LITHOAUTOTROPHIC NITROGEN REMOVAL WITH ANAEROBIC GRANULAR SLUDGE AS SEED BIOMASS AND ITS MICROBIAL COMMUNITY

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Abstract : Autotrophic nitrogen removal and its microbial community from a laboratory scale upflow anaerobic sludge bed reactor were characterized with dynamic behavior of nitrogen removal and sequencing result of molecular technique (DNA extraction, PCR and amplification of 16S rDNA), respectively. In the experiment treating inorganic wastewater, the anaerobic granular sludge from a full-scale UASB reactor treating industrial wastewater was inoculated as seed biomass. The operating results revealed that an addition of hydroxylamine would result in lithoautotrophic ammonium oxidation to nitrite/nitrate, and also hydrazine would play an important role for the success of sustainable nitrogen removal process. Total N and ammonium removal of 48% and 92% was observed, corresponding to nitrogen conversion of 0.023 g N/L-d. The reddish brown-colored granular sludge with a diameter of 1~2 mm was observed at the lower part of sludge bed. The microbial characterization suggests that an anoxic ammonium oxidizer and an anoxic denitrifying autotrophic nitrifier contribute mainly to the nitrogen removal in the reactor. The results revealed the feasibility on development of high performance lithoautotrophic nitrogen removal process with its microbial granulation.
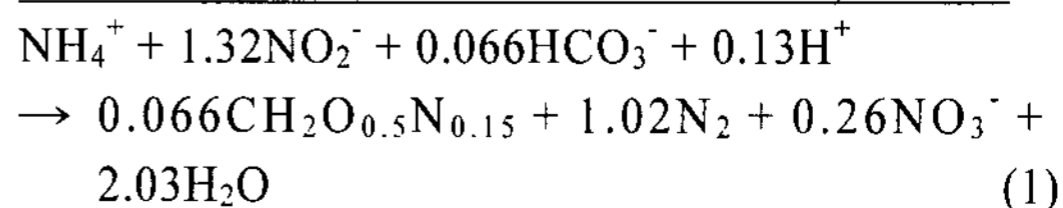
INTRODUCTION

The conventional biological nitrogen removal process consists principally of two sub processes, nitrification and denitrification, resulting in intensive cost requirements due to the need of oxygen sourc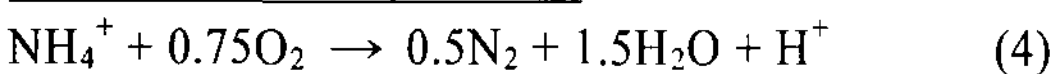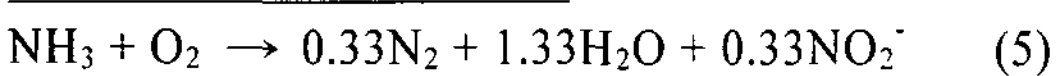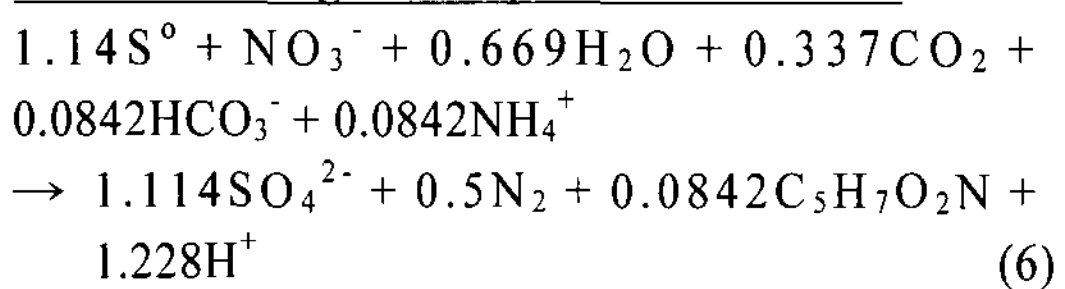e and carbon source during the process. Recently, lithoautotrophic anaerobic ammonium oxidation (Anammox) has been reported to be a powerful piece of technology and that its application for nitrogen removal could lead to quite lower (90% reduction) operational cost.[1] Beside the Anammox, there are some reports on lithoautotrophic organisms contributing to nitrogen removal: anoxic ammonium oxidizer such as *Nitrosomonas eutropha*,[2] denitrifying nitrifier such as *Nitrosomonas europaea*,[3] and sulfur utilizing denitrifier such as *Thiobacillus denitrifican*.[4] Also, Fdz Polance *et al.* described simultaneous sulfate reduction and an Anammox reaction as a possible new process.[5] The lithoautotrophic denitrification utilizing inorganic sulfur compound, hydrogen, ammonia, or nitrite as electron acceptors can be presented as follows:[6-10]

Anammox (anaerobic ammonium oxidation) Process

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+$$
$$\rightarrow 0.066CH_2O_{0.5}N_{0.15} + 1.02N_2 + 0.26NO_3^- + 2.03H_2O \quad (1)$$

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Canon (completely autotrophic nitrogen removal over nitrite) Process

$$NH_4^+ + 0.85O_2$$
$$\rightarrow 0.44N_2 + 0.11NO_3^- + 1.43H_2O + 0.14H^+ \quad (2)$$
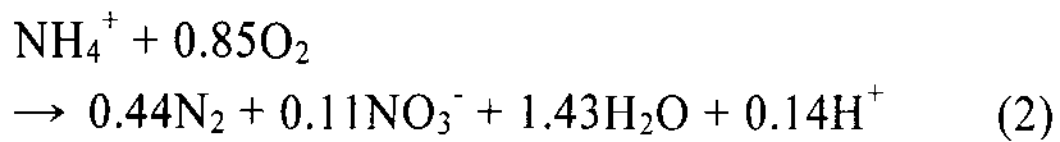
NOx Process (denitrification activity of _Nitrosomonas_-like bacteria with NOx supply)

$$3NH_4^+ + 3O_2 + 2[H] \rightarrow 1.5N_2 + 3[H] + 6H_2O \quad (3)$$
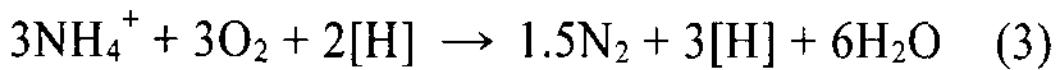
OLAND (oxygen-limited autotrophic nitrification and denitrification) Process

$$NH_4^+ + 0.75O_2 \rightarrow 0.5N_2 + 1.5H_2O + H^+ \quad (4)$$

Aerobic Deammonification

$$NH_3 + O_2 \rightarrow 0.33N_2 + 1.33H_2O + 0.33NO_2^- \quad (5)$$

Sulfur-oxidizing autotrophic denitrification

$$1.14S^\circ + NO_3^- + 0.669H_2O + 0.337CO_2 +$$
$$0.0842HCO_3^- + 0.0842NH_4^+$$
$$\rightarrow 1.114SO_4^{2-} + 0.5N_2 + 0.0842C_5H_7O_2N +$$
$$1.228H^+ \quad (6)$$

Even though there are still many unknown reactions, it appears that these organisms can contribute to sustainable nitrogen removal under anaerobic/or anoxic conditions, depending on the type of electron donor/acceptor in the substrate. Because these lithoautotrophic organisms, as well as the Anammox bacteria, are characterized by a low maximum growth rate, a reactor with high biomass retention, such as immobilization or granulation process (biofilm or UASB) is required. However, the granular sludge reactor for higher nitrogen loading application has a disadvantage of a longer startup time.[11]

The research focuses on the development of an anaerobic lithoautotrophic nitrogen removal process and microbial sludge granulation. The sustainable anaerobic nitrogen removal was investigated by the operation of a lab-scale anaerobic granular sludge bed reactor treating inorganic wastewater. The effect of hydroxylamine and hydrazine as the Anammox intermediates was also estimated.

MATERIALS AND METHODS

Laboratory Reactor Operations

A laboratory scale 1.5L UASB glass reactor (internal diameter 6 cm) with a 0.5 L settling tank was operated at mesophilic (35°C) condition. The reactor was operated using a semi continuous feeding system. The substrate was fed four times daily in a fill-and-draw mode. During the overall operating period, the reactor constantly operated with a hydraulic retention time (HRT) of 5 days. The settled sludge in the settler was recycled into the bottom of the UASB reactor, resulting in the upflow rate of about 1Q. 0.7L of granular sludge (18.6 g VS/L and 65% VS/TS) was inoculated as seed biomass. Originally, the granular sludge was collected from a full-scale UASB reactor treating brewery wastewater.

In the meantime, inorganic synthetic nitrogenous wastewater was used as the substrate. In the initial experiment (Reactor A), the reactor was operated with a high nitrogen concentration (1,000 mg NH_4-N/L and 1,300 mg NO_2-N/L). The influent of this reactor switched to much lower influent nitrogen concentration (100 mg NH_4-N and 50 mg NO_2-N/L) in the second experiment (Reactor B), in order to avoid the nitrite inhibition in the Anammox reaction. To induce the reaction of ammonium oxidation, a little amount of hydroxylamine and hydrazine were added and its effects were estimated. To avoid potential deficiencies in the trace element supply, the mineral medium solution (20 mL per L influent) was added to the synthetic substrate. The medium solution for the enhancement of microbial granulation contained KH_2PO_4 13.61 g/L, NH_4Cl 49.20 g/L, $CaCl_4$ 4.44 g/L, $MgCl_2 \cdot 6H_2O$ 8.13 g/L and 10 mL/L of a trace element solution.[26]

Analysis

The nitrogen (ammonium, nitrite and nitrate) concentration was measured colorimetrically by Standard Methods.[12] In the operation of the reactor, the pH (Orion 720, USA), bicarbonate alkalinity (BA) and gas production (Wet-test gas meter, Sinagawa Model W-NK-0.5A, Japan) were monitored daily. A gas chromatograph (Tremetrics Model 9000, USA) with a TCD detector and a Hayesap Q (80/100) column was utilized to measure gas composition (N_2O, N_2, CO_2, NH_3 and CH_4). The temperature for the column was kept at 35°C, 120°C for the injector, and 120°C for the detector. The helium carrier gas had a flow rate

of 30 mL/min. Data integration was accomplished using a Varian 4270 Integrator. The sludge granulation during the operation was examined by microscopic analysis (Olympus BX60F5, Japan) and by settleability assessment.[13] Hydroxylamine and hydrazine were measured as described by Frear and Burrell[14] and Watt and Chrisp,[15] respectively.

Microbial Community Analysis

Genomic DNA of the isolates was purified by the procedure of Hallin and Lindgren.[16] Genomic DNA extractions from the sludge samples were performed using a modified method of Lee *et al.*[17] The quality of extracted DNA was analyzed by applying the standard agarose gel electrophoresis. DNA concentrations were measured by absorbance level at 260 nm. PCR and amplification of 16S rDNA from chromosomal DNA were carried out in a DNA thermal cycle (Model480; Perkin-Eelmer, Norwalk, Conn. USA) with universal bacterial primers, 27F (AGAGTTTGATCMTGCTCAG) and 1492R (GGTTACCTTTGTTACGACTT). After an initial heating to 94°C for 4 min, 30 cycles consisting of 94°C (1 min), 57°C (1 min), and 72°C (2 min) with a final 10 min extension period at 72°C were performed. The PCR product was purified with a QIAquick PCR purification kit (Qiagen, Germany), cloned into pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer's instruction (Promega, Madison, WI, USA). Clones containing appropriate-sized inserts were identified by agarose gel electrophoresis of PCR products obtained from host lysates by PCR with primers complementary to the vector at sites flanking the insertion site. Unique clones were identified by restriction fragment length polymorphism (RFLP) analysis of the insert.[18] The clones representing the dominant restriction fragment groups were selected from a reactor, respectively. These clones were sequenced by PCR cycling sequencing of quick -prepped recombinant plasmids by using cycling was performed with ABI PRISM BigDye Terminator cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The 16S rRNA partial

sequences of sludge samples obtained in this study are available from the EMBL nucleotide sequence database under accession no.AY609339 to AY609349.

RESULTS AND DISCUSSION

Autotrophic nitrogen removal

Figure 1 represents the operating results of Reactor A, which operated with constant nitrogen loading of 0.5 g T-N/L-day. At the end of phase I (about Day 40), the gradual ammonium removal and nitrite accumulation were observed. The reactor discontinued on Day 43. However, 20 days later (Day 63 - phase II), it could be observed somewhat of a decrease in both ammonium and nitrite, resulting in a nitrogen conversion and removal of 0.02 g N/L-d and 18%, respectively. Based on the positive result of the addition of the Anammox intermediates,[19] a trace amount (3.2 mg/L) of hydroxylamine was injected on Day 63. As shown in the Figure 1, this resulted in a faster decrease of ammonium, whereas nitrite and nitrate was increased at the same rate. Contrary to the results of the literature, a net nitrogen conversion in the end of phase II was almost zero. This means that lithoautotrophic ammonium oxidation to nitrite /nitrate could occur by an addition of hydroxylamine, as described by Böttcher and Koops.[20] Even though the oxygen source under an anoxic condition is unknown, nitrite, NO_2, and NO could theoretically have been used as the oxygen source.[21]
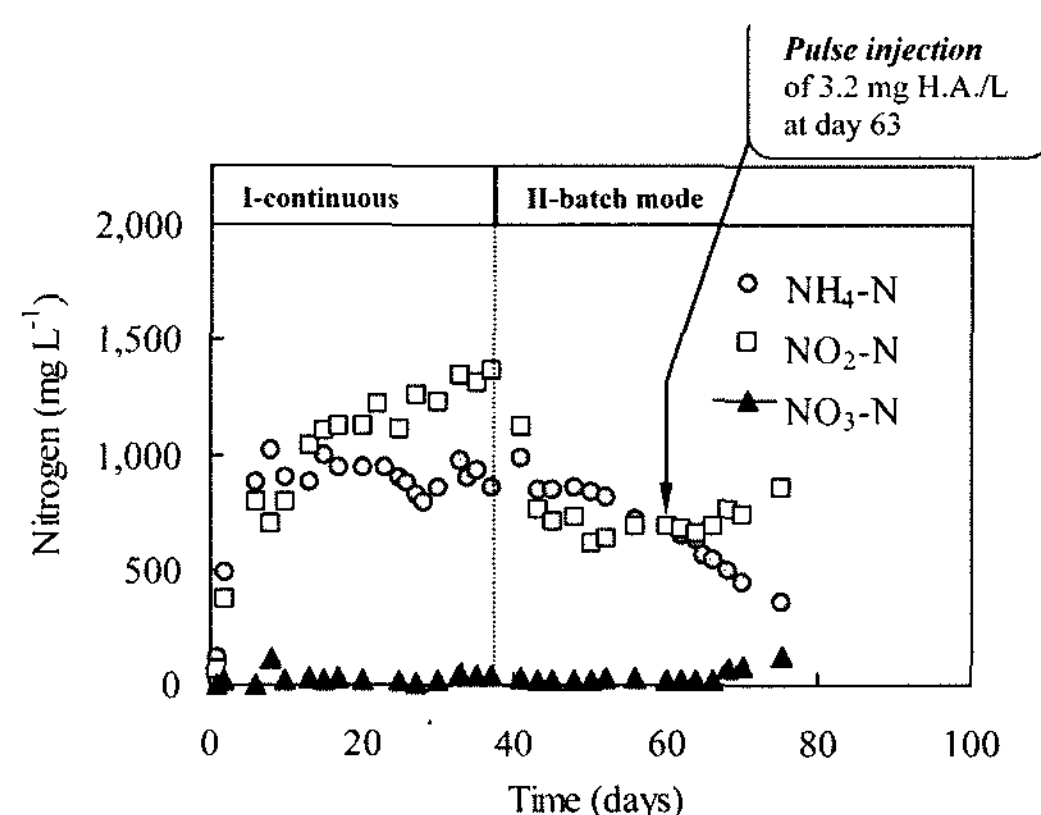

Figure 1. Performance of Reactor A (Influent : 1 g NH_4-N L^{-1}, 1.3 g NO_4-N L^{-1}).

The operating condition of this reactor switched to much lower influent nitrogen concentration (100 mg NH_4-N and 50 mg NO_2-N/L- designated to Reactor B). This change was based on the result that nitrite (> 0.1 g NO_2-N/L) could inhibit the Anammox process.[19] Also, hydroxylamine and hydrazine were constantly added to the influent for Reactor B. As shown in Figure 2, the ammonium over 90% was removed during the overall operating period, and the nitrite showed higher concentration than that of the influent. The results show a similar trend to Reactor A. However, this reactor reveals that the nitrite reduction occurred gradually, meaning that microbial acclimation occurred as well. Particularly, the nitrite and nitrate decreased faster at the end of each operating mode, when the Anammox intermediates (10 mg/L of hydroxylamine and 9 mg/L of hydrazine) were added.
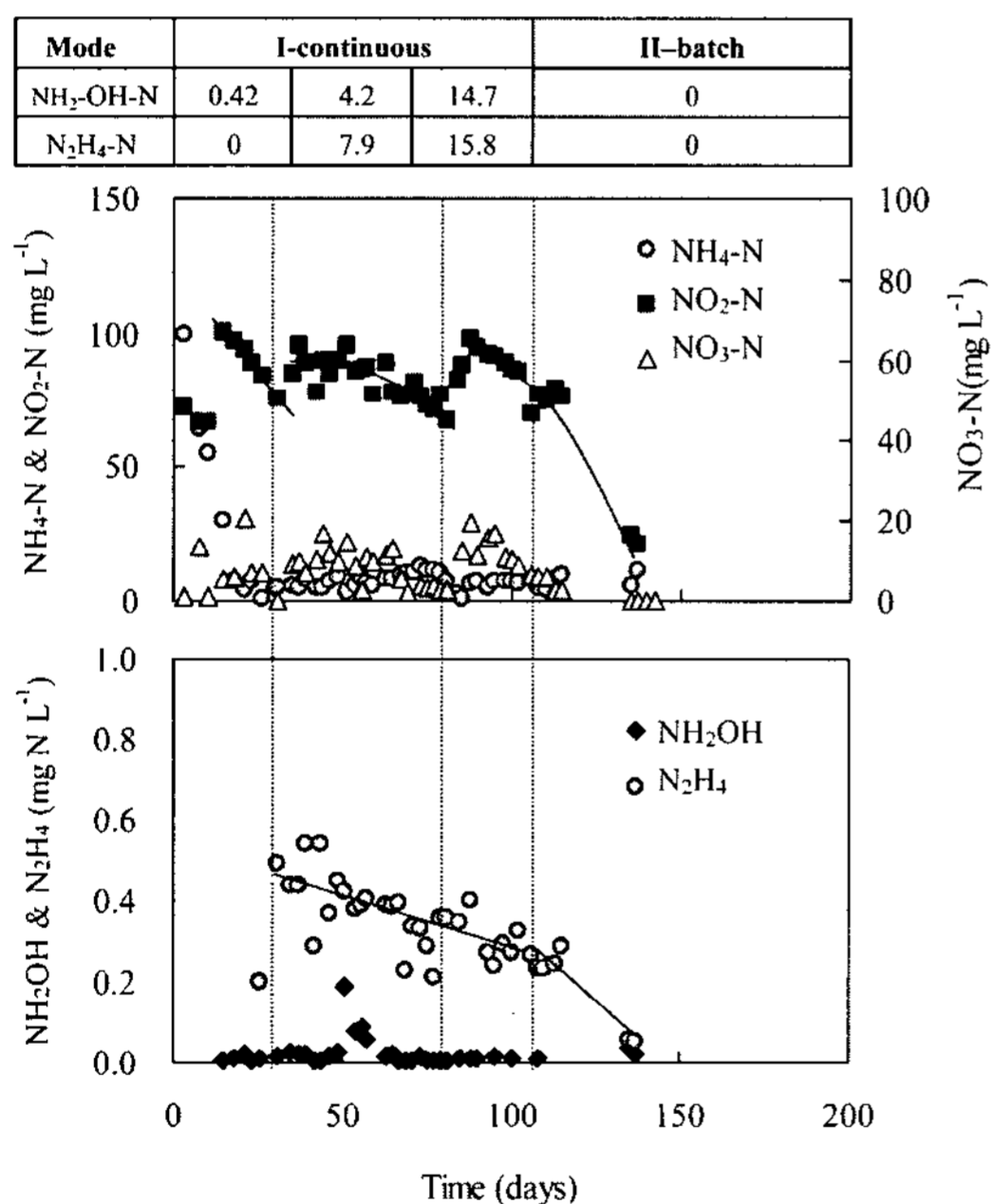
Mode	I-continuous			II–batch
NH_2-OH-N	0.42	4.2	14.7	0
N_2H_4-N	0	7.9	15.8	0

Figure 2. Performance of Reactor B (Influent : 0.1 g NH_4-N L^{-1}, 0.05 g NO_4-N L^{-1}).

When the addition rate of the intermediates increased, the nitrite and nitrate temporarily increased, but was removed faster in phase I. The concentration of the Anammox intermediates was very low compared with those of the influent, and the hydrazine concentration gradually decreased

according to the operation time. Particularly the nitrite and hydrazine was dramatically removed during the batch operation (phase II). These results reveal that the Anammox intermediates (particularly, hydrazine) are important parameters for success of the anaerobic nitrogen removal process. Table 1 represents the summarized results in each phase. The nitrogen loading and conversion rate were 0.03 g/N-d and 0.01~0.02 g N/L-d, respectively. The total nitrogen removal was 33~63.4%.

Table 1. Operating results of Reactor B after start up

Mode	I-Continuous			II-Batch
	day 35	day 81	day 108	108 ~137
Influent (mg L^{-1})				
NH_4-N	100	100	100	100
NO_2-N	50	50	50	50
NH_2OH-N	0.42	4.2	14.7	0
N_2H_4-N	0	7.9	15.8	0
Effluent (mg L^{-1})				
NH_4-N	6	7	5	11
NO_2-N	85	68	77	21
NO_3-N	9.3	2.5	6	0.2
NH_2OH-N	0.03	0.004	0.01	0.02
N_2H_4-N	0.44	0.36	0.23	0.05
BA	2,660	1,950	1,558	2,770
TIN removal (%)	33.0	52.0	51.1	63.4
N conversion rate	0.01	0.02	0.02	0.002

Note: TIN, total inorganic nitrogen; N conversion rate, g N/L-d; BA, bicarbonate alkalinity (mg L^{-1} as $CaCO_3$)

Figure 3 and Table 2 represent the results of the secondary operation for Reactor B. As seen in Figure 3, the results clearly show that ammonium oxidation and nitrite accumulation occurred between Day 130 to 175, resulting in a total N and ammonium removal of 48% and 92%, respectively. In addition, double ammonium loading resulted in a rapid nitrite reduction for the next 10 days. After Day 190, the reactor showed a stable performance, representing that the total N and ammonium removal was 30% and 57%, respectively. However, the nitrogen conversion rate was similar throughout the entire period (0.018~0.023 g N/L-d).
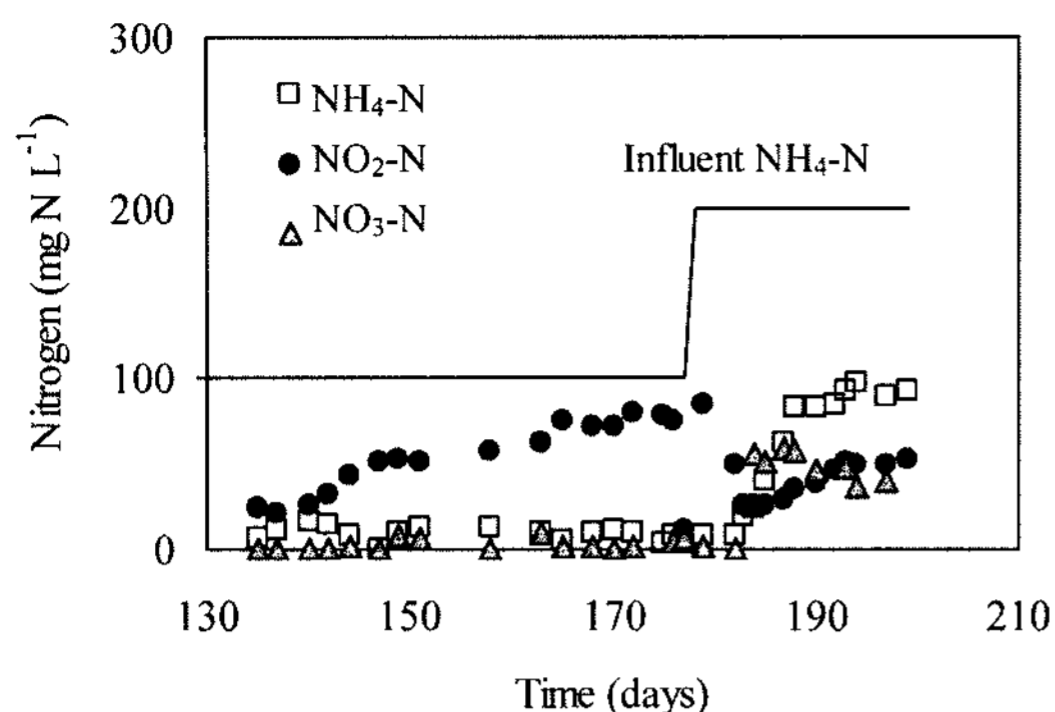
Figure 3. Performance of Reactor B during second operation.

Table 2. Operating result of Reactor B during second operation

Items	Period (day)	
	150~175	190~200
Influent NH$_4$-N (mg L^{-1})	100	200
Influent NO$_2$-N (mg L^{-1})	50	50
Effluent NH$_4$-N (mg L^{-1})	8±4	86±11
Effluent NO$_2$-N (mg L^{-1})	75±11	44±9
Effluent NO$_3$-N (mg L^{-1})	5±6	47±8
T-N loading (g L^{-1} d^{-1})	0.03	0.05
T-N conversion (g L^{-1} d^{-1})	0.02±0.002	0.02±0.003
NH$_4$-N conversion (g L^{-1} d^{-1})	0.018	0.023
T-N removal (%)	48±6	30±5
NH$_4$-N removal (%)	92±5	57±7

According to recent research, there are some results that the ammonium removal at anaerobic (or anoxic) process treating inorganic wastewater, can be performed by anoxic ammonium oxidizer such as *Nitrosomonas eutropha*,[2] lithoautotrophic denitrifying nitrifier such as *Nitrosomonas europaea*,[3] Anammox organism with sulfate reduction bacteria,[5] as well as Anammox organism such as *Brocadia anammoxidans*.[1] The performance of the reactor in this study showed as that of the Canon process.[21] The operating results indicated that the ammonium removal in this reactor would be mainly performed by three-types of nitrogen removal organisms, thus anoxic ammonium oxidizer, denitrifying autotrophic nitrifier and/or Anammox organism, due to substrate composition (thus, absence of gaseous NO$_2$ or sulfate as electron donor) based on the results of the literature described above.

Microbial Granulation and Settleability

During the operation of the reactor, the colour of the sludge bed biomass changed from black to reddish brown, which was accompanied by an increase in the content of cytochrome.[22] From the microscopic analysis at the end of the experiment, the granular sludge with a diameter of 1~2 mm was observed at the lower part of sludge bed, as shown in Figure 4. The physical characteristics of granular sludge can be estimated by using the settleability test,[13] as shown in Figure 5. In Figure 5, a profile with higher upflow velocity indicates better settleability of granular sludge. The settleability assessment of the granular sludge revealed that the granular sludge had a good settleability even though it was worse than that of the initial seed granular sludge.
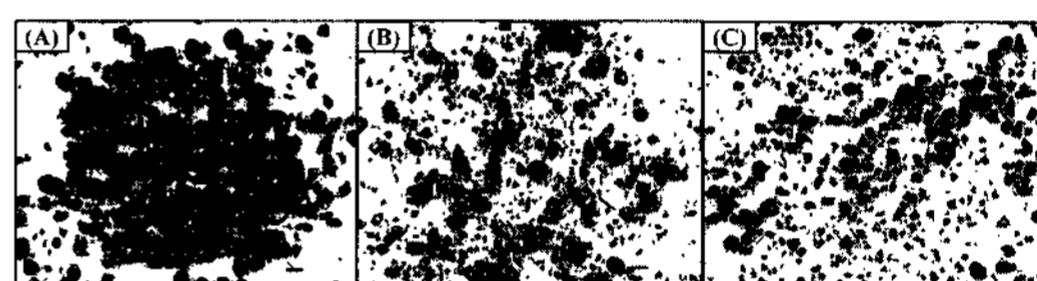

Figure 4. Micrograph of autotrophic nitrogen removal granular sludge (bar=5mm; A, seed granular sludge; B, Day 150; C, Day 250).
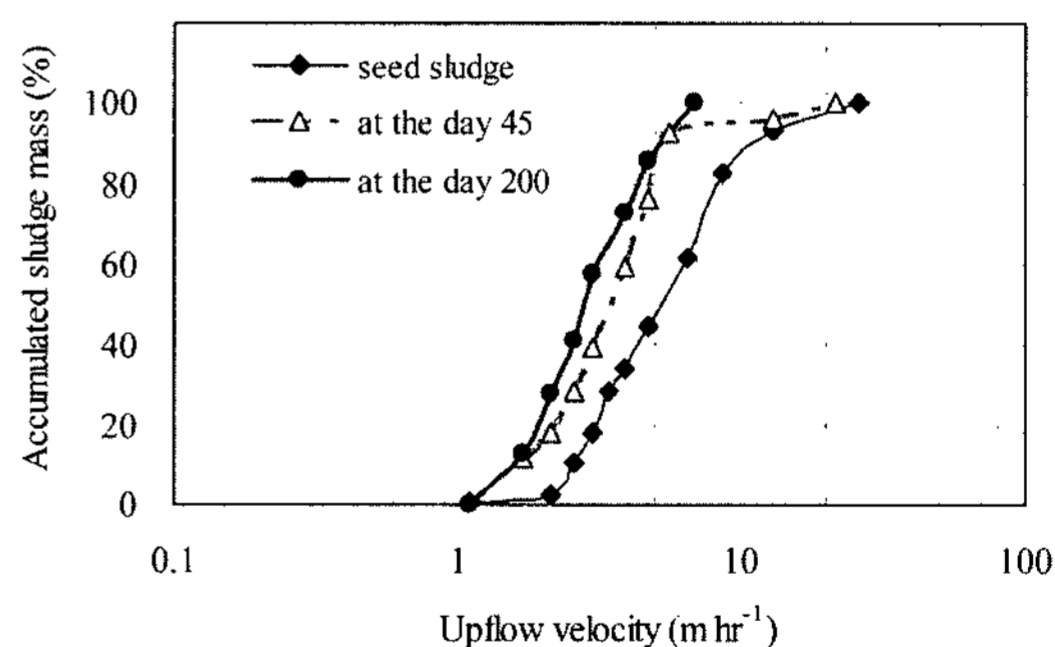

Figure 5. Settleability profiles for granular sludge.

Microbial community analysis

To more fully characterize the bacterial community, 16S rDNA directly extracted from the reactors was cloned and analyzed. Cloned 16S rDNA was analyzed by restriction fragment length polymorphism (RFLP) analysis. Total 129 clones were analyzed and RFLP patterns which appeared more than twice classified into eleven dominants (Table 3 and Figure 6). K2 group

Table 3. Summary of phylogenetic diversity of the domain clones based 16S rDNA sequences identified by BLAST database in the reactor

Clones of Reactor	16S rRNA gene sequencing		
	Closest match	No. of nucleotides compared	% Similarity with Closest match
K1 (3)	*Uncultured bacterium* SHA-25 16S rRNA gene (AJ249101)	844	96
K2 (32)	*Uncultured eubacterium* partial 16S rRNA gene (AJ412669)	877	96
K3 (9)	*Uncultured sludge bacterium* S16 16S rRNA gene (AF234759)	580	95
K4 (8)	*Uncultured eubacterium* partial 16S rRNA gene (AJ412669)	855	97
K5 (13)	*Nitrosomonas eutropha* (AY123795)	794	96
K6 (10)	*Nitrosomonas europaea* (ATCC 19718, BX321856)	767	97
K7 (13)	*Uncultured bacterium* partial 16S rRNA gene (AJ504594)	467	99
K8 (8)	*Uncultured bacterium clone* 265ds10 16S rRNA gene (AY212715)	757	92
K9 (3)	*Uncultured bacterium* mle1-33 (AF280861)	811	93
K10 (5)	*Curacaobacter baltica* OS 140 16S rRNA gene (AJ002006)	859	96
K11 (3)	*Uncultured bacterium* SJA-61 (AJ009469)	909	96

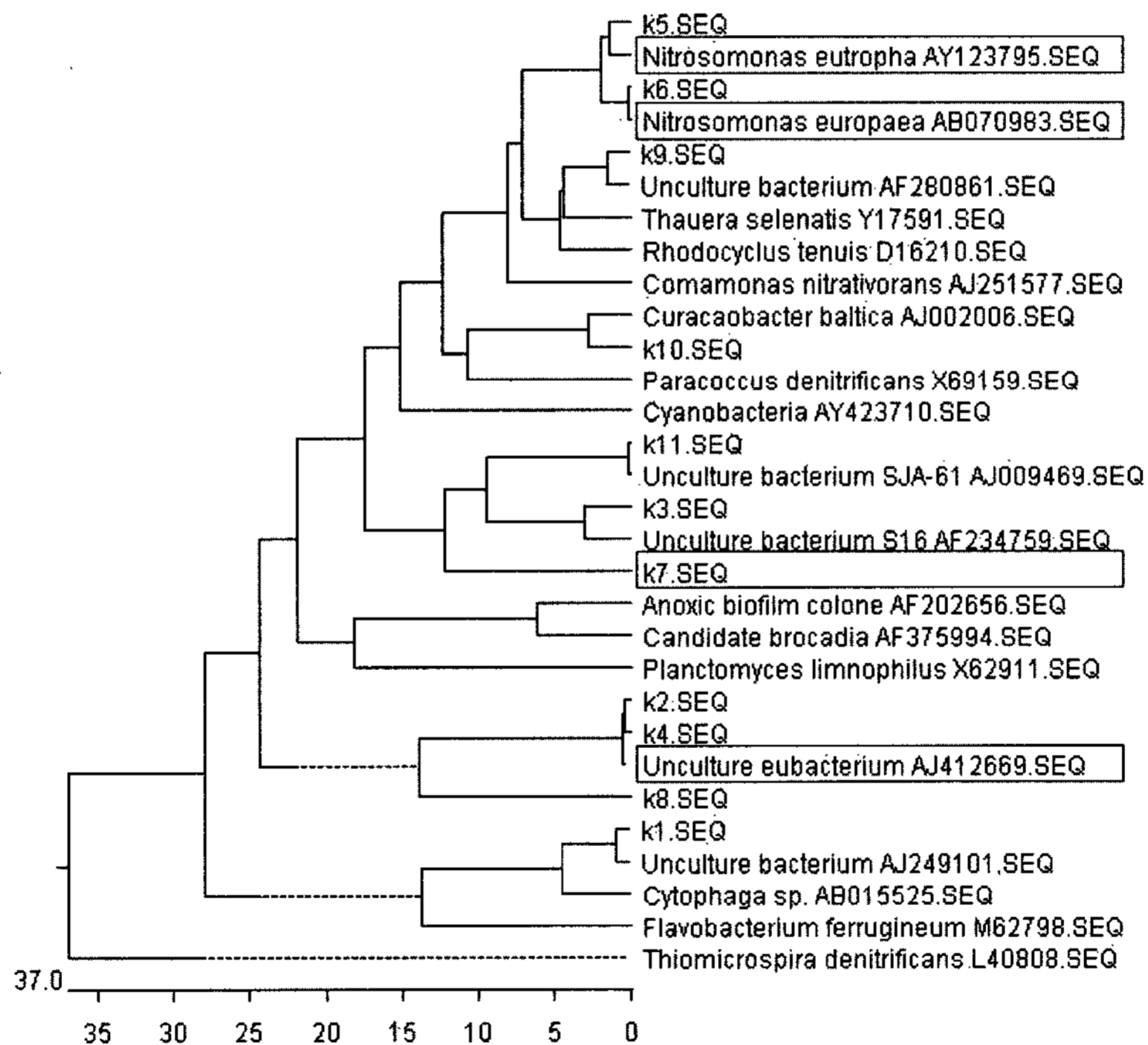* The parenthesis is the number of colonies involved in same group

Figure 6. Phylogenetic tree of the domain clones based 16S rDNA sequences.

was the dominant clones from the clone library. The K2 and K4 groups could be matched to a known sequence of uncultured eubacterium (AJ 412669). Etchebehere *et al.* reported that the denitrifying bacteria, isolated from the anoxic reactor of a combined system treating landfill leachate, belonging to the genera *Thaura, Acido-* *vorax,* and *Alcaligenes.* K5 and K6 groups were most closely matched *Nitrosomonas eutropha* (AY 123795) and *Nitrosomonas europaea* (BX321856), respectively.[23] The other dominant groups of K3 and K4 were related to nitrogen removal. K9 and K10 were characterized of microbes with thermophilic environment[24] and deep sea in the

sediment environment,[25] respectively. All groups as well as K5 and K6 have an ability to remove nitrogen. The microbial identification demonstrated that the anoxic ammonium oxidizer and denitrifying autotrophic nitrifier contributed mainly to the nitrogen removal in the reactor.

CONCLUSIONS

In the operation of a laboratory scale anaerobic nitrogen removal reactor with synthetic wastewater and anaerobic granular sludge as seed biomass, the supplementation of the Anammox intermediates (hydroxylamine and hydrazine) resulted in stimulation of nitrogen elimination activity to nitrogen gas. Particularly, it revealed that the hydrazine would play an important role in anoxic ammonium oxidation process. At the end of the experiment, total N and ammonium removal of 48% and 92% was observed, corresponding to nitrogen conversion of 0.023 g N/L-d.

The colour of the sludge bed biomass changed from black to reddish brown. From the microscopic analysis, the granular sludge with a diameter of 1~2 mm was observed at the lower part of sludge bed. The settleability assessment of the granular sludge revealed that the granular sludge had a good settleability. The results of microbial characterization demonstrated that the anoxic ammonium oxidizer and denitrifying autotrophic nitrifier contributed mainly to the nitrogen removal in the reactor.

ACKNOWLEDGEMENT

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