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Enrichment and characterization of a bacteria consortium capable of heterotrophic nitrification and aerobic denitrification at low temperature

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Abstract

Nitrogen removal in wastewater treatment plants is usually severely inhibited under cold temperature. The present study proposes bioaugmentation using psychrotolerant heterotrophic nitrification-aerobic denitrification consortium to enhance nitrogen removal at low temperature. A functional consortium has been successfully enriched by stepped increase in DO concentration. Using this consortium, the specific removal rates of ammonia and nitrate at 10 ^{o}C reached as high as 3.1 mg-N/(g-SS·h) and 9.6 mg-N/(g-SS·h) respectively. PCR-DGGE and clone library analysis both indicated a significant reduction in bacterial diversity during enrichment. Phylogenetic analysis based on nearly full-length 16S rRNA genes showed that *Alphaproteobacteria*, *Deltaproteobacteria* and particularly *Bacteroidetes* declined while *Gammaproteobacteria* (all clustered into *Pseudomonas* sp.) and *Betaproteobacteria* (mainly *Rhodoferax ferrireducens*) became dominant in the enriched consortium. It is likely that *Pseudomonas* spp. played a major role in nitrification and denitrification, while *Rhodoferax ferrireducens* and its relatives utilized nitrate as both electron acceptor and nitrogen source.

Keywords: low temperature; enrichment; heterotrophic nitrification; aerobic denitrification; 16S rRNA gene;

1. Introduction

During the last few decades, the importance of nutrient removal has increased as a result of the necessity to avoid eutrophication of receiving waters. The most common, efficient, and cost-effective way to remove nitrogen from municipal and industrial wastewaters is biological treatment which normally involves autotrophic nitrification and anoxic denitrification. However, the implementation of biological technology is very challenging in the winter months due to high sensitivity of autotrophic nitrifiers to low temperature. Randall and Buth (1984) demonstrated that both nitrite and nitrate formation were strongly inhibited at temperatures of 10 ^{o}C or less. Studies of various wastewater treatment systems employing nitrification have reported that failures occurred during the winter (Ilies and Mavinic, 2001; Kim et al., 2006). Additionally, the reliability of denitrification on the temperature was increased when temperature fell below 10ºC (Carrera et al., 2003).

Recent studies have highlighted the existence of bacteria which are both heterotrophic nitrifier and aerobic denitrifier, such as *Alcaligenes faecalis* (Joo et al., 2005), *Acinetobacter calcoaceticus* (Zhao et al., 2010), *Pseudomonas stutzeri* (Zhang et al., 2011), and *Bacillus subtilis* (Yang et al., 2011). Under aerobic conditions, these heterotrophic microorganisms are able to oxidize ammonia to hydroxylamine, nitrite, or nitrate and immediately denitrify these products to N_2O and/or N_2. As a result, ammonium compounds can be converted to gaseous products in a single aeration phase. Due to their useful characteristics, heterotrophic nitrifying-aerobic denitrifying

microorganisms have already been applied in the bioaugmentation treatment of nitrogenous wastewater (Bouchez et al., 2009; Joo et al., 2006; Patureau et al., 2001).

Under cold temperatures conditions, bioaugmentation using heterotrophic nitrifying-aerobic denitrifying microorganisms holds obvious advantages over conventional autotrophic nitrification and anoxic denitrification: (1) heterotrophic nitrifier possess better tolerance to low temperature compared with autotrophic nitrifier (Eckenfelder, 2000); (2) hardly any nitrite/nitrate would be present in the effluent of aerobic tank because nitration products are denitrified simultaneously; and (3) heterotrophs grow faster and so should easily be retained in the treatment system. However, such studies have rarely been conducted to date.

The present paper describes enrichment of an aerobic denitrifying consortium and further investigation into its capability of both heterotrophic nitrification and aerobic denitrification at 10 ^{o}C. Microbial community structure analysis was also conducted to understand better the community alternation during enrichment and the functional group in the enriched consortium. This study provides a basis for further applications of the present aerobic consortium in cold bioaugmentation for nitrogen removal.

2. Materials and Methods

2.1. Enrichment of the consortium

Enrichment of aerobic denitrifying organisms was carried out in two identical 1 l reactors (R1 and R2) using inorganic salts medium with the fill-and-draw cultivation .

The seed sludge was taken from the secondary sedimentation tank of a local municipal wastewater treatment plant in Beijing on a cold winter's day (Mid- January). The inorganic salts medium used contains (per liter) : 2.73 g sodium acetate; 0.306 g NH_4Cl; 1 g KNO_3; 0.15 g KH_2PO_4; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.006 g $FeSO_4 \cdot 7H_2O$; pH 7.0-7.3. Both reactors were initially inoculated with the original seed sludge and thereby acclimation commenced. Both systems were operated at a cycle of 24 h, including instant feeding, 20 h of aerobic reaction, 0.5 h of sludge settling, 10 min of effluent discharge and idling. The volumetric exchange ratio was almost 100%, resulting in a hydraulic retention time (HRT) of approximate 24 h and the nitrate loading rate of 0.14 kg/(L·d). The sludge retention time (SRT) was calculated to be around 10 d. Aeration was supplied by an adjustable air pump and porous stone that provided fine bubble aeration. In each cycle, constant aeration was supplied and the dissolved oxygen (DO) concentration fluctuated in a definitive range during most of the aerobic period (only increased obviously when the growth matrix were completely depleted, typically in the last hour of the aerobic period). DO concentrations were measured periodically in each cycle and the average value was used to represent the DO level of the whole day. In different cycles, DO level was controlled by changing air flow rate with the help of a gas flow meter. For R1, high amount of aeration was applied throughout the enrichment process to ensure the DO concentration at ≈ 6.0 mg/L (ranged 4.9-6.7 mg/L). In R2, the aeration amount was raised in two steps to ensure DO concentration increasing from <0.5 mg/L to ≈3 mg/L (ranged 2.2-3.8 mg/L) and

then ≈6.0 mg/L (ranged 5.2-6.9 mg/L). The pH in both systems, which was not controlled during the whole experiment, typically increased from 7.0 to 8.6 over the cycle. The cultivation temperature was decreased from 15 ^{o}C to 10 ^{o}C on the seventh day.

2.2. Nitrogen removal performance

The medium used for performance evaluation contained 2.73 g/L CH_3COONa, 0.15 g/L KH_2PO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, and 0.001 g/L $FeSO_4 \cdot 7H_2O$. Furthermore, 0.577 g/L nitrate was supplied as the sole nitrogen source when nitrate removal performance was investigated; similarly, 0.306 g/L ammonia was used as the sole nitrogen source for assessing the ammonia removal performance. 100 mL of fresh test medium was placed in a 250 mL shake flask, inoculated with the test biomass (washed twice before use, initial MLSS concentration set to 800 mg/L) and then cultivated at 10 ^{o}C and 160 rpm for 12 h. DO concentration fluctuated between 1.5 and 3.2 mg/L during the whole test. Samples were taken at 3-h intervals for measurement of COD, NH_4^+-N, NO_2^--N, and NO_3^--N.

2.3. Analytical methods

NH_4-N, NO_2–N and NO_3-N were measured by Nessler's reagent spectrophotometry, N-(1-naphthalene)-diaminoethane spectrophotometry and ultraviolet spectrophotometric method respectively (APHA, 1998). Dissolved oxygen (DO)

concentration was measured by a DO meter (YSI-550A, YSI, USA). Mixed liquor suspended solids (MLSS) was determined using Methods 2540 D (APHA, 1998).

2.4. DNA extraction

Genomic DNA was extracted from 1 mL samples using a 3S DNA isolation kit (Shenergy Biocolor, Shanghai) according to the manufacturer's instructions. Obtained genomic DNA was examined on an 1% agarose gel to estimate the DNA's size and concentration, and then stored at below -20 ^{o}C.

2.5. PCR-DGGE analysis

PCR was performed using the universal bacterial primers GC-F341 (5'-CCTACGGGAGGCAGCAG -3', attached with a GC clamp on the 5' terminus) and R518 (5'- ATTACCGCGGCTGCTGG -3') to amplify the V3 hypervariable regions of 16S rRNA genes. The total reaction mixture of PCR consisted of 50 μl with the following ingredients: 5 μl 10×buffer, 4 μl dNTP mix (2.5 mM each), 1 μl primer GC-F341 (10 mM), 1 μl primer R518 (10 mM), 0.25 μl recombinant Taq DNA polymerase (TaKaRa, Dalian, China), 5 μl of extracted DNA (diluted to approx. 2ng/ul before use), and sterile Milli-Q water to the final volume. A touchdown PCR program was then performed as described by Vivas et al. (2009) with the initial annealing temperature decreased to 60 ^{o}C and the extension time in amplification reduced to 1 min. PCR products were analyzed by electrophoresis in 2% agarose gel.

DGGE of the PCR product was performed in a polyacrylamide gel (10% w/v) with denaturing gradients from 25% to 55% using a D-code DGGE system (Bio-rad, USA). Electrophoresis was performed in 1×TAE buffer at 160V and 60 ^{o}C for 6h. After electrophoresis, the gel was stained with Gelred™ (Biotium, USA) for 20 min, followed by rinsing with Milli-Q water. The gel was then scanned with a UV transilluminator (Bio-rad, USA) to acquire the DGGE band image.

2.6. Construction of 16S rRNA gene clone libraries and sequence analysis

Two clone libraries were constructed from the original seed sludge and the enriched consortium in R2. PCR was performed with forward primer F27 (5'-AGAGTTTGATCMTGGCTCAG -3') and reverse primer R1492 (5'-TTGGYTACCTTGTTACGACT -3') to get nearly full-length (~ 1500 bp) 16S rRNA genes. The reaction system was consistent with the conditions used for DGGE. PCR was performed under the following conditions: 95 ^{o}C /5 min denaturation step; 35 cycles each of 95 ^{o}C /45 s, 50 ^{o}C /45 s, 72 ^{o}C /90 s; and a final extension step at 72 ^{o}C /10 min.

Triplicate PCR products were pooled and purified with Qiaquick PCR Gel Extraction Kit (QIAGEN, Stanford, CA, USA), cloned using the pGEM-T Easy Vector System (TaKaRa, Dalian, China) with TOP10 competent *E. Coli* cells (Tiangen, Beijing, China), and plated on LB (Luria-Bertani) plates supplied with ampicillin (Sigma). Colonies were randomly picked, cultured overnight in LB broth supplemented with

ampicillin, and then sequenced on both strands using the vector primers M13F-47 and M13R-48 in Invitrogen Inc. (Beijing, China).

Obtained sequences were compared with available sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Phylogenetic reconstructions were performed in MEGA 5.05 using neighbor-joining (NJ) method with 1000 bootstrap replicates and the maximum composite likelihood model. Diversity indices were calculated from sequence data based on the number of OTUs (operational taxonomic units). An OUT was defined as a 16S rRNA gene sequence group in which sequences differ by 5% or less. The Shannon diversity index H (Shannon, 1948) was calculated as follows: $H=-\Sigma(p_i)\ (\ln p_i)$ where p_i is the proportion of an individual OTU relative to all sequences analyzed. Library coverage C was calculated according to the formula $C=(1-(n_1/N))*100\%$, where n_1 was the number of OTUs containing only one sequence, and N the total number of 16S rRNA gene sequences analyzed (Good, 1953).

3. Results and Discussion

3.1. Enrichment of the consortium

Two different aeration modes have been adopted to enrich consortium capable of aerobic denitrification at low temperature. Fig. 1 shows the time-histories of nitrate removal performance and DO concentration during the enrichment process in R1 and R2. Initially, with seed sludge present, nitrate removal took place under anoxic

conditions (R2) rather than aerobic conditions (R1), implying that the seed sludge was only capable of anoxic denitrification. During enrichment with a constant air supply (DO ≈ 6 mg/L) in R1, the nitrate removal efficiency increased over time and eventually saturated at about 65 %. In R2, despite the stepwise increase of DO concentration (from < 0.5 mg/L to ≈ 3 mg/L and then to ≈ 6 mg/L), the denitrification efficiency was consistently above 90 % and saturated at approximately 99 %. This indicates that a consortium with excellent aerobic denitrification capability at low temperature was successfully obtained in R2.

As shown previously, the aeration mode played an important role in the enrichment process. Alternating aerobic/anoxic conditions could lead to a high density of bacteria carrying out aerobic denitrification, as reported in several studies (Frette et al., 1997; Patureau et al., 2000). Moreover, sequential aerobic cultivation in the presence of nitrate was found to be efficient in enriching aerobic denitrifiers (Takaya et al., 2003). In the present study, transition from anoxic conditions to aerobic conditions via a two-step increase in DO concentration (in R2) was found to be more efficient than constant aerobic conditions (in R1) with regard to enrichment of aerobic denitrifiers. Moreover, the reproducibility of the enrichment method and the stability of the established system were verified. Hence the novel cultivation method applied in R2 is convenient, effective and reliable in enriching a consortium with excellent aerobic denitrification capability.

3.2. Nitrate removal performance at low temperature

Nitrate removal performance of the successfully enriched consortium in R2 was investigated under aerobic conditions at 10 ^{o}C; the seed sludge was also tested for comparison purposes. Fig. 2 presents plots of the removal of nitrate and production of nitrite as functions of time for the enriched consortium and the seed sludge cases. A dramatic decrease of nitrate was observed with the enriched culture in R2, whereas hardly any reduction in nitrate concentration occurred for the seed sludge. In the former case, approximately 70mg/L nitrate was completely removed within 9 h with low accumulation of nitrite; in other words, the specific nitrate removal rate was 7.68 mg-N/(L·h) or 9.6 mg-N/(g-SS·h).

In previous studies (Kim et al., 2002; Nakajima-Kambe et al., 2005; Vacková et al., 2011) denitrification activity at 10 ^{o}C was invariably measured under anoxic conditions (see Table 1 for a summary of the specific experimental conditions and corresponding nitrate removal rates). The presence of oxygen has severe inhibition effect on conventional denitrification process. As result, conventional denitrification can only take place under anoxic conditions. However, the consortium obtained in the present study exhibited excellent denitrifying activity under relatively high DO (1.5-3.2 mg/L) condition, which is even higher than that obtained under anoxic conditions in previous studies. With high specific denitrification rate and no restrictions of DO concentration, the present consortium could effectively denitrify total oxidized nitrogen (TON, sum of nitrate and nitrite) during the aeration phase for the realization of simultaneous

nitrification and denitrification (SND) process.

3.3. Ammonia removal performance at low temperature

As mentioned in Section 1, large percentage of aerobic denitrifiers are also heterotrophic nitrifiers, so the heterotrophic nitrification capability of the enriched aerobic denitrification consortium was further investigated. Fig. 3 plots the time histories of ammonia removal and production of total oxidized nitrogen at 10 ^{o}C for the enriched consortium in R2 and seed sludge cases. The enriched consortium exhibited a 90% increase in ammonia removal capability over that of the seed sludge taken from a municipal wastewater treatment plant. During the 12h test period, the concentration of ammonia declined from 80 mg/L to less than 50 mg/L, with a corresponding specific removal rate of 2.44 mg-N/(L·h) or 3.1 mg-N/(g-SS·h). This rate at 10 ^{o}C represented an increase of approximate 100% over previous results from a pilot-scale sequencing batch reactor using real-time control where the ammonia removal rate was 1.54 mg NH_4-N/g MLSS/h at 11.9 ^{o}C (Yang et al., 2007), and > 800% increase over aerobic granular sequencing batch airlift reactor (SBAR) where the specific ammonia removal rate was 0.31-0.35 mg/g/h at 10 ^{o}C (Bao et al., 2009).

Although the present tests show that ammonia removal occurred at 10 ^{o}C, no corresponding accumulation of total oxidized nitrogen was observed under aerobic conditions in either the seed sludge or enriched consortium cases. Aerobic nitrification test with ATU as an inhibitor for autotrophic ammonia oxidation was carried out for

both cultures. The results showed that the oxygen uptake rate was barely depressed by the addition of ATU, which indicated that the autotrophic nitration reaction was severely inhibited in both systems. Considering that seed sludge cannot support aerobic denitrification, the low accumulation of total oxidized nitrogen indicates that nitration reaction (both autotrophic nitration and heterotrophic nitration reaction) was strongly inhibited. And as a result, the ammonia removal in the seed sludge system was mainly due to bacterial assimilation as also reported elsewhere in the literature (Ilies and Mavinic, 2001). The increased amount of removed ammonia and hardly any accumulation of total oxidized nitrogen in the enriched culture system could probably be attributed to heterotrophic nitrification and simultaneous aerobic denitrification (as already observed in Section 3.2).

Consequently, bioaugmentation with the present enriched consortium holds considerable promise for effective ammonia removal and realization of SND at low temperature. Moreover, the relatively high cell yield should make large-scale cultivation and further application a practical proposition. However, other investigators discussed the possibility of slight increase of excess sludge in the treatment system bioaugmented with similar fast-growing microorganisms, provided the added bacteria took a small proportion in the activated sludge system (Patureau et al., 2001). Only in high-strength wastewater treatment, excess sludge could become one of the key issues needed to be considered (Joo et al., 2006). Also the enriched consortium formed large flocs of about 3–4 mm size and exhibited excellent settling and

compaction properties, resulting in a high-quality treated effluent.

3.4. PCR-DGGE analysis

In order to investigate the changes in the microbial community structure during the enrichment process, PCR-DGGE analysis was performed. The results, depicted in Fig. 4, indicate a substantial difference between the microbial community structure of the enriched consortium and that of the original seed sludge, and this difference was more pronounced in R2 enrichment than R1. It can be discerned that the complexity of the microbial community significantly reduced during the enrichment process in R2. For example, bands 4, 8-9 and 12-14, which were dominant in the DGGE profiles of the seed sludge and even in R1 enrichment culture, became less intense or completely disappeared in the profiles of the R2 enrichment. Meanwhile, a small number of dominant bands intensified or emerged during the enrichment process in R2, such as bands 20, 23 and 24. These changes imply that the microorganisms which can effectively utilize nitrate under aerobic conditions became dominant, whereas others, which were unable to adapt properly, declined during the selective enrichment process (Chanika et al., 2011; Hilyard et al., 2008).

3.5. Detailed bacterial community analysis based on 16S rRNA gene clone libraries

To characterize the detailed bacterial community composition of the seed sludge and the R2 enrichment, nearly full-length (~ 1500 bp) 16S rRNA gene clone libraries

were constructed. For each case, 48 clones were randomly picked and sequenced. Analysis of the sequences from the seed sludge clone library revealed 32 distinct OTUs while only 8 distinct OTUs were found in the R2 enrichment clone library. Diversity indices was calculated to be 3.24 for the seed sludge clone library and 1.49 for the enrichment library, indicating a significant reduction in bacterial diversity. Additionally, library coverage was calculated and the results indicated a high coverage in the enrichment clone library (93.18 %) and a much lower coverage (47.92 %) in the seed sludge library. Generally, the outcomes from DGGE and clone library analysis both indicated a significant reduction in bacterial diversity during enrichment. However, some inconsistence of the outcomes was noted. Clone library analysis provided less dominant microbial species for R2 culture than DGGE analysis. One possible explanation is that 1-2 base mutation or mispairing during the PCR amplification could lead to different migration on the DGGE gel and thus generation of extra bands.

Phylogenetic analysis was then performed using BLAST searches at NCBI. Phylum or class-level (for Proteobacteria) phylogenetic affiliations for these sequences are depicted in Fig. 5. It is evident that several dominant groups in the seed sludge declined, such as *Alphaproteobacteria*, *Deltaproteobacteria* and particularly *Bacteroidetes* (from 41.7% in seed sludge clone library to only 4.2% in R2 enrichment). Members of the *Bacteroidetes* phylum are thought to degrade complex organic matters, and in the enrichment process aiming at functional group capable of nitrogen removal, it

declined as expected. In the enriched consortium, *Betaproteobacteria* and *Gammaproteobacteria* have emerged to be two dominate groups, accounting for 47.9% (23 out of 48) and 41.7% (20 out of 48) of the total sequences respectively. Detailed phylogenetic affiliation of the sequences retrieved from R2 enrichment were summarized in table 2 and a neighbor-joining phylogenetic tree was also constructed (Fig. 6).

All the 20 *Gammaproteobacterial* sequences from the enrichment culture were clustered into *Pseudomonas* sp. which originally only accounted for 2.1% (1 out of 48) of the total sequences from the seed sludge. Several lineages of the genus *Pseudomonas* have been reported capable of heterotrophic nitrification and aerobic denitrification, including *P. alcaligenes* (Su et al., 2006), *Pseudomonas putida* (Kim et al., 2008), and *Pseudomonas stutzeri* (Miyahara et al., 2010; Zhang et al., 2011). Moreover, *Pseudomonas* spp. have also been found to adapt well to cold conditions and flourish at relatively low temperature (Margesin and Schinner, 1994; Matsuo et al., 2010). Therefore *Pseudomonas* spp., as the most abundant species in the enriched consortium, potentially played the largest role in nitrification and denitrification under cold temperature. Additionally, *Pseudomonas* spp. were reported to produce mainly N_2 as end product under aerobic conditions in previous studies. For example, *P. alcaligenes* AS-1 was reported to produce 1.5±0.5 mmol L^{-1} N_2 and 0.2±0.0 mmol L^{-1} N_2O from 2.5±0.2 mmol L^{-1} NH_4-N (Su et al., 2006). Moreover, *Pseudomonas stutzeri* TR2 produces little N_2O under all conditions tested and thus possesses potential

to reduce N_2O emissions when applied to sewage disposal fields (Miyahara et al., 2010). As a result, it is reasonable to infer N_2 as the main end product of the enriched consortium.

Most of the 23 sequences affiliated with the class *Betaproteobacteria* cluster under *Comamonadaceae*, a family of bacteria often reported in wastewater treatment systems. Of these, 15 sequences were closely related to *Rhodoferax ferrireducens* T118, which is a psychrotolerant, facultatively anaerobic bacterium capable of utilizing nitrate as the electron acceptor (Finneran et al., 2003). A constraint-based, genome-scale in silico metabolic model and laboratory studies have unveiled the genome of *R. ferrireducens* T118 contains a respiratory nitrate reductase complex (Rfer_2792-95) and assimilatory nitrate reductase (Rfer_2559) (Risso et al., 2009). As a result, *R. ferrireducens* probably utilized nitrate as both electron acceptor and nitrogen source under low temperature.

It is interesting to note that no sequences affiliated with autotrophic nitrosobacteria were available in both clone libraries. Instead, putative heterotrophic nitrification-aerobic denitrification bacteria became most dominant in R2 enrichment. This lends further credence to the proposition that nitrification in the enriched consortium is carried out by heterotrophic rather than autotrophic nitrifiers.

4. Conclusions

By stepwise increase of DO concentration during enrichment, a psychrotolerant

consortium with excellent nitrogen removal capability (specific ammonia and nitrate removal rate being 3.1 mg-N/(g-SS·h) and 9.6 mg-N/(g-SS·h) and optimized community structure has been obtained. Detailed community structure analysis indicated that *Pseudomonas* spp. and the closest relatives of *Rhodoferax ferrireducens* probably played a major role in nitrogen removal. Bioaugmentation with this consortium can potentially improve ammonia removal efficiency with little accumulation of total oxidized Nitrogen during a single aeration phase, and thus offer a promising alternative for nitrogen removal under cold temperature conditions.

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Figure Captions

FIGURE 1. Time-histories of nitrate removal performance and DO concentration during enrichment process in (a) R1 and (b) R2. Symbols: open pentagons, DO concentration; closed stars, nitrate removal efficiency.

FIGURE 2. Nitrate removal performance of the enriched consortium in R2 and seed sludge at 10 ^{o}C. Symbols: closed symbols, enriched consortium in R2; open symbols, seed sludge; squares, NO_3^--N; triangles, NO_2^--N.

FIGURE 3. Ammonia removal performance of the enriched consortium in R2 and seed sludge at 10 ^{o}C. Symbols: closed symbols, enriched consortium in R2; open symbols, seed sludge; circles, NH_4^+-N; diamonds, TON.

FIGURE 4. DGGE analysis of the bacterial community in seed sludge (lanes 0), enrichment consortium in R1 (lanes 1) and R2 (lanes 2).

FIGURE 5. Phylum or class-level (for Proteobacteria) phylogenetic affiliations for sequences retrieved from seed sludge and enrichment consortium in R2.

FIGURE 6. Phylogenetic tree of the enriched consortium in R2 based on nearly full-length 16S rRNA gene sequences.

TABLE 1. Specific nitrate removal rate at low temperature in several studies.

TABLE 2. Closest phylogenetic affiliation of 48 clones in R2 enrichment based on BLAST comparison in the GenBank database.

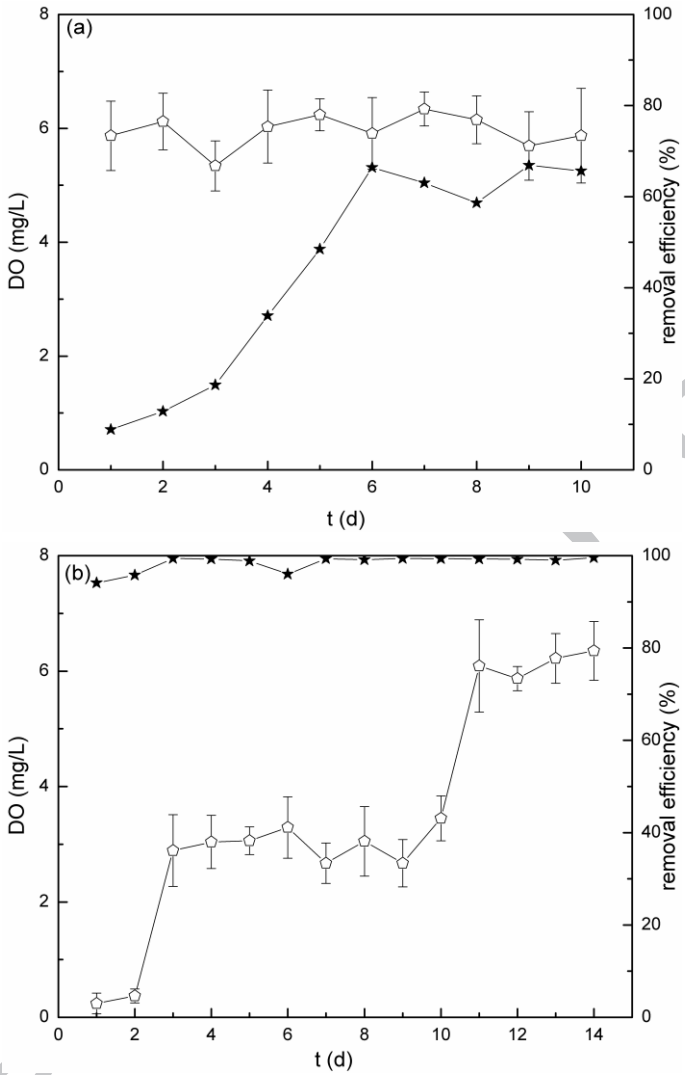

Figure 1

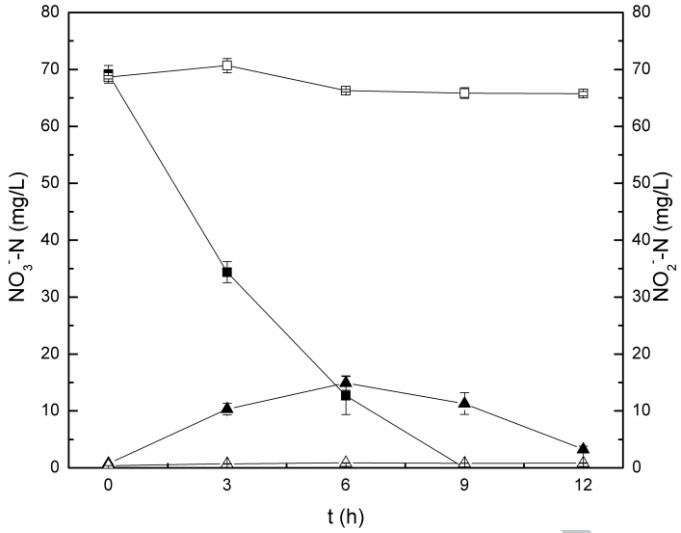

Figure 2

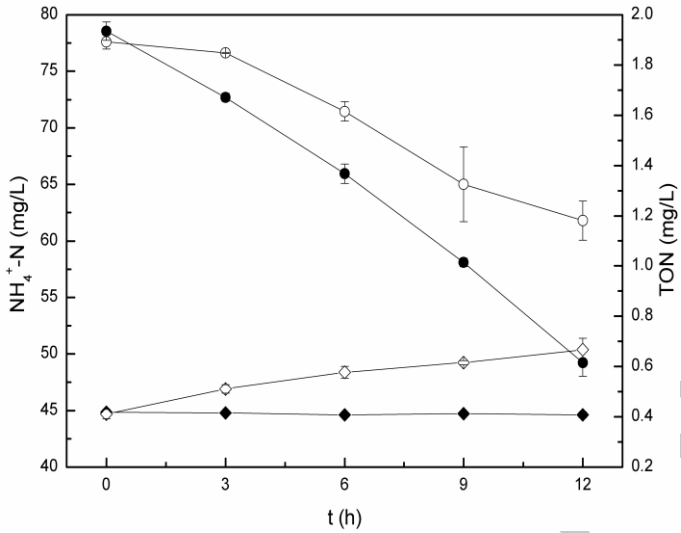

Figure 3

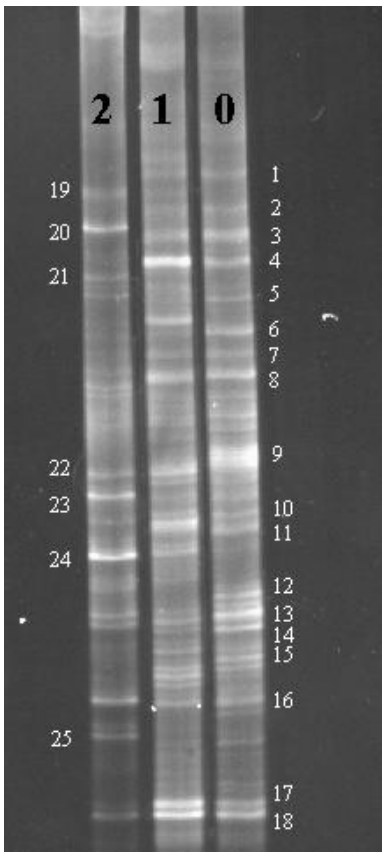

Figure 4

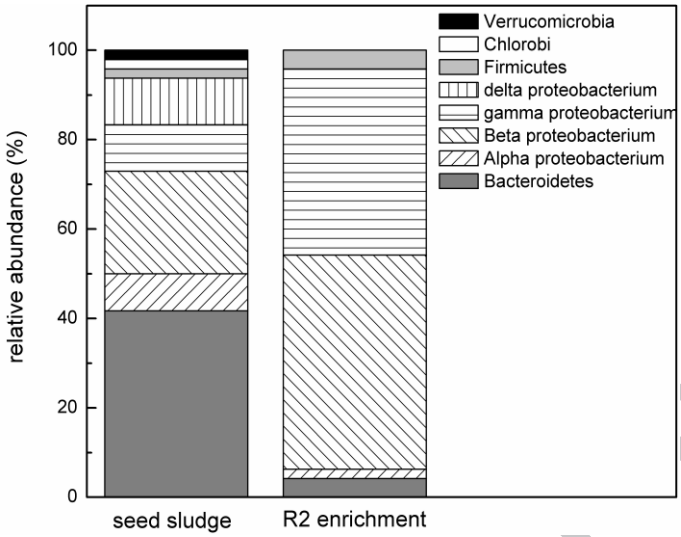

Figure 5

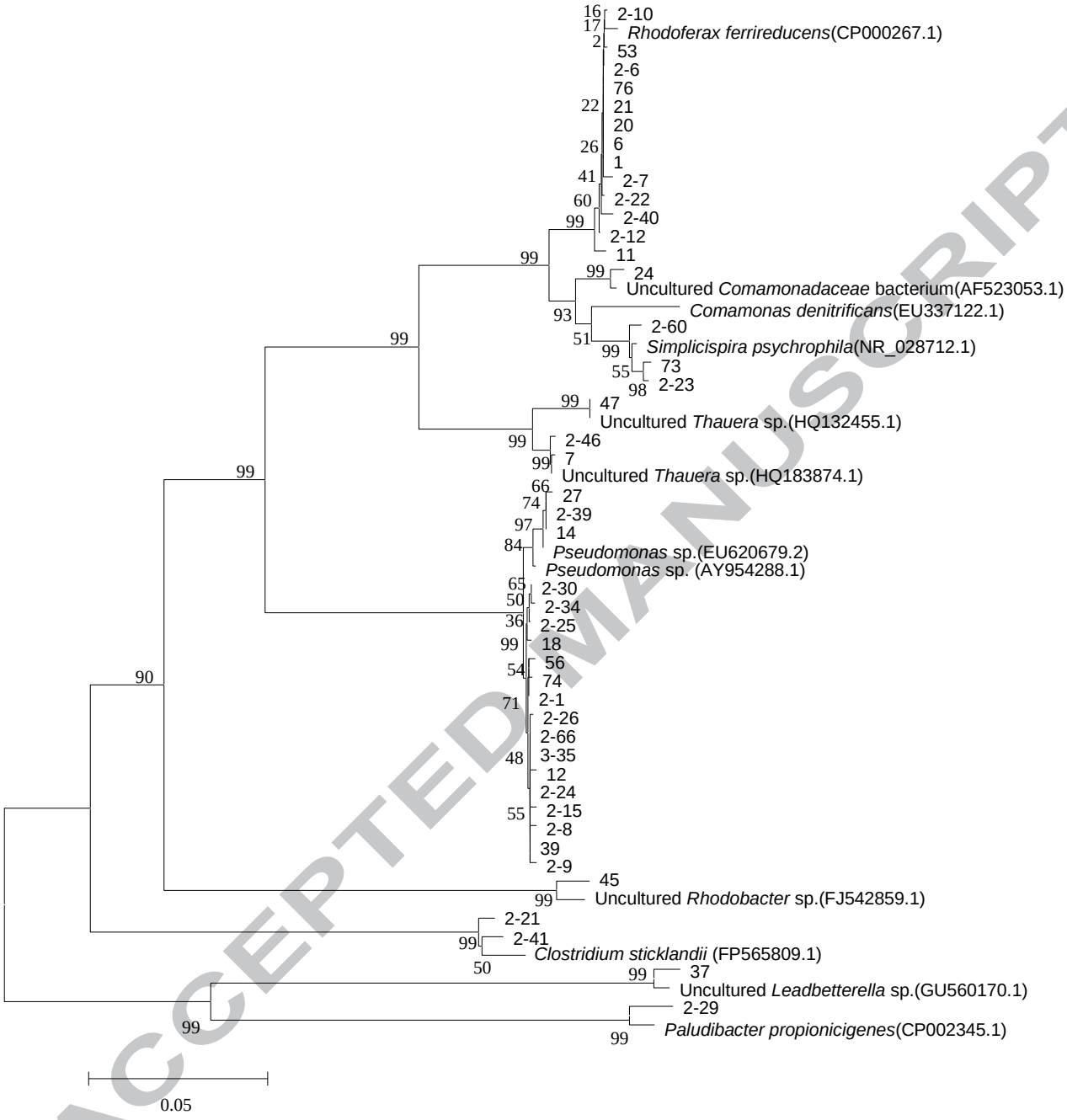

Figure 6

Table 1

Specific rate (mg-N/(g-SS·h))	Biomass	Carbon source	Oxygen condition	Reference
2.38	Pure culture	Soluble starch	Anoxic	Kim et al., 2002
1.0	Pure culture	Glucose	Anoxic	Nakajima-Kambe et al., 2005
0.45	Mixed culture	Glucose	Anoxic	Nakajima-Kambe et al., 2005
0.075	Immobilized enriched consortium	Ethanol	Anoxic	Vacková et al., 2011
0.14	Immobilized pure culture	Ethanol	Anoxic	Vacková et al., 2011
9.6	Mixed culture	Acetate	Aerobic	Present study

Table 2

No. of clones	Closest relative	Accession	% Similarity
18	*Pseudomonas* sp. SKU 16S ribosomal RNA gene, partial sequence	AY954288.1	98-99
2	*Pseudomonas* sp. HY-14 16S ribosomal RNA gene, partial sequence	EU620679.2	99
15	*Rhodoferax ferrireducens* T118, complete genome	CP000267.1	98-99
3	*Simplicispira psychrophila* strain CA 1 16S ribosomal RNA, partial sequence	NR_028712.1	98
1	Uncultured *Comamonadaceae* bacterium clone B-23 16S ribosomal RNA gene, partial sequence	AF523053.1	98
1	*Comamonas denitrificans* strain 2B7 16S ribosomal RNA gene, partial sequence	EU337122.1	99
2	Uncultured *Thauera* sp. clone De168 16S ribosomal RNA gene, partial sequence	HQ183874.1	99
1	Uncultured *Thauera* sp. clone S-123 16S ribosomal RNA gene, partial sequence	HQ132455.1	99
1	Uncultured *Rhodobacter* sp. clone A05-08G 16S ribosomal RNA gene, partial sequence	FJ542859.1	97
2	*Clostridium sticklandii* str. DSM 519 chromosome,	FP565809.1	98-100

	complete genome		
1	*Paludibacter propionicigenes* WB4, complete genome	CP002345.1	97
1	Uncultured *Leadbetterella* sp. clone W4S69 16S ribosomal RNA gene, partial sequence	GU560170.1	98

- Psychrotrophic heterotrophic nitrifying-aerobic denitrifying consortium acclimated.
- Stepwise increase of DO concentration for high efficient consortium enrichment.
- High removal rates of ammonia and nitrate (3.1 and 9.6 mg-N/(g-SS·h)) at 10 ^{o}C.
- Main functional groups identified as *Pseudomonas* sp. and *Rhodoferax ferrireducens*.