

Enrichment of Ammonia-Oxidizing Bacteria for Efficient Nitrification of Wastewater

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Abstract Ammonia-oxidizing bacteria (AOB) were enriched by repeating fed-batch cultivations in an AOB-selective medium of activated sludges from a domestic wastewater treatment plant. Enriched culture showed strong capabilities of ammonia oxidation [0.810 mg NH₄⁺-N/mg mixed liquor suspended solids (MLSS)·day] as well as NO_x⁻-N production (0.617 mg NO_x⁻-N/mg MLSS·day). Degree of enrichment was examined through fluorescent *in situ* hybridization (FISH) analyses using an AOB-specific Cy3-labeled oligonucleotide probe (NSO190) and terminal-restriction fragment length polymorphism (T-RFLP) analyses. FISH analyses confirmed that the fraction of AOB among 4',6-diamidino-2-phenylindole (DAPI)-stained cells increased from about less than 0.001% to approximately 42% after enrichment of AOB, and T-RFLP analyses showed that bacterial community became simpler as enrichment was continued. When the enriched culture of AOB was added (150 mg/l as dry suspended solid) to the normal activated sludge (3,000 mg/l as dry suspended solid), nitrification efficiencies were improved from 0.020 mg NO_x⁻-N/mg MLSS·day to 0.041 mg NO_x⁻-N/mg MLSS·day in a synthetic wastewater and also from 0.0007 mg NO_x⁻-N/mg MLSS·day to 0.0918 mg NO_x⁻-N/mg MLSS·day in a real domestic wastewater. Therefore, it is expected that this enrichment method could be used for improving efficiency of nitrification in wastewater treatment plants.

Key words: Enrichment, AOB, nitrification, FISH, T-RFLP

Nitrification, the oxidation of ammonia to nitrate, is the key process in biological nitrogen removal and an essential component of the wastewater treatment plants (WTPs) [4, 23, 35]. Two distinct groups of bacteria catalyze the two separate oxidation steps involved in nitrification. The first group is composed of the chemolithoautotrophic ammonia-

oxidizing bacteria (AOB), which convert ammonia (NH₃) to nitrite (NO₂⁻), while the second group, nitrite-oxidizing bacteria (NOB), catalyzes the oxidation of nitrite to nitrate (NO₃⁻) [23, 35]. Compared with NOB and other heterotrophic bacteria, AOB grow extremely slowly (μ_{\max} = 0.5 d⁻¹) [13], because AOB obtain energy only from ammonia oxidation and carbon from CO₂ fixation. Furthermore, ammonia monooxygenase (AMO), a membrane-integrated enzyme of AOB, which catalyzes the oxidation of ammonia to hydroxylamine, is known to be sensitive to environmental factors such as pH, temperature, and a wide range of organic compounds [13, 29, 30]. Thus, ammonia oxidation is known to be the rate-limiting step for the removal of nitrogen from wastewater [4, 35] and causes many WTPs to fail in establishing stable nitrification [13, 31]. Although the problem of slow growth of AOB can be alleviated by manipulating the SRT (solid retention time) of the system [23, 31], stability of AMO activity can only be improved by understanding the influent characteristics [4]. For example, AMO is inhibited by toxic compounds such as allylthiourea, thiourea, *p*-cresol, and aniline [20, 28, 30, 31]. Presence of these compounds in the influent fundamentally threatens the nitrification capability of a WTP, since slower ammonia oxidation causes slower growth and consequently washout of nitrifying population from the system. Once this happens, re-establishment of the nitrifying community would take a long time. Operating the WTP under such unfavorable conditions needs precautionary measures to solve the nitrification problems. One method is to introduce enriched nitrifiers into the system.

To date, many enrichment culture methods for treating various pollutants have been studied [9]. Eriksson *et al.* [10] were successful to degrade polycyclic aromatic hydrocarbons by using the culture enriched from soils in a nitrate-reducing condition, Chang *et al.* [7] enriched a culture from a landfill leachate sediment for the degradation of *cis*-1,2-dichloroethylene, and Häner *et al.* [14] applied the enrichment culture method to improve the degradation of

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p-xylene. Similarly, it is likely that enriched AOB might be effective for improving nitrification rate. Thus far, many studies have been carried out to identify the community structure and diversity of AOB, and to isolate dominant species from natural and engineered ecosystems using molecular techniques such as fluorescent *in situ* hybridization (FISH) [4, 6, 22, 29] and terminal restriction fragment length polymorphism (T-RFLP) analyses [1, 8, 16, 24]. However, these studies have been focused on the identification and isolation of AOB rather than the application of enriched AOB at field [11].

In this study, AOB was enriched from a domestic wastewater sludge at AOB selective conditions. FISH and T-RFLP analyses were carried out to identify the AOB and bacterial community structure of enriched cultures. Finally, enriched AOB were bioaugmented with the normal activated sludges to evaluate nitrification efficiency in wastewaters.

MATERIALS AND METHODS

Enrichment of AOB

The selective medium (P medium) used for the enrichment of the AOB from the activated sludge contained 0.7 g/l of KH_2PO_4 , 13.5 g/l of Na_2HPO_4 , 2.5 g/l of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g/l of NaHCO_3 , 0.005 g/l of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.001 g/l of Fe-EDTA. Since the P medium contains NaHCO_3 as a carbon source and no energy source except $(\text{NH}_4)_2\text{SO}_4$, only AOB can grow in this medium. Activated sludge (3,300 mg/l) obtained from the Seonam domestic wastewater treatment plant (Seoul, Korea) were suspended in 1.2 l of P medium in a 2-l Erlenmeyer flask, and temperature of the culture was kept at 30°C with mild agitation in the dark [15]. When the nitrite concentration reached 300–400 mg/l, 80% (v/v) of the culture fluid were discarded and replaced with the same volume of fresh P medium for continuing cultivation. This fed-batch cultivation was repeated five times until the time elapse for the nitrite accumulation was not shortened anymore.

Cell Counting of Heterotrophic and Ammonia-Oxidizing Bacteria During Enrichment

During the enrichment of AOB through the repeated fed-batch cultivation, cells were plated on a solid complex medium (NWR1 agar) containing 3 g/l of peptone, 0.5 g/l of soluble casein, 0.2 g/l of K_2HPO_4 , 0.05 g/l of MgSO_4 , 1.0 g/l of FeCl_3 , and 15 g/l of agar (for heterotrophs), and a minimal medium (HEPES solid) containing 0.5 g/l of KH_2PO_4 , 5 g/l of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g/l of NaHCO_3 , 11.92 g/l of HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 0.005 g/l of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.075 g/l of Fe-EDTA, and 10 g/l of gellan gum for AOB [15, 33]. Grown colonies were counted after 5 days of incubation for heterotrophs and 14 days for AOB.

FISH Analysis

For FISH analysis, 2 ml of enrichment culture were fixed in the solution of 4% paraformaldehyde in phosphate buffered saline (PBS) [2] at 4°C for about 10 h and were then washed with PBS. Washed sample was spotted onto a microscopic hybridization well on a gelatin-coated slide glass, air-dried, and then dehydrated for 3 min each in a graded series of 50, 80, and 100% ethanol. For detecting ammonia-oxidizing bacteria of β -subclass of the *Proteobacteria*, the 16S rDNA-targeted oligonucleotide probe NSO190 (5'-CGATCCCCTGCTTTTCTCC-3') [4, 17] was used. NSO190 was synthesized with Cy3 fluorochrome at the 5'-end (Takara shuzo, Japan). The hybridization of the samples was performed according to the hybridization protocol described by Yoshiteru [4] and Paul *et al.* [6] at 46°C for 2 h in the hybridization buffer containing NaCl (0.9 M), formamide (40%), Tris-HCl (20 mM, pH 7.4), and sodium dodecyl sulfate (0.01%). The probe concentration was 25 ng/ μ l. A stringent washing step followed, using a wash buffer at 48°C for 20 min. The wash solution contained Tris-HCl (20 mM, pH 7.4), NaCl (25 mM), and sodium dodecyl sulfate (0.01%). After washing, the slides were removed and rinsed with distilled water and air-dried. Dual staining of cells with 4',6-diamidino-2-phenylindole (DAPI) and Cy3-labeled NSO190 was performed by a modified method of Hicks *et al.* [19, 25]; i.e., cells were stained with DAPI (0.1 μ g/ml) for 5 min following *in situ* hybridization with Cy3-labeled NSO190. NSO190-hybridized and DAPI-stained samples were examined using a fluorescent microscope (Olympus BX51) with filter sets of WU (for DAPI staining) and IG (for Cy3-labeled probe). For counting the changes of AOB fraction among entire DAPI-stained cells, an image analysis software (Optimas, Media Cybernetics, U.S.A.) was used.

T-RFLP Analysis

Total genomic DNA of enriched culture was extracted using a previously described protocol [27]. For additional purification of DNA, Ultraclean™15 (Mo Bio, U.S.A.) was used [21]. To amplify bacterial 16S rDNA from the genomic DNA extracts, PCR was conducted with a universal primer set, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') [5, 17, 21, 26]. The forward primer 27F was labeled with 6-FAM™ at the 5'-end (Metabion GmbH, Germany) for detecting 5'-terminal restriction fragments (T-RF) with an automatic DNA sequencer. The mixture of the PCR contained 100–150 ng of the template DNA, each primer at 0.1 μ M concentration, a reaction buffer (100 mM Tris-HCl, 400 mM KCl, 1.5 mM MgCl_2 , and 500 μ g/ml of bovine serum albumin, pH 8.3), each deoxynucleotide triphosphate at 200 μ M concentration, and 2.5 units of *Taq* polymerase in a total volume of 50 μ l. PCR was performed using the following temperature program: initial denaturation step at 94°C for 3 min, followed by 30

cycles of denaturation at 94°C for 1.5 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, and a final extension at 72°C for 3 min. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Germany). Amplified 16S rDNAs were digested with two tetrameric restriction endonucleases, *Hae*III and *Hha*I [8, 16], according to the manufacturer's instruction. Afterwards, 3 μ l of the digested solutions were mixed with 2 μ l of loading buffer and 0.5 μ l of an internal length standard (Rox TM Size standard, Applied Biosystems, U.S.A.). The mixture was analyzed with the ABI PRISM-System 3770 automated sequencer (Applied Biosystems, U.S.A.).

Application of Enriched AOB

In order to confirm the efficacy of the enriched AOB on the nitrification efficiency, 1 l of enriched AOB broth was

centrifuged at 7,000 rpm, and the pellet obtained (150 mg as dry weight) was added to 1 l of a synthetic wastewater containing 3,000 mg/l of a normal activated sludge. The synthetic wastewater used in this experiment contained 103 mg/l of $\text{Na}\cdot(\text{CH}_3\text{COO})\cdot 3\text{H}_2\text{O}$, 200 mg/l of peptone, 355.8 mg/l of $(\text{NH}_4)_2\text{SO}_4$, 44 mg/l of KH_2PO_4 , 14 mg/l of KCl, 3 mg/l of yeast extract, and 85 mg/l of Na_2CO_3 [12]. The concentrations of the COD_{Cr} , total nitrogen (TN), and total phosphate (TP) were 270 mg/l, 100 mg/l, and 120 mg/l, respectively. Another experimental set containing the normal activated sludge only was used as a control. To investigate the effects of enriched AOB on nitrification efficiency in a real wastewater, ammonia oxidation tests were carried out as mentioned above using a domestic wastewater obtained from the Bu-kok domestic WTP. One liter of enriched AOB broth was centrifuged at 7,000 rpm, and the pellet

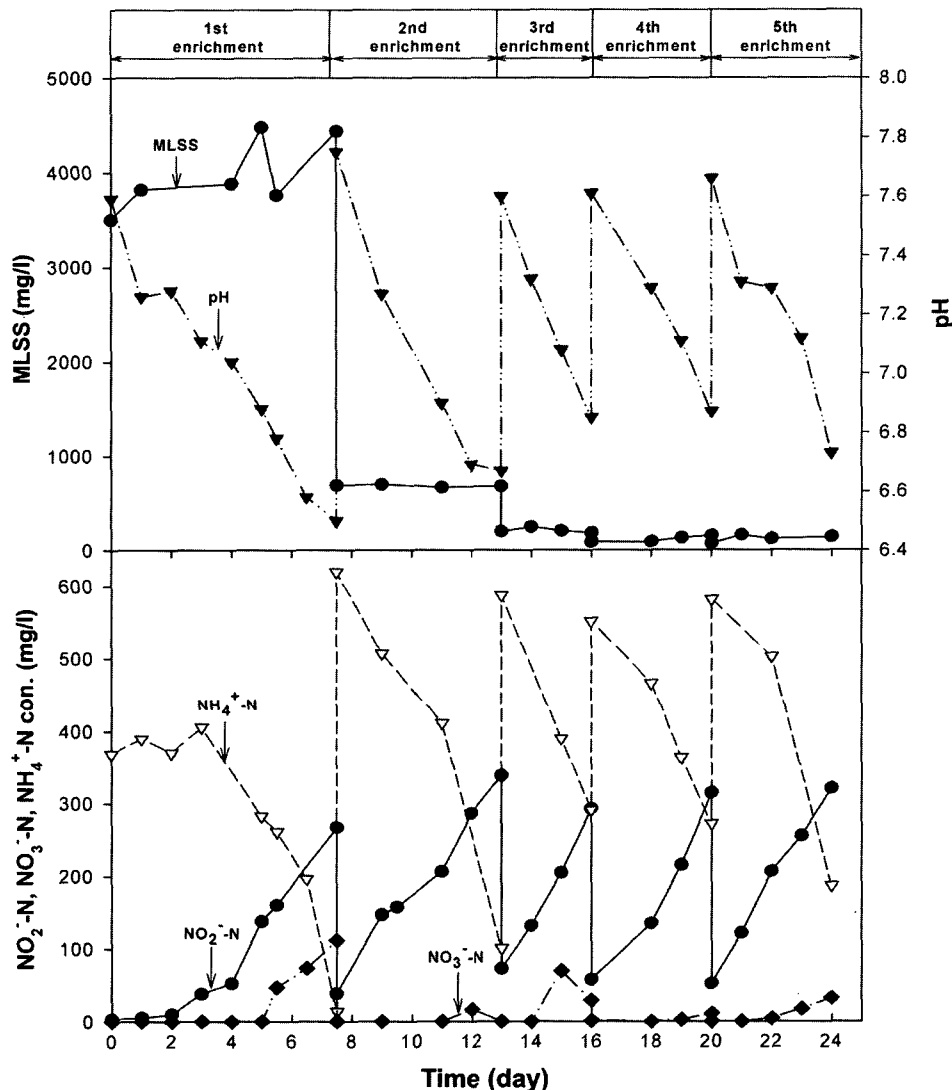


Fig. 1. pH, and MLSS, NO_x^- -N, and NH_4^+ -N concentrations during AOB enrichment in a 2-l flask.

obtained (150 mg) was added to 1 l of Bu-kok wastewater containing 3,000 mg/l of activated sludge of the Bu-kok WTP.

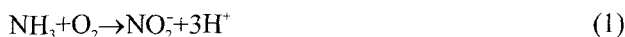
Analysis

Suspended solid (SS) was measured by following the Standard Method [3]. pH was measured with a pH meter (Orion-370). To determine the concentrations of soluble COD (S-COD) and nitrogen compounds, supernatants were obtained by centrifuging sample suspensions at 4,500 rpm for 10 min. COD_{Cr} was measured by the open reflux method [3]. Ammonia and nitrite were measured by the phenate method [3] and colorimetric method [3], respectively. Nitrate was determined by the brucine-sulfanilic acid method [34].

RESULTS AND DISCUSSION

Enrichment of AOB

AOB were enriched from the activated sludge of the Seoman WTP. For enrichment, repeated fed-batch cultivation was carried out in the AOB-selective P-medium by transferring 20% (v/v) of the grown culture into the fresh medium, when the nitrite concentration reached around 300 mg/l, and enrichment was stopped after the fifth cultivation (Fig. 1). Specific oxidation rate of ammonia increased from 0.0171 in the first culture to 0.810 mg NH_4^+-N /mg MLSS-day in the fifth culture. Specific NO_3^- -N production rate also increased from 0.013 in the first culture to 0.617 in the fifth culture (Fig. 2). Decrease of pH was attributed to acid production, accompanied by ammonia oxidation as described in the reaction (1).



The concentrations of AOB and heterotropic bacteria in enriched culture broth are shown in Fig. 3, which was

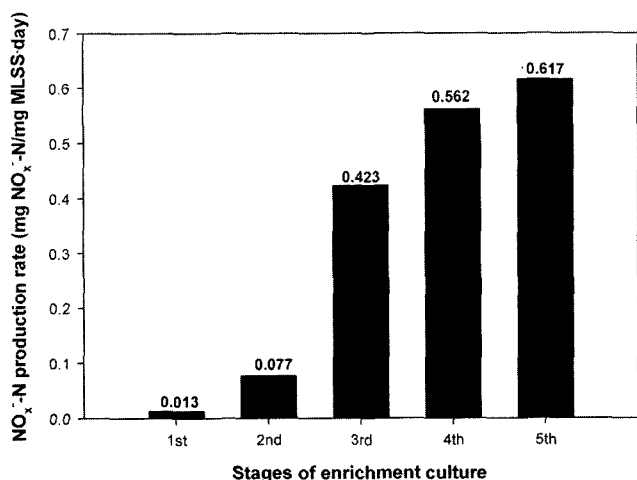


Fig. 2. Specific NO_3^- -N production rate during AOB enrichment in a 2-l flask.

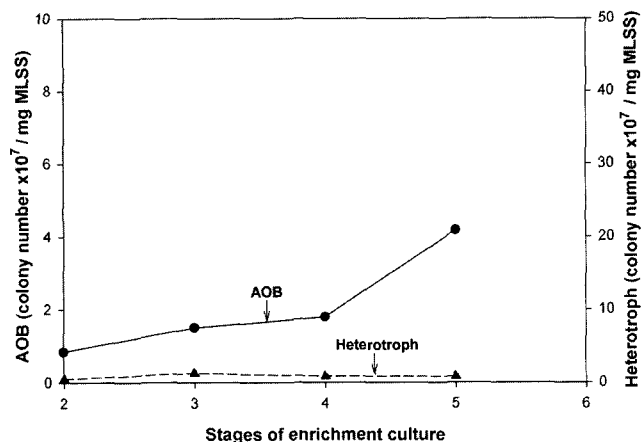


Fig. 3. Cell number changes of heterotrophic bacteria and AOB during AOB enrichment.

obtained by counting the colonies grown on solid media. The number of AOB colonies on HEPES medium increased with the progress of enrichment, whereas the number of heterotrophic bacterial colonies remained unchanged. Percentage of AOB in total colony counting increased due to the enrichment and reached about 83% of entire colonies after the fifth enrichment.

In the present study, FISH analysis was also performed to identify and quantify enriched AOB using an NSO190 probe that can detect AOB of β -subclass of the *Proteobacteria* (Fig. 4). To date, FISH, using oligonucleotide probes targeting signature regions of the 16S rDNA of ammonia oxidizers, has been successfully applied for phylogenetical identification and quantification in natural and engineered ecosystems [4, 6, 35]. In Fig. 4(a), the red color detected by the probe NSO190 (right side) indicates members of AOB of β -subclass of the *Proteobacteria*, and the blue color stained by DAPI (left side) indicates entire cell population of the seed sludge. As shown in Fig. 4(a), the cells detected by the NSO190 probe were sparse compared to the entire cells detected by DAPI staining. This observation was essentially the same as other studies, in that AOB account for less than 10% of the entire cells in the activated sludge [31]. From the first to the fifth stages of enrichment, the amounts of AOB detected by NSO190 continuously increased, as seen in the third [Fig. 4(b)] and the fifth cultures [Fig. 4(c)]. To quantify AOB, a fraction of area occupied by AOB over the total area stained by DAPI was calculated. As shown in Fig. 5, the fraction of AOB increased from about zero to approximately 42%, thus confirming that AOB was successfully enriched by the AOB-selective repeated fed-batch cultivations.

The percentage of cells detected by the NSO190 probe (about 42% in the final stage) was lower than the total cell counting data (about 83%). There are two possible explanations for this discrepancy. First, NSO190 bears a

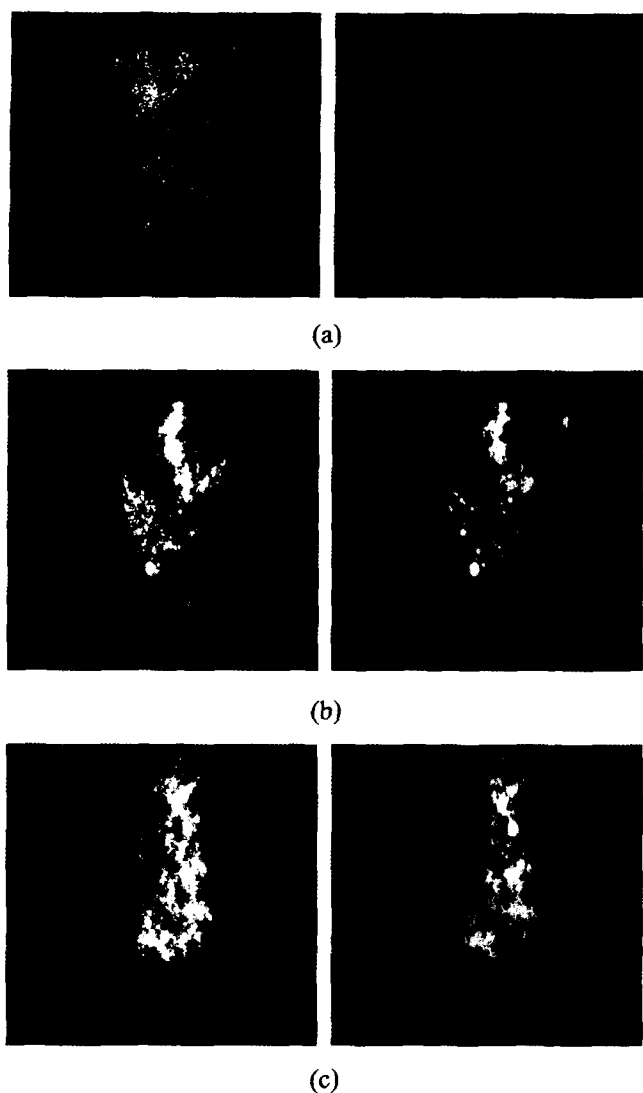


Fig. 4. Visualization of enriched AOB by FISH at each stage of enrichment (using the Cy3-labeled probe NSO190 and DAPI staining).

The left side of each row is the image of DAPI staining and the right side is the image of hybridization with NSO190 probe. (a) Seed sludge, (b) the third culture, (c) fifth culture.

restrictive specificity for AOB of β -*Proteobacteria*. Based on comparative 16S rRNA gene (rDNA) sequence analyses, cultured ammonia-oxidizing bacteria were found to be comprised of two monophyletic groups within the *Proteobacteria*. *Nitrosococcus oceanus* and *N. halophilus* belong to the γ -subclass of the *Proteobacteria*, while the members of the genera *Nitrosomonas*, *Nitrospira*, *Nitrosovibro*, *Nitrosolobus*, as well as *Nitrosococcus mobilis* constitute a closely-related assemblage within the β -subclass of *Proteobacteria* [6, 35]. Since AOB of γ -*Proteobacteria* are not detected by the NSO190 probe, AOB in enriched culture could be underestimated. Secondly, some studies demonstrated that the detection level of a

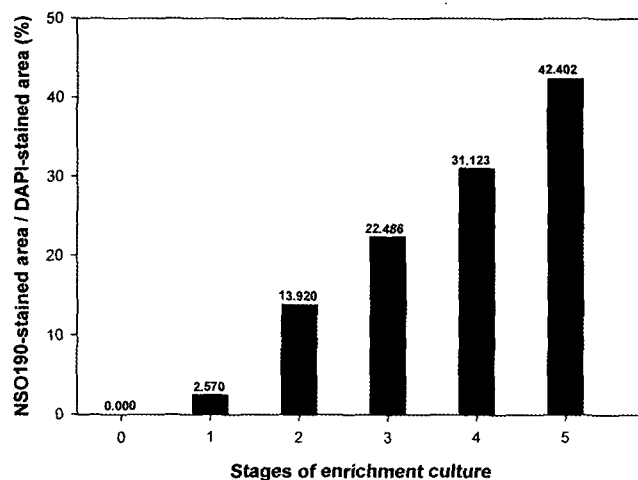


Fig. 5. The changes of fraction of AOB among entire cells. 0 stage indicates the normal activated sludge of the Seonam WTP.

eubacterial probe (EUB338) was between 65% to 85% that of DAPI [2, 32], and this may be due to the fact that DAPI binds to cell debris, including double-stranded RNAs, proteins, and phospholipids, resulting in overestimation of the total cells detected by DAPI staining [18].

T-RFLP Analysis

To identify the changes of microbial community structure during the enrichment of AOB, T-RFLP analysis was conducted. Figure 6 illustrates the T-RFLP patterns of 16S rDNAs amplified from the genomic DNA of enriched cultures, which were digested by *Hae*III. *Hha*I digestion generated patterns similar to those of *Hae*III (data not shown). The horizontal axis indicates the observed size of each T-RF, and the vertical axis indicates the percentage of each T-RF among total T-RFs. It has been shown that 43 and 42 distinct 5'-terminal fragments were generated by *Hha*I and *Hae*III digestions of PCR products of the seed sludge, respectively. It was also observed that the number of T-RFs decreased as enrichment proceeded. Consequently, for the fifth enrichment culture, the numbers of T-RFs for *Hha*I and *Hae*III digestions were 26 and 20, respectively. This decrease can be explained by the fact that AOB became dominant due to selective pressure provided by P-medium. As a result, the diversity of the microbial community in enriched culture broth decreased. To evaluate the changes of diversity and dominance of enriched cultures [8], diversity statistics of each enrichment stage were calculated based on the number and relative abundance of each T-RF (Table 1). From the first to fifth stages of enrichment, it was observed that richness decreased from 43 (*Hha*I) and 42 (*Hae*III) to 26 and 20, respectively. In addition, diversity decreased from 3.996 (*Hha*I) and 4.123 (*Hae*III) to 3.545 and 3.213, respectively. On the other hand, dominance increased from 0.025 (*Hha*I) and 0.028 (*Hae*III) to 0.048 and 0.123,

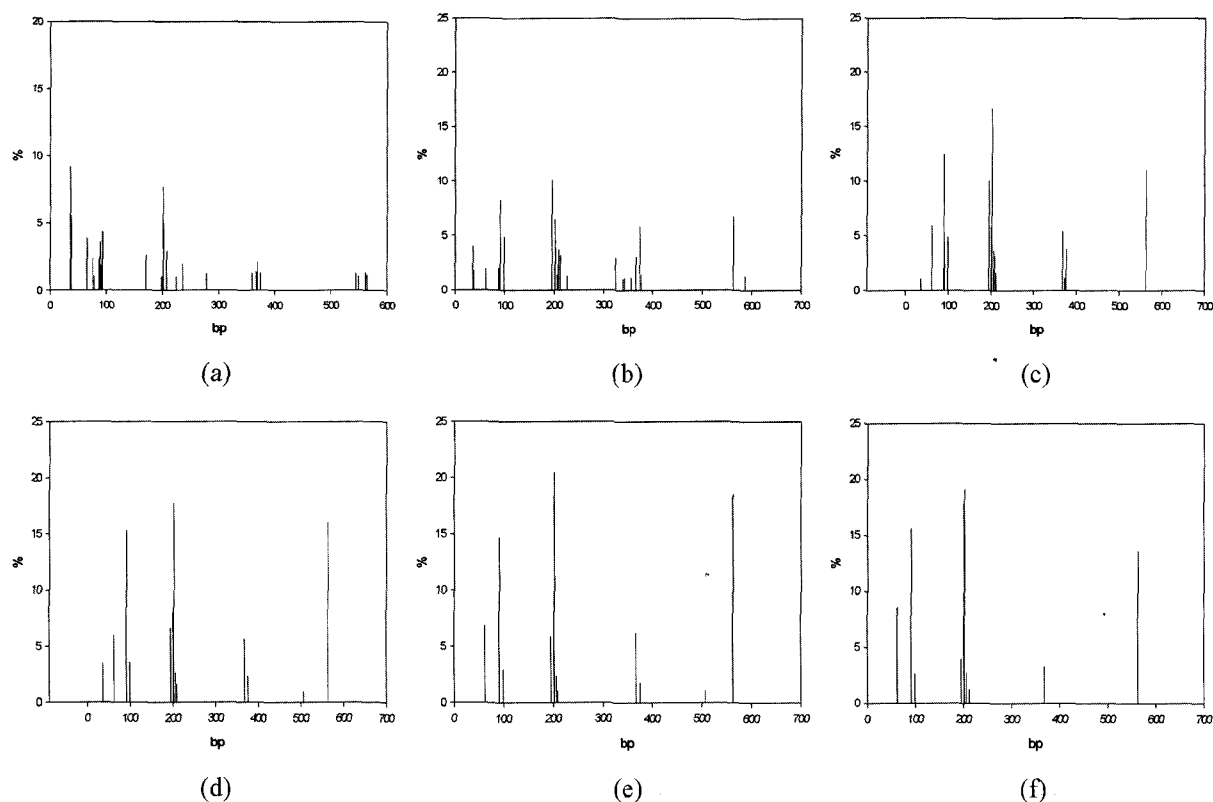


Fig. 6. T-RFLP patterns of 16S rDNAs amplified from the genomic DNAs of enriched culture broth at each fed-batch stage. PCR products were digested with *Hae*III. Graphs of seed sludge (a), the first enrichment (b), the second enrichment (c), the third enrichment (d), the fourth enrichment (e), and the fifth enrichment (f).

respectively. Consequently, richness (S) and diversity (H) decreased as AOB were enriched, while dominance (D) increased.

Application of Enriched AOB

In order to examine the efficacy of the enriched AOB on the nitrification efficiency in wastewaters, batch culture

augmented with enriched AOB was carried out in a synthetic wastewater. As shown in Fig. 7, a significant difference in ammonia oxidation was observed between the control (3,000 mg/l of a normal sludge only) and the augmented culture (3,000 mg/l of a normal sludge+150 mg/l of enriched AOB). Specific nitrification rates of control and the enriched AOB-augmented culture were 0.020 mg NO_x-

Table 1. Diversity indices calculated from T-RFLP patterns during enrichment of AOB.

Parameter	Enzyme	Sample					
		Seed sludge	1st	2nd	3rd	4th	5th
Richness (S)	<i>Hha</i> I	43	32	26	28	26	26
	<i>Hae</i> III	42	34	25	27	26	20
Diversity (H)	<i>Hha</i> I	3.996	3.761	3.535	3.413	3.389	3.545
	<i>Hae</i> III	4.123	4.046	3.795	3.759	3.564	3.213
Evenness (E)	<i>Hha</i> I	0.736	0.743	0.752	0.710	0.721	0.754
	<i>Hae</i> III	0.765	0.765	0.817	0.790	0.758	0.744
Dominance (D)	<i>Hha</i> I	0.025	0.059	0.070	0.065	0.061	0.048
	<i>Hae</i> III	0.028	0.041	0.083	0.103	0.099	0.123

Richness (S), the number of species within a community, was obtained as the total number of distinct T-RFs. The Shannon-Weaver diversity index (H) was calculated as follows: $H = -\sum(p_i)(\log_2 p_i)$, where p_i is the proportion of an individual peak height over the sum of all peak heights. Simpson's index of diversity (D) was calculated as follows: $D = 1 - \sum(p_i)^2$. Evenness (E), the size of species populations within a community, was calculated from the Shannon-Weaver diversity function: $E = H/H_{max}$, where $H_{max} = \log_2(S)$.

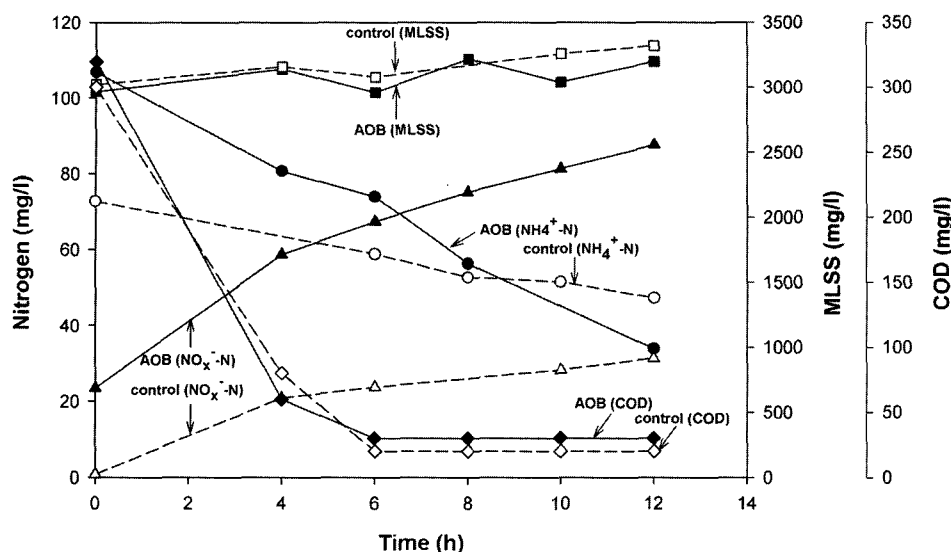


Fig. 7. MLSS, COD, and concentrations of $\text{NH}_4^+\text{-N}$ and $\text{NO}_x^-\text{-N}$ in a synthetic wastewater. High initial concentrations of $\text{NH}_4^+\text{-N}$ and $\text{NO}_x^-\text{-N}$ for AOB were originated from the inoculated broth of the enriched culture.

N/mg MLSS·day and 0.041 mg $\text{NO}_x^-\text{-N}$ /mg MLSS·day, respectively. However, COD removal showed no difference between the control and the augmented culture, as anticipated. The same experiments with a real domestic wastewater, obtained from the Bu-kok domestic WTP, were carried out. As shown in Fig. 8, activated sludge from the Bu-kok wastewater treatment plant showed a low ammonia oxidation rate. However, the nitrification rate was improved from 0.0007 mg $\text{NO}_x^-\text{-N}$ /mg MLSS·day to 0.0918 mg $\text{NO}_x^-\text{-N}$ /mg MLSS·day by adding the enriched AOB, thus indicating that enriched AOB was effective in improving nitrification efficiency even in the real wastewater. Therefore, it is expected that enriched AOB can be used for bioaugmentation of WTP as an emergency measure.

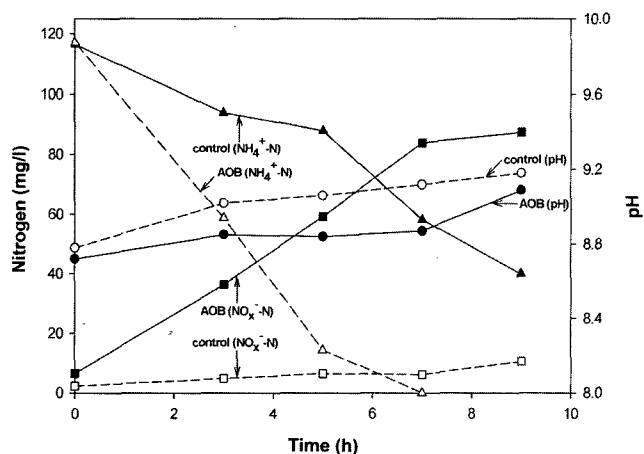


Fig. 8. pH and concentrations of $\text{NH}_4^+\text{-N}$ and $\text{NO}_x^-\text{-N}$ in a domestic wastewater.

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