Nutrient Removal and Biofuel Production in High Rate Algal Pond Using Real Municipal Wastewater

Byung-Hyuk Kim1, Zion Kang1,2, Rishiram Ramanan1, Jong-Eun Choi1,2, Dae-Hyun Cho1, Hee-Mock Oh1,2, and Hee-Sik Kim1,2*

1Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIIBB), Daejeon 305-806, Republic of Korea
2Major of Green Chemistry and Environmental Biotechnology, University of Science and Technology, Daejeon 305-806, Republic of Korea

Introduction

Among the conventional aeration-based secondary wastewater treatment processes, activated sludge treatment (AST) is one of the most widely employed for a wide range of wastewaters [26]. AST has been effective for both high-strength and low-strength wastewaters on a commercial scale, usually in combination with anaerobic treatment [5]. However, this involves high energy inputs associated with O2 supply and does not allow recycling of nutrients present in the wastewater [9]. Moreover, sludge dewatering and disposal remain an environmental concern along with emission of greenhouse gases such as CO2 and CH4 [35].

Municipal wastewater treatment options are limited to a combination of anaerobic and aerobic treatments [31]. The nutrient biodegradability, pathogenicity, and overall quality of anaerobically treated municipal wastewater is rather poor, resulting in use of various post-treatment options [31]. Moreover, municipal wastewater treatment plants have high energy usage because of aeration-related secondary treatment, and render themselves costly and environmentally unsustainable for the above-mentioned reasons.

Recently, microalgae are considered to be one of the most promising feedstocks for biodiesel production owing to their higher growth rates and oil productivity compared with other oil crops. However, the industrial-scale cultivation of microalgae is not currently viable because of nutrient and harvesting costs. Thus, the utilization of wastewater for supply of nutrients, thereby treating wastewater, is considered to be a highly sustainable process [13].

This study evaluated the growth and nutrient removal ability of an indigenous algal consortium on real untreated municipal wastewater in a high rate algal pond (HRAP). The HRAP was operated semicontinuously under different hydraulic retention times (HRT: 2, 4, 6, and 8 days). The average removal efficiencies of chemical oxygen demand, and total nitrogen and phosphate of real municipal wastewater were maintained at 85.44 ± 5.10%, 92.74 ± 5.82%, and 82.85 ± 8.63%, respectively, in 2 day HRT. Algae dominated the consortium and showed high settling efficiency (99%), and biomass and lipid productivity of 0.500 ± 0.03 g/l/day and 0.103 ± 0.0083 g/l/day (2 day HRT), respectively. Fatty acid methyl ester analysis revealed a predominance of palmitate (C16:0), palmitoleate (C16:1), linoleate (C18:2), and linolenate (C18:3). Microalgal diversity analyses determined the presence of Chlorella, Scenedesmus, and Stigeoclonium as the dominant microalgae. The algal consortium provides significant value not only in terms of energy savings and nutrient removal but also because of its bioenergy potential as indicated by the lipid content (20–23%) and FAME profiling.

Keywords: Algal consortium, high rate algal pond, municipal wastewater, Chlorella sp., Scenedesmus sp.
Microalgae-based wastewater treatment presents several advantages over conventional technologies, including recovery of nutrients and reduction in CO₂ and CH₄ emissions, due to their autotrophic metabolism. Furthermore, when microalgae containing microbiota are used, symbiotic relationships can be established since microalgae supply oxygen whereas non-photosynthetic microorganisms degrade complex organic material and produce the CO₂ needed for microalgae growth [10]. Thus, in a microalgal-dominated consortium, mechanical aeration may not be required, resulting in energy savings. In addition, nutrients such as nitrogen and phosphate, causative chemicals of eutrophication, are concomitantly eliminated by algal uptake [6]. Moreover, algal photosynthesis favors the elimination of many pathogens and viruses by increasing the temperature, pH, and dissolved oxygen concentration of the treated effluent [27].

Although the potential of microalgae for wastewater treatment, especially municipal wastewater, has been well established, municipal wastewater is perceived to be heavily contaminated by bacteria and other microorganisms, thus either aerobically/anaerobically treated wastewater or completely sterilized municipal wastewater is used in most studies. This approach nullifies the synergistic effect that the co-cultivation of algae and bacteria could have on municipal wastewater treatment [33]. In this study, the effect of hydraulic retention time (HRT) on the total nitrogen (TN), total phosphorus (TP), chemical oxygen demand (COD), and total suspended solids (TSS) biodegradability by an indigenous algal consortium derived from municipal wastewater in a high rate algal pond (HRAP) was evaluated. The algal consortium responsible for the effective nutrient removal of untreated municipal wastewater (after primary settling) and the promise of this consortium as a potential source of biodiesel were also elucidated.

Materials and Methods

Study Site and Wastewater Source

The HRAP was operated at a greenhouse located at the Daejeon Municipal Wastewater Treatment Facility (latitude: 36°22’48.89”N; longitude: 127°24’27.71”E), which treats the municipal wastewater of Daejeon Metropolitan City, and using the municipal wastewater obtained after primary treatment (primary clarifier, before aeration) in the facility. The average influent municipal wastewater quality (from June 2011 to July 2012) is given in Table 1. The average temperature and rainfall of Daejeon during the operating period were 25.8°C and 266 mm, respectively, whereas the average municipal wastewater temperature and pH were 22.58°C and 7.28, respectively.

Semicontinuous HRAP

HRAP was constructed with a total capacity of ~100 L and a working volume of 60 L at a depth of 30 cm. An optimum horizontal water velocity of approximately 0.3 m/s was obtained after mixing with the aid of paddle wheel. Prior to semicontinuous operation, the HRAP was initially operated in a batch mode for 10 days to enable stabilization and biomass growth of indigenous microalgae. In semicontinuous mode, the HRAP was operated at HRT of 2, 4, 6, and 8 days by removing 30, 15, 10, and 7.5 L of microalgal culture while adding an equal volume of fresh real wastewater every day, respectively. The HRAP was positioned in a greenhouse with average natural light intensity of approximately 600 µmol/m²/s (during the study period; June–August 2012) and natural light/dark cycles.

Determination of Chlorophyll a and Dry Cell Weight

Dry cell weight was measured according to the standard methods [2]. Chlorophyll a concentration was determined by the spectrophotometric method described earlier [12]. The chlorophyll a was calculated using the following equation:

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\text{Chlorophyll } a \text{ (mg } m^{-2}) = \left(11.85 \times (E_{645} - E_{664}) - 0.08 (E_{664} - E_{750})\right) \times V_{e} / L \times V_{f}.
\]

where

\[
L = \text{Cuvette light-path in centimeter}
\]

\[
V_{e} = \text{Extraction volume in milliliter}
\]

\[
V_{f} = \text{Filtered volume in liter}
\]

\[
E = \text{optical density}
\]

Total Lipid and Fatty Acid Methyl Ester (FAME) Analysis

The total lipids were extracted by a chloroform-methanol solvent mixture, and FAME analysis was performed as described previously [29]. FAME composition analysis was determined using a gas chromatograph (Shimadzu GC-2010, Japan) by comparing their retention times and fragmentation patterns with those of the standards.

Denaturing Gradient Gel Electrophoresis (DGGE)

The DNA of algae was extracted using the EMNE method [15]. The algal-specific 18S rRNA gene was obtained using primers SR4F-GC (5’-CGC CGG CGG CCG CCG CGG GGC GGC GCG GGC GGC GGA CCG GGG GAG CCG CGG TAA TTC CAG CT-3’), SR7R (5’- TCC TTG GGC AAA TGC TTT GC-3’) to obtain an amplicon of ~500 base pair. Each 30 µl of the PCR mixture contained the following: 1x PCR buffer, 200 nmol of MgCl₂, 30 nmol of dNTP mixture, 10 pmol of each primer, 2.5 U of Taq polymerase, and approximately 100 pg of the template DNA.

The touchdown thermocycling program was as follows: initial denaturation for 5 min at 94°C; 20 cycles of 94°C for 45 sec, annealing for 45 sec, and 72°C for 45 sec (with the annealing temperature decreasing from 65°C to 50°C at -0.5°C/cycle); an additional 15 cycles with annealing at 50°C; and a 5 min final extension at 72°C. DGGE conditions were as mentioned in a previous
study [14]. Portions of the selected DGGE bands were excised from the DGGE gels for nucleotide sequence determination. The gel pieces were placed in 1.5 ml EP tubes containing 50 µl of sterile water. After incubation overnight at 4°C, 2 µl of the solution containing the eluted DNA was used for a PCR and DGGE analysis, as described above. The PCR products were then cloned and sequenced as described above. All products were cloned with the T-Blunt vector (Solgent, Daejeon, Korea) according to the manufacturer’s protocol and sequenced. Nearly full-length 18S rRNA gene sequences were assembled with SeqMan II (DNastar) using the best match sequence of each operational taxonomic unit (OTU) on GenBank (www.ncbi.nlm.nih.gov/BLAST) as a template.

**Chemical Analysis**

All samples were filtered through 0.2 µm pore size membranes (Minisart, Sartorius Stedim Biotech, Germany) before the analysis. The concentrations of TN and NH₃-N were determined using a second-derivative method. The concentration of TP was measured according to the ascorbic acid method prescribed in standard methods [2]. Culture pH and water temperature were measured using a pH electrode fitted with digital thermometer (pH 3110 SET 2/SenTix 41, WTW, Germany). The COD was measured according to standard methods [2]. TOC was analyzed using a TOC analyzer multi N/C 3100 (Analytik Jena AG, Germany).

The microalgal consortium settleability was determined by the sludge volume index (SVI) test, carried out on biomass samples taken from the HRAP, according to standard methods [2].

**Results and Discussion**

The municipal wastewater treatment is highly aeration dependent, as anaerobic digestion is known to be ineffective at lower temperature and at lesser organic rates [1]. There are approximately 2,500 municipal wastewater treatment facilities in South Korea treating >16 million liters per day of municipal wastewater. A Korean Ministry of Environment report attributes >1,000,000 MWh of electricity is being consumed annually for aeration processes in wastewater treatment. Aeration processes, principally AST, consume electricity as well as generate >8,400 tons/day of sludge, which have disposal issues [23]. One of the most promising alternative municipal wastewater treatment options emerging recently is microalgae-based treatment. Furthermore, algal cultivation is attracting worldwide attention for its value-added products, making wastewater a sensible nutrient source. In this context, the use of an algal consortium to achieve these twin goals with a HRAP using real municipal wastewater derived from a wastewater treatment facility was evaluated.

**Nutrient Removal of Untreated Municipal Wastewater**

**Nitrogen and phosphorus removal in HRAP**

The indigenous algal consortium was allowed to grow in
conditions favoring algal growth [25] first in batch mode for 10 days to allow stabilization and biomass growth of at least 1 g/l and later in semicontinuous mode. The mild aeration provided by the paddle wheel helps to establish an algal-dominated consortium, as high oxygen levels inhibit algal growth and encourage bacterial growth, a condition analogous to AST [24]. This approach probably results in an algal community that synergistically helps in degrading the carbon and available nutrients. Experimental results obtained clearly revealed that the nutrient removal efficiency (TN and TP) under all HRT conditions was well below legally allowed discharge levels (Figs. 1A and 1B); however, the AST plant present in the municipal wastewater treatment facility had better TP removal efficiency and much less retention time. The average TN effluent concentrations of HRAP were maintained 3.28 ± 0.84, 2.36 ± 0.17, 2.60 ± 0.35, and 2.19 ± 0.43 ppm for a HRT of 2, 4, 6, and 8 days, respectively, corresponding to average TN removal efficiencies of 92.74 ± 5.82%, 94.69 ± 4.13%, 94.11 ± 2.36%, and 95.05 ± 1.30%. The TN removal efficiencies were better than the results obtained at the AST plant (Fig. 1A).

The ammoniacal nitrogen removal efficiencies under all HRT conditions were nearly 100% (Fig. 1C) suggesting that ammoniacal nitrogen is the preferred nitrogen source [10]. The remaining nitrogen is attributed to residual nitrate, as ammoniacal nitrogen has been utilized and even free ammonia would not persist longer in an open system because of volatilization, as the pH also remained high (10.5).

The average TP effluent concentrations maintained were 0.79 ± 0.09, 0.85 ± 0.01, 1.04 ± 0.08, and 0.88 ± 0.10 ppm for a HRT of 2, 4, 6, and 8 days, respectively, corresponding to average TP removal efficiencies of 82.65 ± 8.63%, 81.23 ± 11.24%, 76.93 ± 13.42%, and 95.49 ± 13.81% (Fig. 1B). At higher pH, phosphate in the wastewater is known to precipitate; however, this does not seem to be the case in this study as even increasing HRT, which should increase the cumulative precipitation, did not result in a linear increase in phosphate removal efficiency, and hence phosphate removal is likely because of algal assimilation. In addition, the pH of the system remained in the range of 10.4–10.6 across various HRTs, whereas phosphorus removal efficiency remained variable, indicating that chemical precipitation is not a possibility. Earlier studies have modeled and validated that phosphorus assimilation by algae is much higher than chemical precipitation in wastewater treatment [11]. A recent study on nutrient removal demonstrated a phosphate removal efficiency of 78% (83.16% in this study) when the pH was maintained constant at 7.0, indicating the role of algal assimilation [3].

Although the phosphorus removal efficiency was relatively less when compared with the AST plant results, the values were well within the international standards for surface waters. Moreover, a major factor for high phosphorus removal in the conventional wastewater treatment is the use of chemical treatment after AST, whereas in HRAP, phosphate removal is chiefly because of algal assimilation. Phosphate removal efficiency was also less when compared with nitrogen removal efficiencies, as nitrogen is a limiting nutrient in the municipal wastewater and phosphorus is not a limiting nutrient with an influent N:P ratio of 10:1, whereas the desirable N:P ratio ranges from 4:1 to 40:1 depending upon the algal species [7]. It must also be noted that no additional nutrient had been added to the municipal wastewater to compensate this imbalanced N:P ratio. Since both nitrogen and phosphorus serve as major nutrients for phytoplankton, inefficient treatment results in eutrophication of surface waters [20]. Both nitrogen and phosphorus removal in AST is always laden with limitations and complications of environmental pollution and various inputs increasing overall operation cost [20]. High rate algal ponds provide very high nutrient removal efficiency,
resulting in good effluent quality and lesser chances of eutrophication. This study has one of the highest nutrient and carbon removal efficiencies when compared with other recent studies on municipal wastewater treatment using microalgae (Table 1).

**Carbon removal**

Analysis of COD removal in time-course experiments in batch-semicontinuous operation revealed a direct correlation between decreasing COD removal efficiency and increased HRT. The average COD concentration in the effluent of the HRAP was maintained at 16.03 ± 3.92, 27.72 ± 1.46, 33.55 ± 2.56, and 38.74 ± 3.63 ppm for a HRT of 2, 4, 6, and 8 days, respectively, corresponding to COD removal efficiencies of 85.44 ± 5.10%, 74.27 ± 5.19%, 68.41 ± 6.63%, and 63.59 ± 8.58% (Fig. 2A). A similar trend was also observed in the TOC levels, with TOC removal efficiencies corresponding to 77.65 ± 13.25%, 53.42 ± 10.53%, 42.85 ± 10.79%, and 34.03 ± 14.87% for a HRT of 2, 4, 6, and 8 days, respectively (Fig. 2B). The best removal efficiency for both COD and TOC was obtained at a HRT of 2 days. The most plausible reason for the increase in carbon content of treated effluent is the release of organic carbon by algae. Previous studies have demonstrated that algae release small organic molecules such as glycolic acid, especially during the stationary phase in autotrophic growth [36]. It is possible that the HRAP operated at higher HRTs (>2 days) might have been exhausted of utilisable organic carbon from the wastewater and switched to autotrophic growth, with available CO₂ supply due to paddling. The COD removal efficiency at 2 days HRT was comparable to AS treatment, suggesting that all values (TN, TP, and COD) are well within prescribed standards stipulated in Korea (Fig. 2) [23]. Furthermore, the total suspended solids level was also comparable to AST plant effluent, as algae autoflocculated in the HRAP (Fig. 2C). Overall, a HRT of 2 days seem to be ideal in this study for both nutrient and carbon removal; however, this could be reduced further with a better design and by using a continuous system.

**Algal Biomass Production**

One of the other disadvantages of AST is production of sludge, which needs to be dewatered and treated. On the contrary, high rate algal ponds not only treat wastewater efficiently but also produce biomass that can be utilized commercially. In this study, algal biomass production was evaluated by measuring both dry cell weight (DCW) and chlorophyll a. The biomass growth increased with increasing HRT at 0.999 ± 0.07, 1.260 ± 0.08, 1.448 ± 0.10, and 1.741 ± 0.16g/l for 2, 4, 6, and 8 days HRT, respectively (Fig. 3A). However, the biomass productivity reduced significantly at 0.500 ± 0.03, 0.315 ± 0.02, 0.241 ± 0.02, and 0.218 ± 0.02g/l/day for 2, 4, 6, and 8 days HRT, respectively. Chlorophyll a results also showed a similar trend of increase with increasing HRT (Fig. 3B). Taken together, the results indicate that a lower HRT is favorable for both wastewater treatment and biomass productivity, indicating that this system can be scaled up to continuous mode and would yield similar results under lower HRTs.

Thus, a system that encourages an algal consortium for the nutrient removal of municipal wastewater seems to be ideal for both wastewater treatment and biomass production. However, high algal growth is essential for efficient functioning of an HRAP, as a reduction in algal numbers would eventually destabilize the system because of lack of adequate oxygen for bacteria [21]. Moreover, this fine balance in this treatment system is usually indicated by pH. A typical algal culture system would result in higher DO and bicarbonate levels, lower CO₂ levels (because of active photosynthesis), and hence subsequently higher pH, which collectively discourage bacterial growth, whereas

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**Fig. 2.** Time course of COD (A), TOC (B), and TSS (C) concentrations in the HRAP.

■ Influent; ◆, HRT 2 days; ●, HRT 4 days; ★, HRT 6 days; ●, HRT 8 days; and ◇, AST. TSS concentrations have been presented for the 2 day HRT only. The dotted line represents the effluent water quality standard of Korea.
vice versa would discourage algal growth [34]. In the HRAP, a healthy pH of 10.5 was maintained because of the activity of this algal consortium (data not shown), resulting in a predominance of algae, whereas bacterial growth (data not shown) was sufficient to support algal growth and for efficient municipal wastewater treatment. Moreover, stabilization of the algal community through batch cultivation prior to semicontinuous operation was also critical for overall system stability.

Settleability of Microalgal Consortium

As widely acknowledged, harvesting consumes about 30% of the total cost of microalgal cultivation [18]. Hence, the settleability of microalgal biomass was investigated. The total solids in the municipal wastewater decreased from 780 to 8 mg/l in the treated effluent, which was mainly attributed to the excellent settling characteristics of the biomass that settled within 2 min, as shown in Fig. 4, with a overall sludge volume index (SVI) of ~10%, which implies its good sedimentation [32]. The presence of bacteria in the algal consortium seems to not only synergistically affect but also aid in autoflocculation, as proved recently [24]. This autoflocculation has several advantages apart from cost reduction. Firstly, elimination of secondary pollutants during operation and biomass harvest and, secondly, a long-term sustainable solution for biomass harvest and valorization.

Microalgal Diversity in HRAP

Microscopic analysis indicated that the Chlorophyceae class, including *Chlorella* sp., *Scenedesmus* sp., and *Stigeoclonium* sp., were the dominant microalgae (Fig. S1). Additionally, PCR-DGGE was performed using the universal green algae 18S rRNA gene (Fig. S1A) for each HRAP run with different HRTs as well for real municipal wastewater. A total of 26 different bands were observed on the DGGE, and some dominant algae observed in DGGE results corroborated with microscopic observations (*Chlorella* sp. (KC416209), and *Scenedesmus* sp. (FR865738)) (Figs. S2A and S2B, Table S1). However, the most dominant strain, *Stigeoclonium* sp., as per microscopic analysis, could not be detected in DGGE analysis with the universal 18S rRNA primer set because of the limitations of a PCR-based approach and inadequate information in the existing databases [14]. Further examination of the DGGE results revealed a slow transition from fungi, protozoa, and algae dominated municipal wastewater to a largely algae-dominated community with increasing HRT. The 2- and 4-day HRT cultures seem to have a mixture of eukaryotes, but were predominated by algae, resulting in maximum municipal wastewater treatment efficiency (Figs. 1, 2, 3, Figs. S2A and S2B, Table S1).

Microalgae are known to remove nitrogen and phosphorus from water through uptake into biomass. The most widely studied microalgal species for nitrogen and phosphorus removal are *Scenedesmus* [37] and *Chlorella* [8]. *Chlorella* was
widely applied for wastewater treatment and had proven abilities of removing nitrogen, phosphorus, and COD [8]. A freshwater microalga, *Scenedesmus* sp. LX1 was tested for its ability to remove nutrients and accumulate lipid while growing in secondary effluent. *Scenedesmus* sp. LX1 adapted better to secondary effluent and achieved the highest biomass (0.11 g/l, dry weight) and lipid content (31–33%, dry weight) [37]. The most dominant algae obtained in this study, *Stigeoclonium*, is a common genus of freshwater algae with filaments that are usually branched and have short, lateral branches that end in an acute apex or hair-like extension, although the development of these multicellular “hairs” depends on nutrient limitation, particularly of phosphate. *Stigeoclonium* species are tolerant of a wide range of water conditions, with ability to grow in waters polluted by heavy metals and/or organic materials [28], but has not been reported for wastewater treatment so far. Overall, the indigenous microalgal community obtained from the HRAP seems to not only possess the ability to completely utilize municipal wastewater for growth (*Chlorella*, *Scenedesmus*, and *Stigeoclonium*) but also to aid in efficient settling of biomass (*Stigeoclonium*).

**Lipid Content and FAME Profiling of Biomass**

It is widely held that lipid accumulation takes place at the late exponential growth phase of algae [29]. Hence, it is rather unsurprising that the lipid content directly correlated with the increase in HRT, with a maximum lipid content of 23.33 ± 1.29% on 8 days HRT (Fig. 5). This may be attributed to the dearth of utilizable nutrient in the 8 day samples, resulting in a nutrient stress condition [29]. However, lipid productivity showed a decreasing trend with increased HRT, corresponding to 0.103 ± 0.0083, 0.066 ± 0.0075, 0.051 ± 0.0030, and 0.051 ± 0.0026 g/l/day for 2, 4, 6, and 8 days HRT, respectively. Hence, it is desirable to have a lower HRT for both high lipid and biomass productivity.

In general, lipids produced from microalgae usually constitute C16 and C18 fatty acids and are thus suitable for biodiesel production [22]. Biodiesel fuels enriched in oleic acid are desirable, because a high oleic acid content increases
the oxidative stability, enabling longer storage [16]. The FAME profiling of biomass from the SSRP showed that the major fatty acids constituted laurate (C12:0), myristate (C14:0), palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0), oleate (C18:1), linoleate (C18:2), and linolenate (C18:3) (Fig. 6). There was little change in the FAME profile of samples with different HRTs, indicating that neither HRT nor a difference in microbial community structure affected the FAME composition. The composition of fatty acids obtained in this study matched that of the dominant algal species. It is partially helped by the fact that the maximum lipid content in bacteria is <5% [38]. *Chlorella* sp. and *Scenedesmus* sp. are known to have a high oleate, linolenate, and unsaturated fatty acid content, which are more suitable as a biodiesel precursor [17]. Hence, the lipid content and FAME profile obtained from this highly settleable biomass in HRAP seem to be an ideal precursor for sustainable biodiesel production.

The algal consortium used for the nutrient removal of untreated municipal wastewater maintained an effluent quality as prescribed by international standards for surface waters. Moreover, the best results were obtained at 2 days HRT, indicating the possibility of further reducing the HRT, and higher scalability in the long-term algal-bacterial consortium might serve as a competitive alternative to AST for municipal wastewater treatment. The system also resulted in high biomass and lipid productivity, which hold promise for the biofuel sector. In conclusion, the algal consortium approach is a simple, economical, and sustainable system that has yielded comparable results to AST in terms of treatment efficiency, whereas it is much superior in terms of its environmental and economic-value additions.
Acknowledgments

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