments

This research was supported by a grant from the Advanced Biomass R&D Center (ABC), a Global Frontier Program funded by the Korean Ministry of Education, Science & Technology, and the Water Industry Development Program funded by K-water of Korea (Project No. WI11STU02).

References

1. Amaro, H. M., A. Guedes, and F. X. Malcata. 2011. Advances and perspectives in using microalgae to produce biodiesel. *Appl. Energ.* **88:** 3402–3410.
2. APHA. 1995. *Standard Methods for the Examination of Water and Wastewater*, 19th Ed. APHA, Washington DC.
3. Aslan, S. and I. K. Kapdan. 2006. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. *Ecol. Eng.* **28:** 64–70.
4. Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol. Adv.* **25:** 294–306.
5. Christenson, L. B. and R. C. Sims. 2012. Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products. *Biotechnol. Bioeng.* **109:** 1674–1684.
6. Godos, I. D., S. Blanco, P. A. Garcia-Encina, E. Becares, and R. Munoz. 2009. Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. *Bioresour. Technol.* **100:** 4332–4339.
7. Goldberg, I. K. and Z. Cohen. 2006. The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. *Phytochemistry* **67:** 696–701.
8. Graham, L. E. and L. W. Wilcox. 2000. *Algae*. Prentice Hall, New Jersey.
9. Hua, G. H., F. Chen, D. Wei, X. W. Zhang, and G. Chen. 2010. Biodiesel production by microalgal biotechnology. *Appl. Energ.* **87:** 38–46.
10. Jeffrey, S. W., M. Sielicki, and F. T. Haxo. 1975. Chloroplast pigment patterns in dinoflagellates. *J. Phycol.* **11:** 374–384.
11. Johnson, M. B. and Z. Wen. 2010. Development of an attached microalgal growth system for biofuel production. *Appl. Microbiol. Biotechnol.* **85:** 525–534.
12. Kapdan, I. K. and S. Aslan. 2008. Application of the Stover–Kincannon kinetic model to nitrogen removal by *Chlorella vulgaris* in a continuously operated immobilized photobioreactor system. *J. Chem. Technol. Biotechnol.* **83:** 998–1005.
13. Lepage, G. and C. C. Roy. 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J. Lipid Res.* **25:** 1391–1396.
14. Marincas, O., P. Petrov, T. Ternes, V. Avram, and Z. Moldovan. 2005. The improvement of removal effects on organic pollutants in wastewater treatment plants (WWTP). *J. Phys. Conf. Ser.* **182:** 12–40.
15. Markou, G. and D. Georgakakis. 2011. Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: A review. *Appl. Energ.* **88:** 3389–3401.

16. McGinn, P. J., K. E. Dickinson, K. C. Park, C. G. Whitney, S. P. MacQuarrie, F. J. Black, *et al*. 2012. Assessment of the bioenergy and bioremediation potentials of the microalga *Scenedesmus* sp. AMDD cultivated in municipal wastewater effluent in batch and continuous mode. *Algal Res*. **1:** 155−165.
17. Mulbry, W., S. Kondrad, C. Pizarro, and E. Kebede-Westhead. 2008. Treatment of dairy manure effluent using freshwater algae: Algal productivity and recovery of manure nutrients using pilot-scale algal turf scrubbers. *Bioresour. Technol.* **99:** 8137−8142.
18. Mulbry, W., S. Kondrad, and J. Buyer. 2008. Treatment of dairy and swine manure effluents using freshwater algae: Fatty acid content and composition of algal biomass at different manure loading rates. *J. Appl. Phycol.* **20:** 1079−1085.
19. Oswald, W. J. 1961. Fundamental factors in stabilization pond design. *Int. J. Air Water Pollut.* **5:** 357.
20. Oswald, W. J. 2003. My sixty years in applied algology. *J. Appl. Phycol.* **15:** 99−106.
21. Park, J. B. K. and R. J. Craggs. 2010. Wastewater treatment and algal production in high rate algal ponds with carbon dioxide addition. *Water Sci. Technol.* **61:** 633−639.
22. Ratledge, C. and S. G. Wilkinson. 1988. An overview of microbial lipids, pp. 3−22. *In* C. Ratledge and S. G. Wilkinson (eds.). *Microbial Lipid*s. Academic Press, London.
23. Wijffels, R. H. and M. J. Barbosa. 2010. An outlook on microalgal biofuels. *Science* **329:** 796−799.
24. Xin, L., H.-Y. Hu, K. Gan, and Y.-X. Sun. 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. *Bioresour. Technol.* **101:** 5494−5500.
25. Xu, N., X. Zhang, X. Fan, L. Han, and C. Zeng. 2001. Effects of nitrogen source and concentration on growth rate and fatty acid composition of *Ellipsoidion* sp. (Eustigmatophyta). *J. Appl. Phycol.* **13:** 463−469.
26. Yang, J., M. Xu, X. Z. Zhang, Q. Hu, M. Sommerfeld, and Y. Chen. 2011. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. *Bioresour. Technol.* **102:** 159−165.