J. Microbiol. Biotechnol. (2002), 12(4), 643–648

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Isolation and Identification of Photosynthetic Bacterium Useful for Wastewater Treatment

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Received: March 28, 2002
Accepted: July 23, 2002

Abstract For wastewater treatment and utilization of the biomass, a photosynthetic bacterium was isolated based on its cell growth rate, cell mass, and assimilating ability of organic acids. The isolate was a Gram-negative rod-shaped bacterium that contained a single polar flagellum and formed a lamellar intracytoplasmic membrane (ICM) system, including bacteriochlorophyll *a*. The major isoprenoid quinone component was identified as ubiquinone Q-10, and the fatty acid composition was characterized as to contain relatively large amount of C-16:0 (18.74%) and C-18:1 (59.23%). Based on its morphology, phototrophic properties, quinone component, and fatty acid composition, the isolate appeared to be closely related to the *Rhodopseudomonas* subgroup of purple nonsulfur bacteria. A phylogenetic analysis of the isolate using its 16S rRNA gene sequence data also supported the phenotypic findings, and classified the isolate closely related to *Rhodopseudomonas palustris*. Accordingly, the nomenclature of the isolate was proposed as *Rhodopseudomonas palustris* KUGB306. A bench-scale photosynthetic bacteria (PSB) reactor using the isolate was designed and operated for the treatment of soybean curd wastewater.

Key words: Identification, photosynthetic bacterium, wastewater treatment

Photosynthetic bacteria have become increasingly important for the treatment of various wastewater and biodegradable solids. These bacteria are Gram-negative prokaryotes and use light as their energy source. Among them, purple non-sulfur bacteria have a relatively simple nutritional requirement and can grow actively, regardless of the oxygen diffusion rate under aerobic/anaerobic conditions in the light or aerobic conditions in the dark. They also have flagella, and thus can absorb substrates rapidly under wastewater treatment conditions. As such, these characteristics are very useful for treating wastewater with a high concentration of organic substances [4, 5, 17, 18].

Wastewater treatment processes using photosynthetic bacteria can result in an increased reduction of biochemical oxygen demand (BOD) and higher biomass production [17]. Photosynthetic bacteria can be recovered by simple precipitation with bioflocculant (e.g., hydrolyzed chitin solution) from the wastewater treatment process, and thus have commercial value as single-cell protein in feedstock supplements or as a fertilizer [9, 11, 19]. Wastewaters from various food industries including soybean processing factories can be used as sources for biomass production of photosynthetic bacteria as single-cell protein [14, 15].

Accordingly, for wastewater treatment and utilization of the biomass, a photosynthetic bacterium was isolated from soils and ponds on the basis of its cell growth rate, cell mass, and assimilating ability to organic acids. The present paper reports on its properties, genetic relationship, and application to the treatment of soybean curd wastewater.

MATERIALS AND METHODS

Organisms

About 150 strains of photosynthetic bacteria were first isolated on isolation media using samples from Korean eutrophic areas, rivers, lakes, rice fields, and damp soils. Among them, several strains were selected based on active initial cell growth rate. Subsequently, the cell growth, assimilating ability of organic acids, and maximum specific growth rate were compared between the strains using isolation media.

Media and Growth Conditions

The medium [1, 16] used for isolating the bacterium contained the following components per liter of distilled

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water; 1.0 g sodium acetate, 1.0 g sodium propionate, 1.0 g sodium butyrate, 1.0 g yeast extract, 1.0 g NH_4Cl, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.3 g $NaHCO_3$, 1.0 g KH_2PO_4, and 1 ml of a trace metal solution. The trace metal solution contained 2.5 g EDTA, 1.54 g $MnSO_4 \cdot 5H_2O$, 10.95 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $CuSO_4 \cdot 5H_2O$, 7.0 g $FeSO_4 \cdot 7H_2O$, and 0.2 g $CoCl_2 \cdot 6H_2O$ per liter. The pH of the medium was adjusted to 6.8–7.0 and then sterilized by autoclaving. A modified Ormerod medium [12] used for further cultivation of the bacterium contained 0.6 g KH_2PO_4, 0.9 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.075 g $CaCl_2 \cdot 2H_2O$, 0.0118 g $FeSO_4 \cdot 7H_2O$, 0.02 g EDTA, 15 μg biotin, 6 g malic acid, 2 g MOPS, 5.61 g sodium glutamate, and 1 ml of a trace element solution per liter. The trace element solution contained 2.8 g H_3BO_3, 2.1 g $MnSO_4 \cdot 4H_2O$, 0.75 g $Na_2MoO_4 \cdot 2H_2O$, 0.24 g $ZnSO_4 \cdot 7H_2O$, and 0.04 g $Cu(NO_3)_2 \cdot 3H_2O$ per liter. The pH of the medium was adjusted to 6.8–7.0 and then sterilized by autoclaving. The cultures were grown phototrophically (anoxic/light) in aluminum-capped bottles with autoclavable rubber seals. The cultures were incubated at 28–30°C at light intensity of 3,000 lux from a tungsten lamp. The phototropic plates were incubated in a GasPak anaerobic jar. The growth was monitored by measuring the optical density at 660 nm.

Bacteriochlorophyll Analysis

The bacteriochlorophyll was extracted from the cell pellet obtained by centrifugation of the culture in acetone/methanol (7:2, v/v). The absorption spectra were recorded using a UV-2401PC double beam spectrophotometer (Shimadzu Co., Kyoto, Japan).

Quinone Analysis

The quinones were extracted from the cell pellet washed with 50 mM phosphate buffer (pH 7.0) using chloroform/methanol (2:1, v/v), and analyzed by thin-layer chromatography (TLC) on Kieselgel $60F_{254}$ plate (Merck, Darmstadt, Germany) using a mixture of hexane:diethyl ether (85:15, v/v) as the developing solvent. The quinone bands were detected under UV wavelength, scraped from the TLC plate, and recovered in acetone. High-performance liquid chromatography (HPLC) was used to determine the isoprenoid quinone composition. The analytical conditions were as follows: column, Spherisorb 5 μm ODS2 (4.6×250 mm, Waters Associates, Milford, MA, U.S.A.); eluent, methanol/isopropyl ether (3:1, v/v) for ubiquinones, methanol/isopropyl ether (4:1, v/v) for menaquinones; flow rate, 1 ml/min. The ubiquinones and menaquinones were detected by monitoring at 275 nm and 270 nm, respectively, using a UV detector.

Cellular Fatty Acid Analysis

The bacterial cells were harvested, saponified, and methylated. The fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (HP 6890, Hewlett-Packard Co., CA, U.S.A.), and identified using a Microbial Identification System (MIDI, Inc., Newark, DE, U.S.A.) to determine the fatty acid composition.

Electron Microscopy

For negative staining, the cells were treated with 1% phosphotungstic acid. For ultrathin sections, the cells were prefixed in glutaraldehyde, fixed in a OsO_4 solution, dehydrated using a graded series of aqueous acetone solutions, embedded in EPON 812, sectioned, and stained with uranyl acetate and lead citrate. Electron micrographs were taken using a TFNM (CM20, Philips, Einthoven, The Netherlands) electron microscope.

Analysis of 16S rRNA Gene Sequence

For the sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Corp., Madison, U.S.A.). Amplification of the 16S rDNA was performed on the isolated DNA using primers (5'-AGAGTTTGATCMTGGCTCAG-3') and (5'-TACGGYTACCTTGTTACGACTT-3'). The amplified products were then resolved on a 1.5% (w/v) agarose gel, excised from the gel, and purified. The purified products were cloned into a pGEM-T Easy vector (Promega Corp., Madison, U.S.A.) and subsequently sequenced using Big Dye Terminator chemistry (ABI, Foster, CA, U.S.A.). The program CLUSTAL X was used to create a multiple-sequence alignment of the sequences and construct a tree from the alignment.

Design and Operation of a Bench-Scale PSB Reactor

A bench-scale photosynthetic bacteria (PSB) reactor using the isolate was designed and operated for the treatment of soybean curd wastewater. The flow chart of the purification process is shown in Fig. 6. The operating conditions of the PSB reactor were as follows: flow rate, 2.8 l/day; initial inoculum of seed culture, 10% (v/v); working volume, 30 l; hydraulic retention time (HRT), 3 days; seeding rate, 5% (v/v/day); temperature, 30°C; and light intensity, 2,500 lux; pH 7.0.

Organic Acid Analysis

The samples were centrifuged at 7,500 ×g for 5 min, and to the supernatant, oxalic acid was added to final concentration of 0.03 M. Organic acids were analyzed by gas chromatography (GC-14A, Shimadzu Co., Kyoto, Japan) equipped with flame ionization detector (FID) and glass column (3.2 mm ID×1.5 m) packed with column material, Carbopack B-DA 80/120 4% CW20M. The chromatographic conditions were as follows: column temperature, 175°C; injection temperature, 200°C; detector temperature, 200°C; carrier gas, argon (1.8 kg/cm^3); hydrogen, 0.4 kg/cm^3; air, 0.5 kg/cm^3; integrator, Shimadzu C-R6A Chromatopac; injection volume, 1 μl.

Other Methods

Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were determined by the standard methods [10].

RESULTS AND DISCUSSION

Isolation of Photosynthetic Bacterium

To select the strain that was best in adaptability and efficiency for wastewater treatment, the cell growth, assimilating ability of organic acids, and maximum specific growth rate were compared between the isolated strains.

Organic wastewater treatment using photosynthetic bacteria can result in a reduction in the BOD together with biomass production, because the bacteria utilize a variety of organic acids, especially acetic acid, propionic acid, and butyric acid, as a carbon source. Therefore, the assimilating ability of organic acids was compared between the strains.

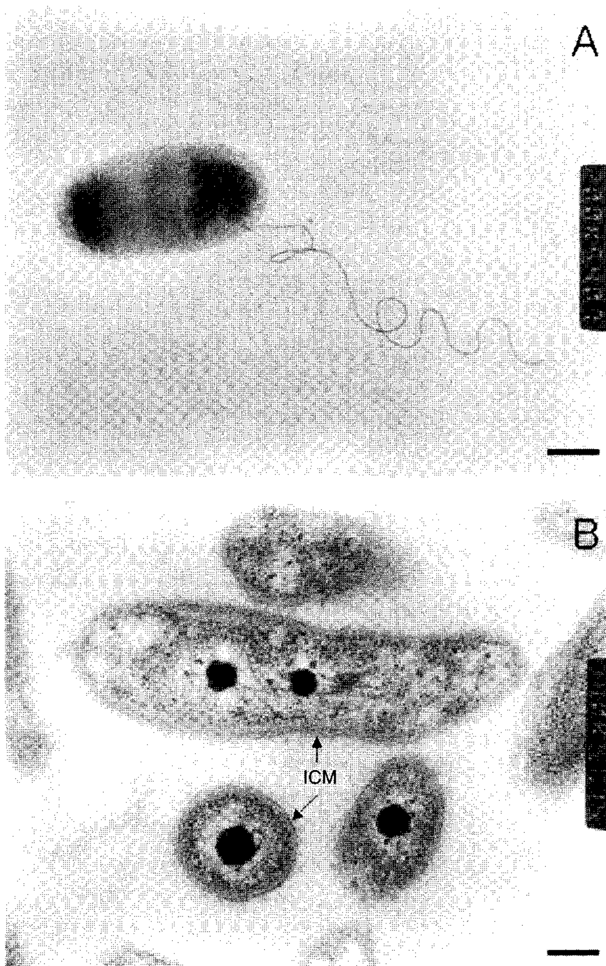

Fig. 1. Transmission electron micrograph of isolate P17.
A: Intact form (×20,000); bar 2 μm. B: Section form (×38,000), ICM (intracytoplasmic membrane); bar 3.8 μm.

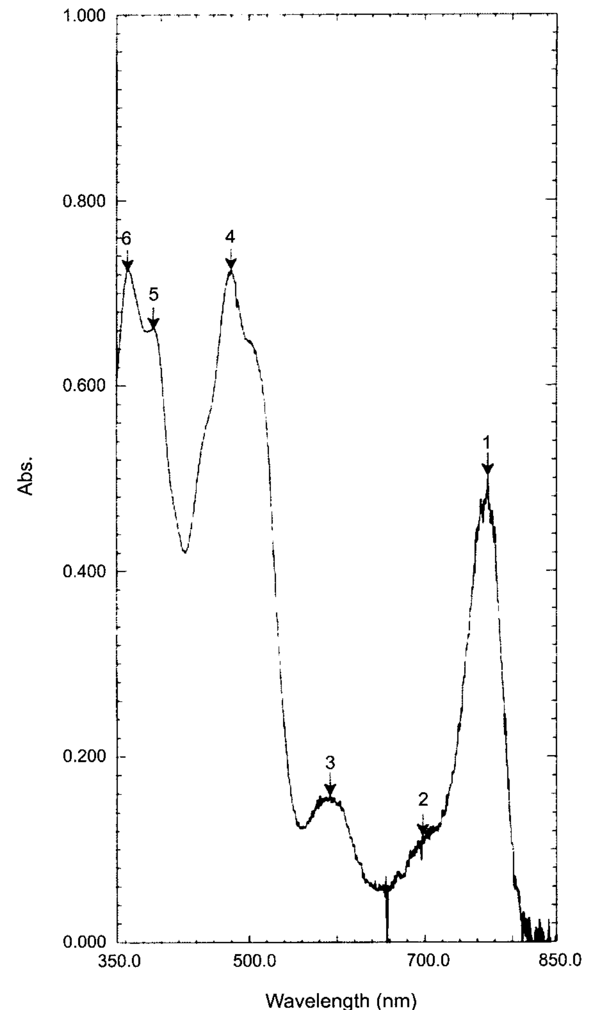

Fig. 2. Absorption spectrum of acetone/methanol extracts (7:2, v/v) of isolate P17.
Absorption maxima were observed at 363 nm (arrow 6), 392 nm (arrow 5), 480 nm (arrow 4), 592 nm (arrow 3), and 772 nm (arrow 1).

Finally, strain P17 was selected as superior in its cell growth, cell mass, and assimilating ability to organic acids among the isolated strains (S. O. Kang. 1993. *Thesis for the degree of master, Korea University*, Seoul, Korea, pp. 13–17).

Morphology, Phototrophic, and Physiological Properties

The isolate P17 was found to be a Gram-negative bacterium, and under an electron microscope, the cells were observed as rod-shaped containing a single polar flagellum (Fig. 1A). Electron micrographs of thin sections of phototrophically grown cells also showed a lamellar intracytoplasmic membrane (ICM) system (Fig. 1B). The bacterium formed purple colonies on an agar medium and purple cell suspensions in liquid media under phototrophic conditions. The strain also formed photosynthetic pigments when grown under anoxygenic phototrophic conditions. Acetone/methanol extracts of the cells exhibited absorption

maxima at 363, 392, 480, 592, and 772 nm (Fig. 2). Based on the long-wavelength absorption maximum at 772 nm, bacteriochlorophyll *a* was confirmed to be present in strain P17 [3]. Growth occurred under aerobic/anaerobic conditions under both light and dark, and biotin was required as a growth factor. Accordingly, when taken collectively, these morphological data, and phototrophic and physiological properties suggested that strain P17 was closely related to the *Rhodopseudomonas* subgroup of purple nonsulfur bacteria [6, 8].

Quinone Component

The major isoprenoid quinone component of strain P17 was found to be ubiquinone Q-10 (Fig. 3). All known purple nonsulfur bacteria contain ubiquinones. Some species include only ubiquinones as the sole quinone component and lack menaquinones and rhodoquinones, while others contain ubiquinone and rhodoquinone, ubiquinone, and menaquinone, or ubiquinone, menaquinone, and rhodoquinone. Some species of the *Rhodopseudomonas* subgroup of purple nonsulfur bacteria (e.g., *Rhodopseudomonas palustris*, *Rhodopseudomonas rutila*, and *Rhodopseudomonas blastica*) also contain Q-10 as the major isoprenoid quinone component [2, 7, 8].

Fatty Acid Composition

The fatty acid composition of strain P17 was characterized by a high content of C-16:0 (18.74%) and C-18:1 (59.23%), and low amount of C-16:1 (7.62%) and C-18:0 (2.65%) (Fig. 4), which is a uniquely characteristic feature among phototrophic purple bacteria [7, 13].

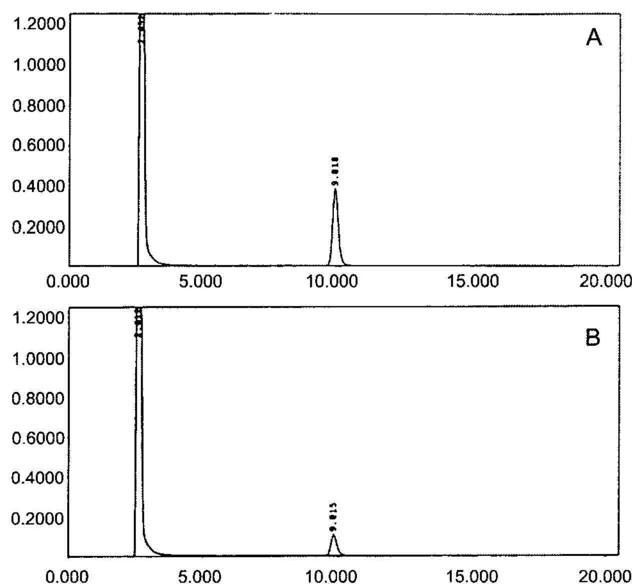

Fig. 3. HPLC chromatogram of the isoprenoid quinone composition of ubiquinone Q-10 standard (A) and quinones extracted from isolate P17 (B).

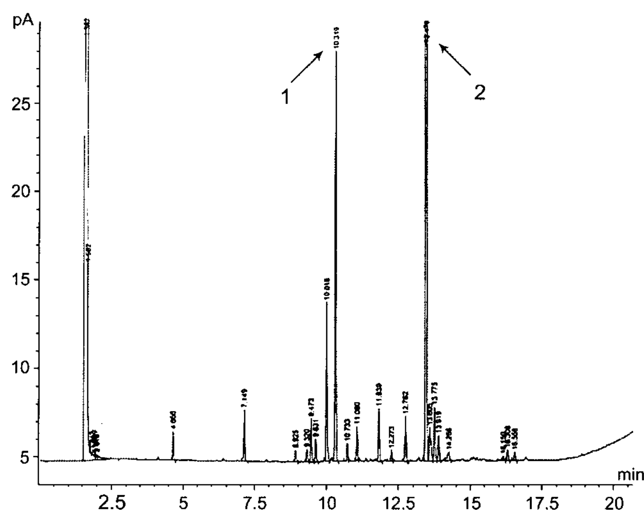

Fig. 4. Gas chromatogram of FAMEs from isolate P17 grown in modified Ormerod medium.
Arrow 1 indicates $C_{16:0}$ and arrow 2 indicates $C_{18:1}$.

16S rRNA Gene Sequence

The 1,429 bp sequence obtained from strain P17 was aligned with all the presently available 16S rRNA gene sequences in the GenBank databases. As a result, a phylogenetic tree was constructed (Fig. 5). The sequence of strain P17 was deposited in the GenBank under accession no. AY084079. The phylogenetic analysis of strain P17 using its 16S rRNA gene sequence data suggested that the strain was most closely related to *Rhodopseudomonas palustris*.

In summary, the strain was found to be closely related to the *Rhodopseudomonas* subgroup of purple nonsulfur bacteria based on its morphology, phototrophic properties, physiological properties, quinone component, and fatty acid composition. The phylogenetic analysis of strain P17 using its 16S rRNA sequence data also supported the phenotypic findings, and classified the isolate closely related to *Rhodopseudomonas palustris*. Accordingly, the isolated bacterium P17 was named as "*Rhodopseudomonas palustris* KUGB306".

Application of the Isolate to the Treatment of Wastewater

A bench-scale PSB reactor using the isolate was designed and operated for the treatment of soybean curd wastewater. Physicochemical data of samples taken from the purification stages (Fig. 6) are summarized in Table 1.

When a soybean curd wastewater (initial BOD, 3,310 mg/l; initial COD, 3,240 mg/l; total volatile acids, 2,540 mg/l) was adapted to the PSB reactor, BOD, COD, and total volatile acids levels of the wastewater were reduced by 86%, 86%, and 83% after 5 days treatment, respectively. These data showed a very high efficiency of wastewater treatment during short periods.

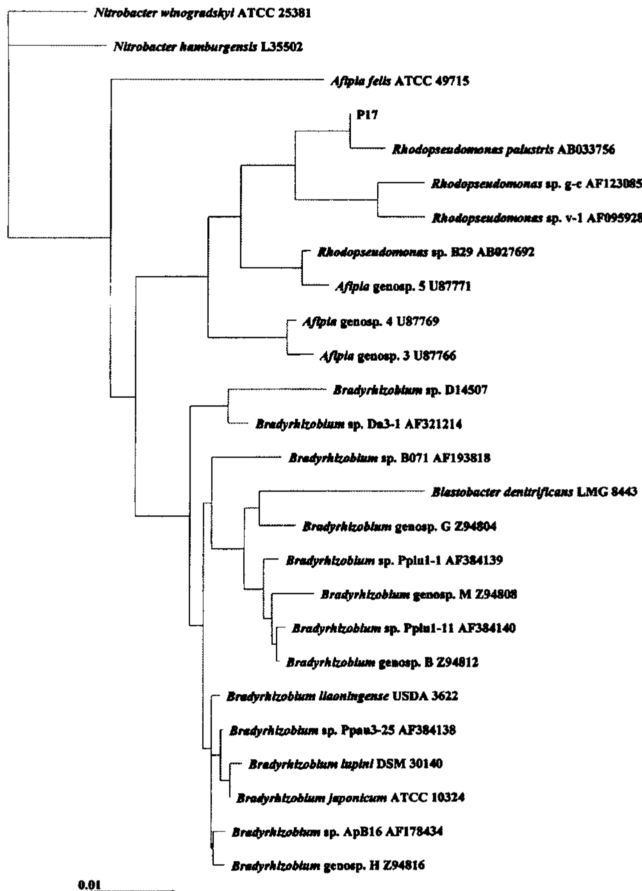

Fig. 5. Phylogenetic comparison between isolate P17 and most closely related bacteria (bar 0.01 fixed changes per nucleotide base).

It was also found that the process has been operated stably, because the concentration of mixed liquor suspended solids (MLSS) was almost constant in each PSB reactor. An offensive odor of the effluent was considerably removed due to reduction of an ammonia-nitrogen (NH_3-N) concentration. Also, it was assumed that reduction of total phosphorus (P) concentration would prevent eutrophication problems in natural waters. Therefore, the isolate might be very useful for wastewater treatment, since it was successfully applied to the treatment of soybean curd wastewater.

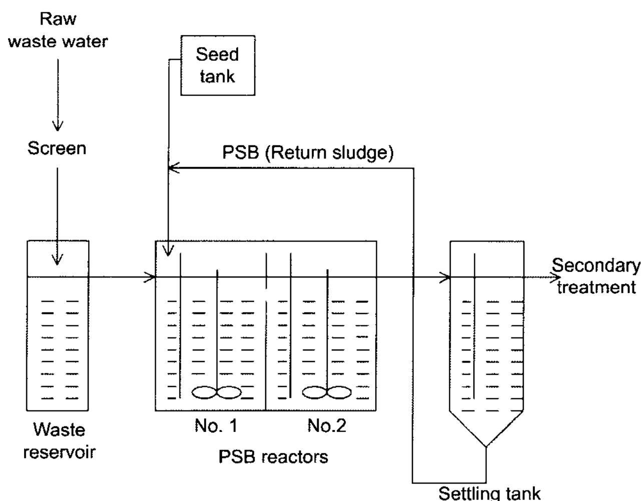

Fig. 6. Flow chart of the photosynthetic sludge process for treatment of wastewater from soybean curd industry.

Table 1. Purification of soybean curd wastewater by the bench-scale PSB reactor.

Factors	Raw waste water	PSB reactor No. 1	PSB reactor No. 2	Settling tank
pH	4.2	7.1	7.3	7.4
COD_{cr} (mg/l)	3,240	920	730	450
BOD_5 (mg/l)	3,310	940	740	460
MLSS (mg/l)	570	3,810	3,860	1,210
Total volatile acids (mg/l)	2,540	790	510	440
Total nitrogen (mg/l)	140	98	84	82
NH_3-N (mg/l)	6.9	–	–	4.8
Total phosphorus (mg/l)	130	–	–	3.8

Acknowledgments

This research was supported by a Brain Korea 21 project grant from the Ministry of Education & Human Resources Development, Republic of Korea, and also supported by a grant from Korea University.

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