JOURNAL
OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Analysis and Quantification of Ammonia-Oxidizing Bacteria Community with *amo*A Gene in Sewage Treatment Plants

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The analysis and quantification of ammonia-oxidizing bacteria (AOB) is crucial, as they initiate the biological removal of ammonia-nitrogen from sewage. Previous methods for analyzing the microbial community structure, which involve the plating of samples or culture media over agar plates, have been inadequate because many microorganisms found in a sewage plant are unculturable. In this study, to exclusively detect AOB, the analysis was carried out *via* denaturing gradient gel electrophoresis using a primer specific to the *amo*A gene, which is one of the functional genes known as ammonia monooxygenase. An AOB consortium (S1 sample) that could oxidize an unprecedented 100% of ammonia in 24 h was obtained from sewage sludge. In addition, real-time PCR was used to quantify the AOB. Results of the microbial community analysis in terms of carbon utilization ability of samples showed that the aeration tank water sample (S2), influent water sample (S3), and effluent water sample (S4) used all the 31 substrates considered, whereas the AOB consortium (S1) used only Tween 80, D-galacturonic acid, itaconic acid, D-malic acid, and L-serine after 192 h. The largest concentration of AOB was detected in S1 (7.6×10^6 copies/µl), followed by S2 (3.2×10^6 copies/µl), S4 (2.8×10^6 copies/µl), and S3 (2.4×10^6 copies/µl).

Keywords: AOB, *amo*A gene, DGGE, CLPP, real-time PCR, sewage wastewater treatment plant

The removal of nitrogen is an integral part of the sewage treatment process. A great part of the nitrogen content in sewage is in the form of ammonia, and this nitrogen is removed biologically with the help of microorganisms. The biological removal of nitrogen proceeds *via* two stages, nitrification and denitrification [12]. In particular, nitrification is very important as it represents the initial stage of biological nitrogen removal in wastewater treatment [5, 31]. Nitrification involves two steps: first, NH_4^+ is oxidized to NO_2^- by ammonia-oxidizing bacteria (AOB), and then NO_2^- is oxidized to NO_3^- by nitrite oxidizing bacteria (NOB) [20]. Therefore, a great quantity of AOB is required in the sewage treatment process [23] However, it is difficult to maintain the AOB population during wastewater treatment because they grow slowly and are sensitive to environmental factors [4, 8]. The dynamics of the microbial community in a wastewater treatment plant is greatly influenced by changes in the influent characteristics of wastewater and operational variables of the plant [38]. Reports indicate that the microbial community dynamics is closely connected to the effective management of the wastewater treatment process [3, 28]. This makes the analysis and quantification of the AOB community in sewage treatment plants of crucial importance.

In most previous studies, the microbial community structure has been determined by growing colonies either by directly plating samples over agar plates, mainly using the microbial culture method, or by plating culture medium obtained by performing enrichment culture on broth culture media over agar plates. These methods were, however, inadequate to define the complete community structure because many microorganisms in sewage treatment plants are unculturable [37]. To supplement these methods, a molecular method could be used to analyze microorganisms not cultured in the solid media. Among these methods, denaturing gradient gel electrophoresis (DGGE), which analyzes a microbial community using DNA, is widely used. DGGE has been shown to detect differences in the melting behavior of small DNA fragments that differ by as little as a single base-pair substitution. In DGGE, DNA, which has various sequences, forms bands at different

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positions, and the more the same sequences exist, the more the brightness of the corresponding band increases [22, 25]. However, the DGGE method could not enable the monitoring of the quantitative changes of the particular microorganism that we wanted to analyze. To overcome this limitation, real-time PCR, which is a new technology used to amplify and simultaneously quantify a targeted DNA molecule, was suggested as an alternative. Note that in order to monitor the AOB community structure within a sewage treatment plant using DGGE or real-time PCR, it is important that only the AOB, and no other microorganism, be detected, because AOB differ from other microorganisms in a sewage plant in terms of physiological function and form [32]. To achieve this, a primer specific to the *amo*A gene, which is one of the functional genes known as ammonia monooxygenase (*amo*), had to be used.

Accordingly, in this study, the AOB community in a sewage treatment plant was examined by DGGE with a primer specific to the *amo*A gene, and the AOB were quantified with the *amo*A gene using real-time PCR.

MATERIALS AND METHODS

Sampling, Storage, and Enrichment Culture

Wastewater samples were taken from the aeration tank water (S2), influent water (S3), and effluent water (S4) in the S Sewage treatment plant of H City in Gyeonggi Province. The samples were taken at 17°C and immediately transported to a laboratory under refrigeration at 2°C. An enrichment culture medium for separating AOB was also stored and used during the experiment, and the sample corresponding to the enrichment culture was denoted S1. An artificial environment was prepared to generate ammonia gas under aerobic condition, in order to estimate the attenuation of ammonia by the AOB in the enrichment culture. The ammonia gas was artificially generated as follows: 0.2 g of finely ground sesame dregs [containing 4% (w/v) nitrogen, 2% (w/v) phosphoric acid, 1% (w/v) potassium, and greater than 70% (w/v) organic matter] and 10 ml of tap water were mixed; the mixture was then poured into a 70 ml vial, which was kept in an incubator at 30°C with shaking at 178 rpm. Ammonia gas was generated usually in the 3rd or 4th day of incubation. The concentration of the generated ammonia gas was measured on the 3rd day, and then sewage sludge as an inoculum was inoculated into the culture. Enrichment culture was performed by transferring preculture to a fresh medium 20 times. Cells were collected from S1 by centrifugation, because this sample was considered to have much biomass, and from S2, S3, and S4 by filtration, because these samples were considered to have less biomass. For centrifugation, 200 ml of the sample was poured into a sterile 250 ml centrifuge tube and then centrifuged for 10 min at 710 ×*g*. Then, the supernatant was drained and the cells were collected. These cells were placed in a sterile 1.5 ml tube and resuspended with sterile water, and centrifugation was performed again for 10 min at 1,260 ×*g* to collect the cells. Influent water and effluent water were considered to have less biomass, so filtration was applied to collect cells from each sample; each sample was filtered with membrane filters (pore size: 0.2 μm), and the cells were washed off with sterile water to be

collected with filter paper. Then, the collected suspension was placed in a 1.5 ml tube for centrifugation for 10 min at 1,260 ×*g* to collect cells. From this 0.5 g (wet weight) of collected cells, genomic DNA was extracted by using a BIO101 Fast DNA SPIN Kit (Q-Biogene, USA). The extracted DNA was refrigerated at −20°C.

Evaluation of Ammonia Gas Removal by Ammonia-Oxidizing Bacteria Consortium

As described above, the enrichment consortium was inoculated in a 70 ml vial. Then, ammonia gas concentration was measured every 24 h using a Kitagawa odor detector tube (Kitagawa, Japan). Each experiment was repeated three times.

Community-Level Physiological Profiling (CLPP) Analysis

Each sample was shaken for 10 min at 200 rpm after accumulation for 1 h. The 150 μl suspension sample was inoculated in an EcoPlate (Biolog, USA), and the plates were incubated at 20°C for 14 days. The absorbance was recorded every day at 595 nm with a Multiskan Ascent photometric plate reader (Thermo Labsystems, Finland). Data for statistical analysis were obtained by calculating the average well color development (AWCD), as below:

$$AWCD = \sum (C - R)/n \qquad (1)$$

C: OD$_{595nm}$ value of each well
R: OD$_{595nm}$ value of control well
n: number of substrates utilized (31)

Data for statistical analysis were obtained using the Shannon Index.

$$H = -\sum P_i \ln P_i \qquad (2)$$

H: Shannon index
P$_i$: (absorbance of each well)/$\sum_{i=1 \text{ to } n}$(well)

The OD$_{595nm}$ values of each well on the 4th, 8th, and 14th day of cultivation were calculated using the formula below. In addition, principal component analysis (PCA) was performed using an SPSS 18.0ko software (SPSS Inc. Korea).

$$(C_{id}-R_{id})/AWCD_{id} \qquad (3)$$

C$_{id}$: OD$_{595nm}$ value of each well on day i of cultivation
R$_{id}$: OD$_{595nm}$ value of control well on day i of cultivation
AWCD$_{id}$: AWCD value on day i of cultivation

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DNA extraction from S1, S2, S3, and S4 samples was performed using a TaKaRa PCR Thermal Cycler (TAKARA Biotechnology Co. Ltd, Japan). The following were used as primers for PCR: 341f-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3'); Br518 (5'-ATT ACC GCG GCT GCT GG-3'); *amo*A-2R (5'-CCCCTCKGS AAAGCCTTCTTC-3'); and *amo*A-1F (5'-GGGGTTTCTACTGGT GGT-3') [33].

PCR amplification was performed by first carrying out denaturation at 95°C for 5 min, followed by 28 cycles of annealing at 60°C for 30 s and extension at 72°C for 30 s; subsequently, a final extension step was carried out at 72°C for 5 min. DGGE analysis was conducted using a DCode system (BIO-RAD, USA). Samples of PCR product (20 μl) were loaded onto 6% (w/v) polyacrylamide gels in 1× TAE buffer. The polyacrylamide gels were made with a

linear denaturing gradient ranging from 40% at the top to 60% at the bottom. The electrophoresis was run at 50 V and 60°C for 14 h. To obtain a clear image, the gel was analyzed with UVIBand Map v.12.14 (UVItec Ltd., Cambridge, UK).

DNA was extracted by from the gel cutting nine representative bands from the DGGE fingerprints using a universal primer and 19 bands from the DGGE fingerprints using a special primer. Then, PCR was performed using the 341f, Br518, *amo*A-1F, and *amo*A-2R primers. The PCR product was cloned by a pGEM-T Vector System (Promega, USA) and transformed to *E. coli* DH5α [1]. Plasmid was extracted from nine transformed clones for analyzing the sequencing [1]. The analyzed sequence was checked against the GenBank database with the Basic Local Alignment Search Tool (BLAST) algorithm. The sequence was edited by the BioEdit program and sorted by the Clustal X program to enable the construction of a phylogenetic tree. The phylogenetic tree was constructed by the MEGA4 program [1, 10].

Quantitative Analysis of Ammonia-Oxidizing Bacteria with *amo*A Gene Using RT-PCR

An RT-PCR method was used for the quantitative analysis of ammonia-oxidizing bacteria with the *amo*A gene. The *amo*A gene was amplified using the *amo*A-2r and *amo*A-1f primers [33]. The DNA of standard samples was purified with a plasmid DNA purification kit (Cosmo Genetech, Korea). The purified DNA was used as the standard curve of the RT-PCR sample. The DNA concentration of the standard samples was measured using a spectrophotometer (DR5000, HACH, USA) at 260 nm. Standard samples were diluted $10^5 - 10^8$-fold. RT-PCR (Illumina Real-Time PCR System, USA) was performed using 20 μl samples in the MicroAmp 48-well reaction plate and MicroAmp cap (Biolog, USA). Nineteen μl of PCR master mix was added to 1 μl DNA template. The PCR reaction mixture consisted of 10 μl of 2× Quantimix SYBR PCR master mix (JMC R&D, Korea), 1 μl of each primer (0.5 pmol), and 8 μl of distilled water. PCR was conducted by first heating at 95°C for 15 min and then repeating 40 cycles of heating at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s. Subsequently, a final cycle was performed by heating at 95°C for 15 s, 53°C for 15 s, and 95°C for 15 s to complete the RT-PCR. Then, the DNA concentration was measured with a standard curve (DNA concentration vs. C_t). The concentration was converted to a copy number using the linear equations below:

Microgram per picomole (μg/pmole) = A × 649 g/mole × 10^{-12} moles/pmole × 10^6 μg/g   (4)

Copies = (C/(B)) × 10^{-12} moles/pmole × 10^{-3} mg/g × 6.022 × 10^{23} copies per mole   (5)

A: length of DNA (bp)
B: microgram per picomole (μg/pmole)
C: values measured by spectrophotometer at 260 nm

The purified DNA was amplified with the *amo*A-1F primer and *amo*A-2R primer and transformed with a vector into *E. coli*. Plasmid DNA was extracted from the transformed DNA using a plasmid purification kit, and its DNA sequence was analyzed (Microgen, Korea). The analyzed sequence was compared with the GenBank database using the BLAST algorithm and it was confirmed to be an uncultured *Nitrosomonas* sp. This plasmid's C_t value was used to plot a standard curve to change to a DNA concentration.

RESULTS AND DISCUSSION

Removal of Ammonia Gas by Ammonia-Oxidizing Bacteria Consortium

The ammonia-oxidizing ability of the consortium obtained by enriching with a substrate, sesame dreg, was evaluated, and the results are presented in Fig. 1. As mentioned earlier, ammonia gas was generated in a vial at 3rd day after mixing the sesame dregs with tap water, at which point the AOB consortium was inoculated into the mixture. It was found that the removal efficiency of the AOB consortium was 100%; in vials containing the AOB consortium, the generation of ammonia gas was reduced to 0.00 ± 0.00 ppmv within 24 h and remained at this level for 168 h (7th day), whereas in vials containing the control group (where no AOB was inoculated), the ammonia gas generation continued for 168 h to 29.75 ± 1.06 ppmv.

Yan *et al.* [39] measured the degree of ammonia oxidation in a bioreactor containing AOB consortia and 20 mM of ammonia, and measured the ammonia level of its effluent. The measured value was 2.4 mM, which implies a removal efficiency of 88% after 7 days [39]. Paungfoo *et al.* [29] enriched AOB consortia from waste shrimp farm water and sediment. The ammonium removal efficiency of these consortia was reported to be 85% after 7 days [29]. As mentioned above, nitrification involves the oxidation of NH_4^+ to NO_2^- by AOB. Accordingly, a nitrifying bacteria consortium in the sewage treatment process contains a large amount of AOB [13]. The ammonia removal abilities of nitrifying bacteria consortia have been reported in some studies. Jiang *et al.* [11] reported 99% ammonia removal efficiency for a 30-day experiment, in which a nitrifying bacteria consortium enriched from active sewage sludge generated in the wastewater treatment process was cultured and its ammonia removal efficiency measured [11]. The ammonia removal efficiency of a bioreactor that was
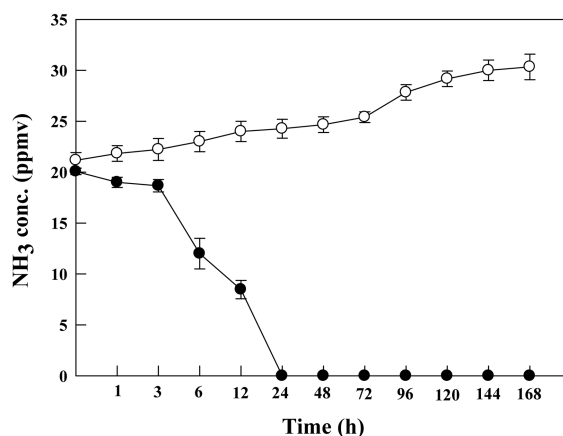

Fig. 1. Biodegradation of ammonia gas by consortia developed in this study (○: control; ●: S1).

inoculated with both AOB consortia and a nitrifying bacteria consortium was reported to be 78% [19]. In addition, the ammonia removal efficiencies of nitrifying bacteria consortia enriched from wood chips [17], from granulated sludge [24], and from exhausted carbon [11] were reported as 92%, 80%, and 96%−98%, respectively. The removal efficiency of an ammonia-oxidizing consortium and a nitrifying bacteria consortium has been reported as ranging from 71% to 99%. In this study, enriched ammonia-oxidizing consortia completely removed ammonia gas in 1 day and their efficiency was maintained for 7 days (Fig. 1). The ammonia removal efficiency of the S1 sample in this study was very superior to any other results reported earlier. The S1 sample was also considered as a powerful consortium that has a potential to be used to reduce the ammonia continuously generated from activated sludge in the wastewater treatment process. It can be considered as a good source for separating nitrification bacteria and ammonia bacteria from a consortium.

Community-Level Physiological Profiling (CLPP) Analysis
CLPP analyses of the S1, S2, S3, and S4 samples were conducted to investigate the microbial community characteristics in terms of carbon availability (Fig. 2).

The results showed that the S2, S3, and S4 samples used all of the 31 substrates included in the study, whereas the S1 sample utilized only Tween-80, D-galacturonic acid, itaconic acid, D-malic acid, and L-serine after 192 h (8 days) of incubation.

Surfactants, such as Tween-80, can improve the microbial efficiency of substrate utilization by distributing fatty acid evenly on the solution [9] and help microbial growth by increasing the biodegradability of organic matter [15]. Tween-80, which is a nonionic surfactant and formally known as polyethylene glycol sorbitan monooleate, was reported to have the ability to improve the production of several bioconversion products, such as 10-hydroxystearic acid and ketostearic acid, in a water/oil-two-phase system [5]. In this study, the S1 sample (*i.e.*, the enriched AOB consortium) used Tween-80 specifically, so it can be deduced that the S1 sample had many microorganisms that could oxidize ammonia. This implies that the S1 sample used Tween-80 as an accelerator that enhances the ability of AOB to oxidize ammonium.

D-Galacturonic acid and D-malic acid are types of carboxylic acids, which are generated from ammonia oxidation. It was hypothesized that the S1 sample used D-galacturonic acid and D-malic acid specifically, because
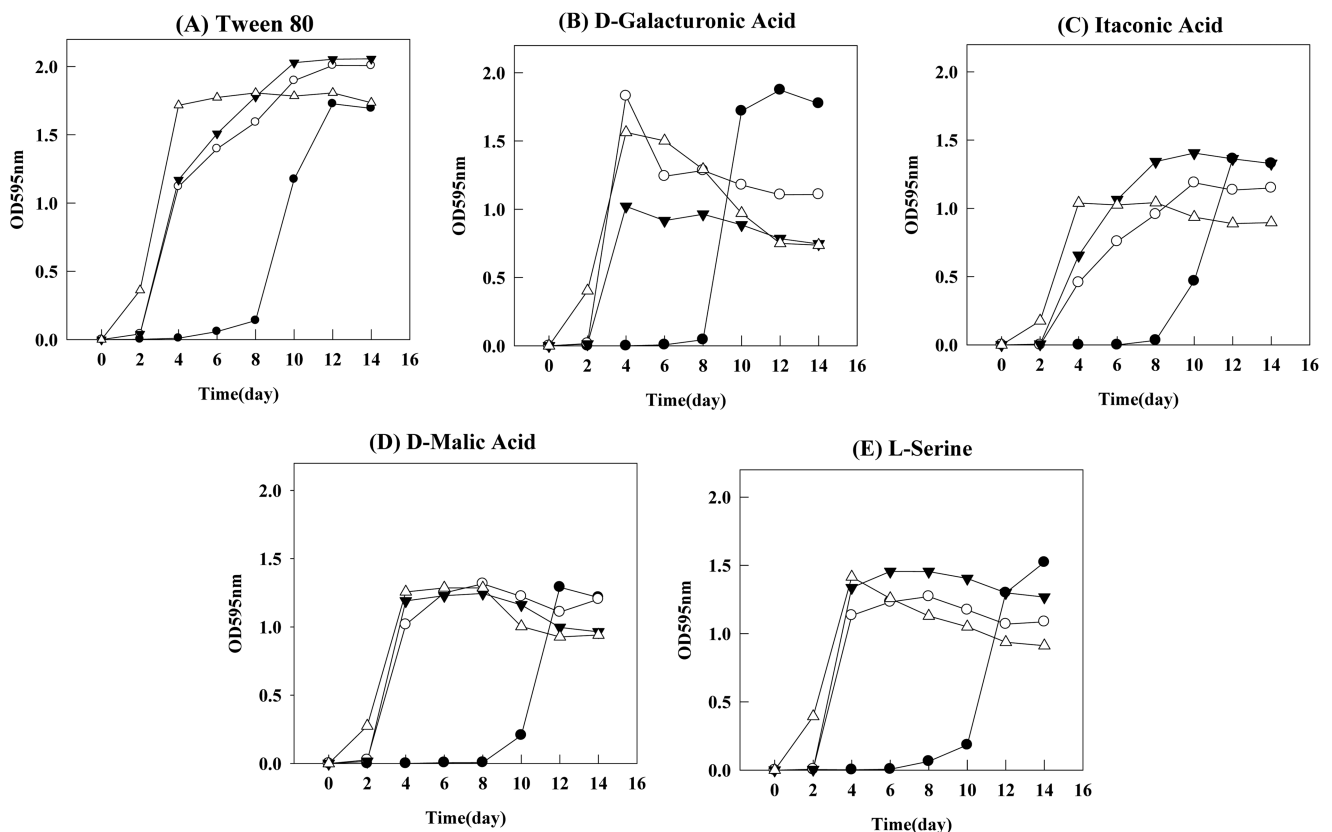

Fig. 2. Time-course of substrate utilization of various samples (●, S1; ○, S2; ▼, S3; ▽, S4).
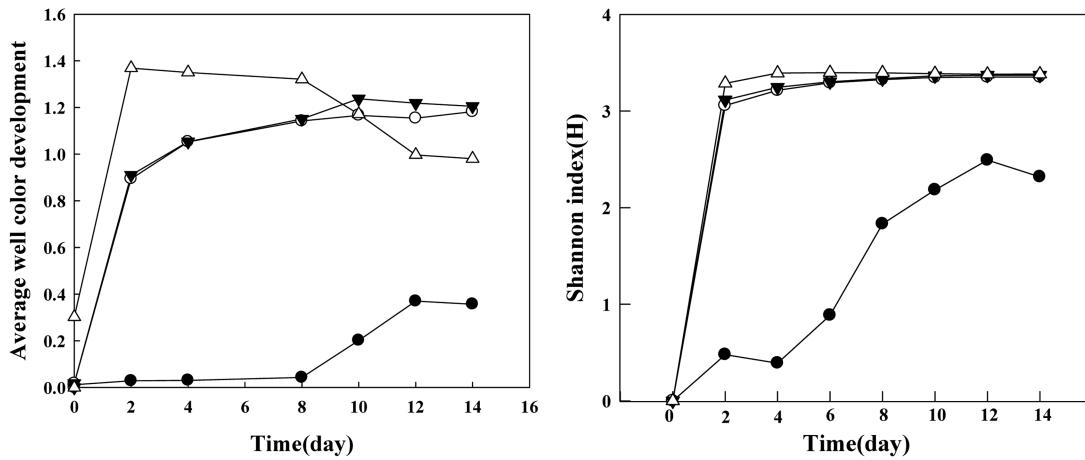
Fig. 3. Variation in average well color development over time in ECO plate and comparison of Shannon index (n = 3) (●, S1; ○, S2; ▼, S3; ▽, S4).

many microorganisms using the carboxylic acid produced by AOB were present in the S1 sample.

L-Serine is a substrate that belongs to amino acids containing organic nitrogen compounds that exist in nature [35]. Many microorganisms that oxidize (nitrify) ammonia would be produced in the process of culturing AOB, so many microorganisms using N_2O as a substrate would also be expected. Therefore, microorganisms in the S1 sample were considered to use L-serine as a nitrogen source.

Itaconic acid (citric acid), which is an organic compound, is involved in the TCA (citric acid) cycle. The addition of ammonia to itaconic acid generates various glutamic acids, which react with transaminase to produce many amino acids [35]. α-Keto acids are generated from the biochemical conversion of amino acids in the TCA cycle and generate ATP. Therefore, it is reasonable to consider that the S1 sample consortium enriched by artificially adding ammonia specifically uses itaconic acid during the above metabolic process.

The use of substrates by the other samples (S2, S3, and S4) taken from the sewage treatment plant increased over 48 h after inoculation, whereas that by the S1 sample increased over 198 h. This difference is explained by the fact that the growth rate of ammonia oxidizing bacteria is usually slow [13]. Hence, the substrate utilization rate of the AOB consortium was slower than that of the microorganisms in other samples. Results of the calculation of AWCD of each well on the 14th day of cultivation indicate that the substrate utilization was the highest for the S2 sample (0.36 for S1, 1.18 for S2, 1.12 for S3, and 0.98 for S4) (Fig. 3). This was attributed to the abundant supply of oxygen in the aeration tank (from which the S2 sample was obtained), which facilitated microbial survival and resulted in the generation of plentiful microbial floc.

To determine the diversity of the microbial species in the samples, the Shannon index of each sample was calculated on the last day (14th) of cultivation, with the following results: S1, 2.31; S2, 3.35; S3, 3.37; and S4,
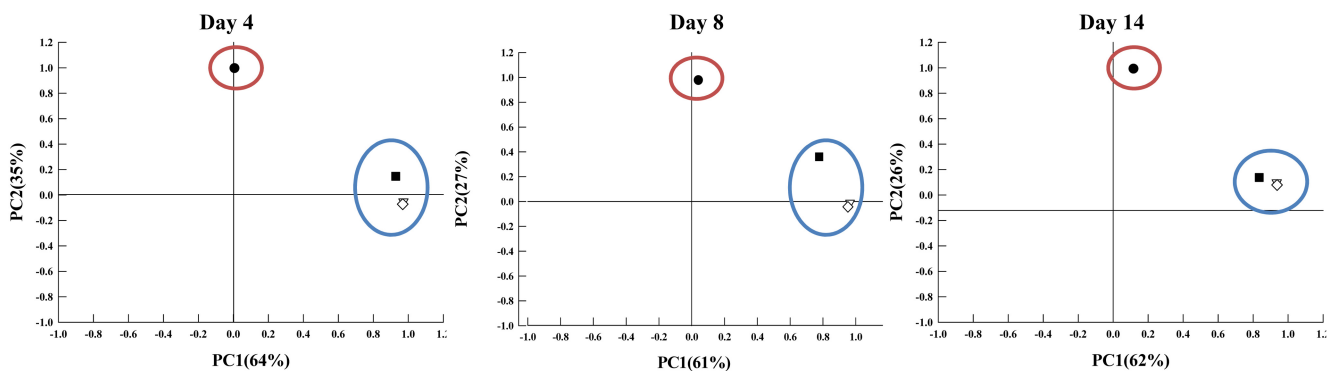

Fig. 4. Principal component analysis of average well color development based on an integrated metric of color development at 14 days. (●, S1; ○, S2; ▼, S3; ▽, S4).
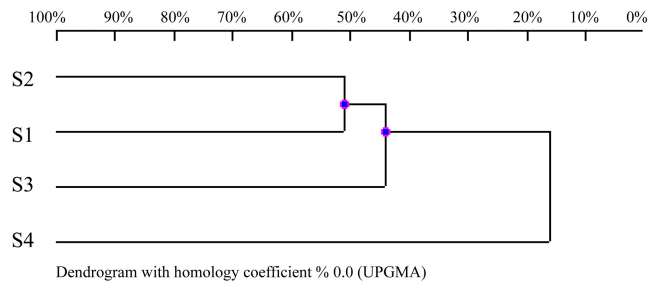
Fig. 5. Similarity analysis of sample based on DGGE fingerprints.

3.38. The Shannon diversity index values for the S2, S3, and S4 samples were very similar, whereas that for the S1 sample was lower than the values for the other samples. This was because AOB were dominant in the S1 sample.

The correlation between samples in terms of the AWCD was determined by using principal component analysis (PCA) (Fig. 4). PCA is a mathematical technique that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal compounds. The results for the 4th, 8th, and 14th days were 64%, 61%, and 62%, respectively, for PC1 and 35%, 27%, and 26%, respectively, for PC2. The S2, S3, and S4 samples could be grouped because their substrate utilization was similar, but the S1 sample could not be grouped with the other samples.

Denaturing Gradient Gel Electrophoresis Analysis
The genomic DNA of each sample was extracted, and the DGGE fingerprint of 16S rDNA PCR is displayed in Fig. 5. The relationships of each strain were shown in the phylogenetic tree by selecting and identifying bands in DGGE fingerprints that were shared among samples (Fig. 6A).

PCR was conducted with a universal primer that could amplify general bacteria and with a special primer that could amplify only AOB. The results showed that the use of the universal primer afforded more variations in band trends than that of the special primer (data not shown).

Subsequently, DGGE was conducted using the PCR product amplified by the universal primer. The overall similarity among the samples was analyzed in terms of the position of the DGGE bands, as shown in Fig. 5. The similarity of the S1 and S2 samples was the highest at 51%, and that of the S3 and S4 samples was the lowest at 9%. Band similarity of the S1 (selectively cultured AOB consortium) and S3 samples was 44%, and that of the S1 and S4 (effluent water) samples was 16%. These results
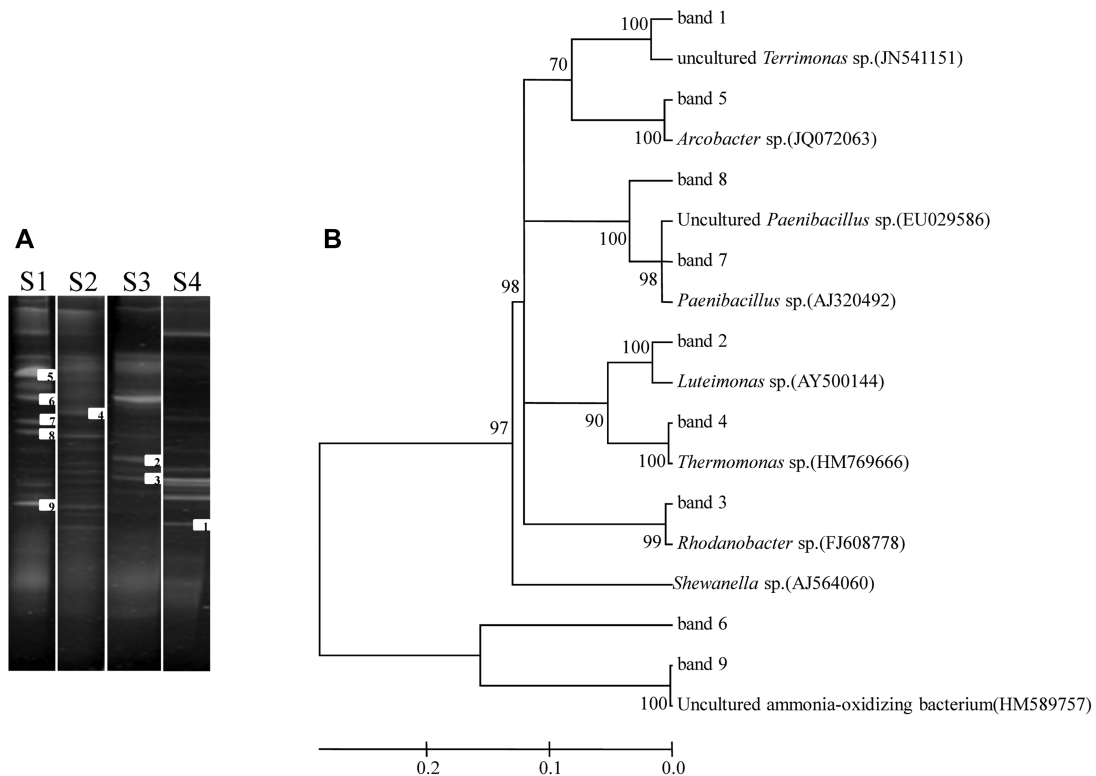

Fig. 6. Results of microbial community analysis by DGGE with universal primer [6% (w/v) polyacrylamide gel, urea concentration 35%−70%, 50 V, 60°C, 14 h]: DGGE band pattern (**A**) and phylogenetic tree illustrating the relationships among the closest relatives in the GeneBank database and the clones (**B**).

suggest that there are large quantities of AOB in the sewage treatment process, and in particular, there are much more AOB in an aeration tank sample (S2) than in the other samples. In addition, the fact that band similarity of the influent water sample (S3) and the effluent water sample (S4) was the lowest shows that many changes in the microbial community structure occur during a sewage wastewater treatment process.

When DGGE was conducted using the PCR product amplified by the primer specific to AOB, the overall band similarity among the samples was high (data not shown). DGGE is a technique that analyzes a microbial ecological community by amplifying the V3 region of 16S rDNA that shows the most dramatic variation among microbial species. It analyzes the microorganisms in a sample on the basis of the difference in the mobility of their DNA by adding a GC-clamp composed of G+C sequences to the 5' end of one of the primers. Comparison of the band positions of the samples was based on the data from gel analysis software or a visual comparison [30]. Since *amo*A-2R and *amo*A-1F were used to specifically detect AOB, the DNA of the microorganisms amplified by these primers might show similar mobility.

Fig. 6 displays phylogenetic trees constructed by selecting specific DGGE bands. Microorganisms identified from the DGGE band amplified using a universal primer were mostly those that are often found in sewage wastewater sludge or the wastewater treatment process. In particular, band 4 [cut from microorganisms in an aeration tank water sample (S2)] showed 99% similarity with *Thermomonas* sp. (Accession No. HM769666), which appeared in enrichment culture of anaerobic AOB in the wastewater sludge for removing nutritional nitrogen. Band 6 [cut from AOB consortium sample (S1)] showed high similarity with *Shewanella* sp. (AJ564060), a microorganism found in paper mill wastewater. This suggests that there are many paper mills around the sewage treatment plant of H city, from where the sample was taken. Band 9 did not appear in the influent water sample (S3) and effluent water sample (S4), appeared dimly in the aeration tank water sample (S2), and was more pronounced in the AOB consortium sample (S1). This band showed 100% similarity with uncultured AOB (HM589757) that is commonly found in wastewater treatment plants. It can be considered that the uncultured AOB were generated in the aeration tank and increased during enrichment culture.

Identification of the DGGE bands using PCR product amplified with the *amo*A-2R and *amo*A-1F primers showed that all the bands were uncultured bacteria that are generally found in the wastewater treatment process. Among these, there were microorganisms for which it was unusual to have an *amo*A gene, and some microorganisms were identified as uncultured AOB (data not shown).

Quantitative Analysis of Ammonia Oxidizing Bacteria with *amo*A Gene using RT-PCR

In order to quantify plasmid DNA, DNA from DGGE bands was diluted serially from 10^2 to 10^8 and a calibration curve of C_t value versus plasmid DNA was obtained (y = $18.80 - 0.31259x$, $R^2 = 0.997$). RT-PCR was performed using SYBR Green2 dye to quantify AOB in the S1, S2, S3, and S4 samples. AOB were detected in all samples, and the C_t values of the S1, S2, S3, and S4 samples were 7.09, 13.78, 15.07, and 14.43, respectively. To convert C_t value to DNA concentration and then to copy number, the Illumina DNA copy number calculator (Illumina, USA) was used. The results were as follows: 7.6×10^6 copies/µl for S1, 3.2×10^6 copies/µl for S2, 2.4×10^6 copies/µl for S3, and 2.8×10^6 copies/µl for S4; hence, the largest amount of AOB was detected in the S1 sample, followed by S2, S4, and S3. A large concentration of AOB was detected in S1, which was the sample of cultured bacteria that had the ability to oxidize ammonia. In the case of the wastewater treatment process, the largest concentration of AOB was found in the aeration tank water, followed by effluent water. Generally, in the process of oxidizing ammonia by AOB, NO_2, hydrogen, and oxygen are generated when NH_4^+ combines with oxygen in the aeration tank [34]. The microorganism involved in this process is *Nitrosomonas* sp., which oxidizes ammonia-nitrogen [7]. Therefore, it is considered that there is a larger concentration of *Nitrosomonas* sp. in an aeration tank using oxygen compared with that in the other parts of a sewage treatment plant.

As regards the analysis of the nitrifying bacteria community in the biological wastewater treatment plant, the molecular method is more suitable than traditional cultivation method because of slow growth rate, small biomass, unpredictable lag phase, and sensitive response to organic compound of AOB [13, 14, 16]. In most studies so far, the general communities of AOB in the wastewater treatment process have been analyzed by using DGGE and terminal restriction fragment length polymorphism [26]. Recently, several researchers have reported quantification of AOB by real-time PCR assay based on the quenching-primer PCR method [2, 21, 36]. Baek *et al*. [2] reported quantification of AOB in livestock, food, paper, sewage, and textile wastewater (AOB were detected in concentrations ranging from 1.7×10^6 to 2.7×10^{10} copies/L). Layton *et al*. [21] reported 4.8×10^{12} copies/L of AOB in industrial wastewater.

Several kinds of AOB can be found in the wastewater treatment process, such as *Nitrosomonas* sp., *Nitrosospira* sp., *Nitrobacter* sp., *Cenarchaeum* sp., and *Candidatus* sp. [13, 34]. Among these, *Nitrosomonas* sp. and *Nitrobacter* sp. are known to be involved in the biological reaction of ammonia-nitrogen [18]. In this study, a smaller amount of AOB was detected than in other studies. This is because a

gene of *Nitrosomonas* sp. was selected as the target gene for the quantification.

In nature, the nitrogen source and the energy source available in the environment determine the microbial species that dominates the environment. Therefore, these environmental factors should be considered when quantifying AOB using real-time PCR. In addition, real-time PCR estimates biomass using microbial-specific DNA. This gives real-time PCR an advantage over the traditional culture method for cell counting and detection of a specific microorganism, which is based on the genus of the microorganism, making it difficult to determine the exact amount of that microorganism [27]. A database of the plasmid DNA of target microorganisms can play an important role in facilitating the monitoring of both AOB and other microorganisms in the wastewater treatment process.

Acknowledgment

REFERENCES

1. An, Y. J., Y. H. Joo, I. Y. Hong, H. W. Ryu, and K. S. Cho. 2004. Microbial characterization of toluene-degrading denitrifying consortia obtained from terrestrial and marine ecosystems. *Appl. Microbiol. Biotechnol.* **65:** 611–619.
2. Baek, K., C. Park, H. Oh, B. Yoon, and H. Kim. 2010. Diversity and abundance of ammonia-oxidizing bacteria in activated sludge treating different types of wastewater. *J. Microbiol. Biotechnol.* **20:** 1128–1133.
3. Briones, A. and L. Raskin. 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr. Opin. Biotech.* **14:** 270–276.
4. Chih-Ju, G. J. and G. C. Huang. 2003. A pilot study for oil refinery wastewater treatment using a fixed film bio-reactor. *Adv. Environ. Res.* **7:** 463–469.
5. Dalmau, E., J. L. Montesinos, M. Lotti, and C. Casas. 2000. Effect of different carbon sources on lipase production by *Candida rugosa. Enzyme Microb. Technol.* **26:** 657–663.
6. Egli, K., C. Langer, H. R. Siegrist, A. J. B. Zehnder, M. Wagner, and J. R. Van Der Meer. 2003. Community analysis of ammonia and nitrite oxidizers during start-up of nitritation reactors. *Appl. Environ. Microbiol.* **69:** 3213–3222.
7. Fang, H. Y. and M. S. Chou. 1993. Nitrification of ammonia nitrogen in refinery wastewater. *Water Res.* **27:** 1761–1765.
8. Hagopian, D. S. and J. G. Rily. 1998. A closer look at the bacteriology of nitrification. *Aquacult. Eng.* **18:** 223–224.
9. Heo, S. H. and B. S. Kim. 2009. Production and recovery of oxygenated fatty acids from oleic acid by *Flavobacterium* sp. strain DS5. *Korean Chem. Eng. Res.* **47:** 620–623.
10. Hong, S. H., K. C. Shin, and E. Y. Lee. 2010. Characterization of a nitrogen fixing bacteria *Mycobacterium hominis* sp. AKC-10. *Kor. J. Microbiol. Biotechnol.* **38:** 302–307.
11. Jiang, X., R. Yan, and J. H. Tay. 2009. Simultaneous autotrophic biodegradation of H_2S and NH_3 in a biotrickling filter. *Chemosphere* **75:** 1350–1355.
12. Joo, H. S., M. Hirai, and M. Shoda. 2005. Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. *J. Biosci. Bioeng.* **100:** 184–191.
13. Junier, P., V. Molina, C. Dorador, O. Hadas, O. S. Kim, T. Junier, *et al.* 2010. Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. *Appl. Microbiol. Biotechnol.* **85:** 425–440.
14. Kelly, J. J., S. Siripong, J. McCormack, L. R. Janus, H. Urakawa, S. E. Fantroussi, *et al.* 2005. DNA microarray detection of nitrifying bacterial 16S rRNA in wastewater treatment plant samples. *Water Res.* **39:** 3229–3238.
15. Kim, D. J. 2007. *Environmental Pollution and Remediation*, 1st Ed. Korea University Press, Korea.
16. Kim, D., D. Lee, and J. Keller. 2006. Effect of temperature and free ammonia on nitrification and nitrite accumulation in landfill leachate and analysis of its nitrifying bacterial community by FISH. *Bioresour. Technol.* **97:** 459–468.
17. Kim, H. S., Y. J. Kim, J. S. Chung, and Q. Xie. 2002. Long-term operation of a biofilter for simultaneous removal of H_2S and NH_3. *J. Air Waste Manage.* **52:** 1389–1398.
18. Kim, D., H. Kim, J. Kim, K. Suh, and I. Kong. 1997. Isolation and characterization of ammonia oxidizing bacteria, *Nitrosomonas* sp. PK1. *Kor. J. Life Sci.* **7:** 107–111.
19. Kumar, V. J., C. Achuthan, N. J. Manju, R. Philip, and I. S. Singh. 2009. Activated packed bed bioreactor for rapid nitrification in brackish water hatchery systems. *J. Ind. Microbiol. Biotechnol.* **36:** 355–365.
20. Laia, C. L. and G. G. Jesus. 2004. Use of *amoA* as a new molecular marker for ammonia-oxidizing bacteria. *J. Microbiol. Methods* **57:** 69–78.
21. Layton, A. C., H. Dionisi, H. W. Kuo, K. G. Robinson, V. M. Garrett, A. Meyers, and G. S. Sayler. 2005. Emergence of competitive dominant ammonia-oxidizing bacterial populations in a full-scale industrial wastewater treatment plant. *Appl. Environ. Microbiol.* **71:** 1105–1108.
22. Li, H., M. Yang, Y. Zhang, T. Yu, and Y. Kamagata. 2006. Nitrification performance and microbial community dynamic in a submerged membrane bioreactor with complete sludge retention. *J. Biotechnol.* **123:** 60–70.
23. Limpiyakorn, T., P. Sonthiphand, C. Rongsayamanont, and C. Polprasert. 2011. Abundance of *amoA* genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants. *Bioresour. Technol.* **102:** 3694–3701.
24. Malhautier, L., C. Gracian, J. C. Roux, J. L. Fanlo, and P. Le Cloirec. 2003. Biological treatment process of air loaded with an ammonia and hydrogen sulfide mixture. *Chemosphere* **50:** 145–153.
25. Muyzer, G. and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leuwenhoek* **73:** 127–141.

26. Nunan, N., T. J. Daniell, B. K. Singh, A. Papert, J. W. McNicol, and J. I. Prosser. 2005. Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. *Appl. Environ. Microbiol.* **71:** 6784–6792.

27. Palomares, C., M. J. Torres, A. Torres, J. Aznar, and J. C. Palomares. 2003. Rapid detection and identification of *Staphylococcus aureus* from blood culture specimens using real-time fluorescence PCR. *Diagn. Microbiol. Infect. Dis.* **45:** 183–189.

28. Park, H. D. and D. R. Noguera. 2004. Evaluating the effect of dissolved oxygen on ammonia-oxidizing bacterial communities in activated sludge. *Water Res.* **38:** 3275–3286.

29. Paungfoo, C., P. Prasertsan, N. Intrasungkha, L. L. Blackall, and R. Bhamidimarri. 2003. Enrichment of nitrifying microbial communities from shrimp farms and commercial inocula. *Water Sci. Technol.* **48:** 143–150.

30. Porcellato, D., H. Grønnevik, K. Rudi, J. Narvhus, and S. B. Skeie. 2012. Rapid lactic acid bacteria identification in dairy products by high-resolution melt analysis of DGGE bands. *Lett. Appl. Microbiol.* **54:** 344–351.

31. Prosser, J. I. 1989. Autotrophic nitrification in bacteria. *Adv. Microb. Physiol.* **30:** 125–181.

32. Purkhold, U., A. Pommerening-Röser, S. Juretschko, M. C. Schmid, H. Koops, and M. Wagner. 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: Implications for molecular diversity surveys. *Appl. Environ. Microbiol.* **66:** 5368–5382.

33. Rotthauwe, J. H., K. P. Witzel, and W. Liesack. 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* **63:** 4704–4712.

34. Siripong, S. and B. Rittmann. 2007. Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plant. *Water Res.* **41:** 1110–1120.

35. Sylvia, D. M., J. J. Fuhrmann, P. G. Hartel, and D. A. Zuberer. 2009. *Principles and Applications of Soil Microbiology*, pp. 401–409. 2nd Ed. Prentice Hall, Pearson.

36. Urakawa, H., H. Maki, S. Kawabata, T. Fujiwara, H. Ando, T. Kawai, *et al*. 2006. Abundance and population structure of ammonia-oxidizing bacteria that inhabit canal sediments receiving effluents from municipal wastewater treatment plants. *Appl. Environ. Microbiol.* **72:** 6845–6850.

37. Wagner, M. and A. Loy. 2002. Bacterial community composition and function in sewage treatment systems. *Curr. Opin. Biotechnol.* **13:** 218–227.

38. Wells, G. F., H. D. Park, C. H. Yeung, B. Eggleston, C. A. Francis, and C. S. Criddle. 2009. Ammonia-oxidizing communities in a highly aerated fullscale activated sludge bioreactor: Betaproteobacterial dynamics and low relative abundance of Crenarchaea. *Environ. Microbiol.* **11:** 2310–2328.

39. Yan, J., J. M. Huub, O. den Camp, M. S. M. Jetten, Y. Y. Hu, and S. C. M. Haaijer. 2010. Induced cooperation between marine nitrifiers and anaerobic ammonium-oxidizing bacteria by incremental exposure to oxygen. *Syst. Appl. Microbiol.* **33:** 407–415.