Engineering Journal issn: 0125-8281

Article

Persistence of Nitrite-Oxidizing Bacteria in Entrapped Cell-Based Partial Nitrifying Reactor Treating Ammonia-Rich Wastewater

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Abstract. A phosphorylated-polyvinyl alcohol (PPVA) entrapped cell-based reactor was employed to promote partial nitrification for ammonia-rich wastewater treatment. High partial nitrification (66% of nitrite accumulation in average) was achieved along the 165 days of operation indicating that the majority of nitrite-oxidizing bacteria (NOB) activity was suppressed probably as a result of low oxygen environment created within the PPVA gel matrix. However, some portion of nitrate (5-17.6% of the influent ammonia) always appeared in the reactor throughout the operation period. Next-generation sequencing and clone library techniques revealed that NOB with different substrate affinities including *Nitrobacter*, *Nitrospira* lineage I and II existed within the gel matrix. The finding speculates that substrate gradient-like microenvironment within the gel matrix probably serves the different physiological groups of NOB to maintain their cells and activities in the reactor. Therefore, instead of using low oxygen environment in gel matrix as a sole control strategy, an additional strategy like promoting free ammonia inhibition in reactor is also needed to affirm the stability of long-term partial nitrification.

Keywords: Nitrite-oxidizing bacteria, persistence, partial nitrification, entrapped cell-based reactor, bacterial community, next-generation sequencing.

ENGINEERING JOURNAL Volume 23 Issue 2
Received 22 July 2018
Accepted 7 December 2018
Published 31 March 2019
Online at http://www.engj.org/
DOI:10.4186/ej.2019.23.2.1

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1. Introduction

Shortcut biological nitrogen removal (SBNR) relies on the oxidation of ammonia to nitrite, followed by the reduction of nitrite to nitrogen gas. As compared to conventional biological nitrogen removal (BNR), SBNR can reduce amounts of oxygen required for nitrification and organic matters needed for heterotrophic denitrification. Partial nitrification, the oxidation of ammonia to nitrite without nitrate as the end product, is an inevitable step of SBNR. The process can be promoted by providing environmental conditions that suppress the activity of nitrite-oxidizing bacteria (NOB) while allowing the activity of ammonia-oxidizing bacteria (AOB) to occur. Such conditions include maintaining high pH [1], high temperature (>35°C) [2], and/or low oxygen (0.3-0.5 mg l^{-1}) [3, 4].

Cell entrapment has recently been applied to promote partial nitrification for treating ammonia-rich wastewater [5]. Besides retaining high cell density, the cell entrapment provides microenvironment inside the gel matrix that promotes partial nitrification. Oxygen concentration gradient generated along the depth of the gel matrix creates oxygen-limited zone somewhere inside the gel matrix leading to the oxidation of ammonia with the remaining nitrite, not nitrate, as the end product. Natural and synthetic polymers, such as alginate, phosphorylated polyvinyl alcohol (PPVA), and polyethylene glycol (PEG) have been employed to form gel matrices for cell entrapment [2, 5, 6]. PPVA is a synthetic polymer that requires short time for cell entrapment and is nontoxic to microorganisms [7]. Previously, partial nitrification was successfully achieved in a PPVA entrapped cell-based reactor operated at a bulk DO concentration of 2 mg l^{-1} [5]. However, the NOB activity was not fully suppressed in the oxygen-limited zone of the gel matrix since a small amount of nitrate (up to 13%) was still accumulated along the operation period [5]. The finding leads to the question of how the community structure of NOB arise in order to survive under low oxygen environment in the gel matrix leading to the deterioration of partial nitrification.

For the reason above, this study aims to investigate the community structure of NOB after long-term operation of an entrapped cell-based partial nitrification reactor. Two 16S rRNA DNA sequencing techniques including Illumina MiSeq sequencing and Sanger sequencing were used to observe the relative abundance of microorganisms and the NOB community within the gel matrix. Realizing the importance of the nitrite-oxidizing bacterial persistence on the stability of partial nitrification is the major implication of this study.

2. Materials and Methods

2.1. Preparation of Entrapped Cells

Mixed liquor from a Bangkok municipal wastewater treatment plant was entrapped using PPVA technique as introduced by [7]. Briefly, the collected mixed liquor was concentrated via centrifugation at a speed of 500 rpm for 15 min. Then, one liter of polyvinyl alcohol (PVA) gel solution, prepared by 100 g of PVA powder in de-ionized (DI) water, was mixed with the concentrated sludge to achieve an initial cell-to-matrix ratio of 4% w/v. The formation of spherical gel matrices was conducted by dropping the mixture into a saturated boric acid solution at a flow rate of 0.83 ml min^{-1}. Then, the gel matrices were immersed into 1 M of phosphate buffer solution at pH 7.0 for 2-3 h to harden the gel matrices.

2.2. Synthetic Wastewater

The synthetic ammonia-rich wastewater which contained ammonia at 700 mg N l^{-1}, was prepared by mixing $(NH_4)_2SO_4$ (3.3 g), Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $CaCl_2 \cdot 2H_2O$ (0.01 g), $FeSO_4 \cdot 7H_2O$ (0.09 g), H_3BO_3 (30 mg), $MnCl_2 \cdot 4H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.19 g), $NiCl_2 \cdot 6H_2O$ (0.024 g), $CuCl_2 \cdot 2H_2O$ (0.02 g), $ZnSO_4 \cdot 7H_2O$ (0.144 g), $Na_2MoO_4 \cdot 2H_2O$ (0.36 g), and $NaHCO_3$ (6.89 g) in one liter of DI water. The composition was modified from [5].

2.3. Setup and Operation of an Entrapped Cell-Based Reactor

A continuous stirred tank reactor with an effective volume of 2 l was inoculated with the prepared entrapped cells with a cell-to-matrix ratio of 4% w/v. The final concentration of cells in the reactor was 2000 mg SS. Bulk DO concentration in the reactor was maintained at 3 mg l^{-1} using DO controllers

(HI8410 DO controller, Hanna, USA). Air flow rate was controlled at 1 l min^{-1}. pH in bulk solution was maintained at 7.8±0.2 to ensure that partial nitrification only occurred by oxygen-limiting condition using pH controllers (Alpha pH 560, Thermo scientific, USA) with electrodes (Eutech instrument, USA). The fluctuation of pH was automatically reduced by adding 0.2 M NaOH solution through peristaltic pumps (505U, Watson Marlow, United Kingdom). To maintain an ammonia loading rate of 500 mg N $l^{-1}d^{-1}$, hydraulic retention time was controlled at 1.4 days. Water temperature was at 24-28 ºC and complete-mixing condition was provided by mechanical mixing (R20, IKA, Germany) at 250 rpm.

2.4. Chemical Analysis

Water samples taken from the reactor were filtered through 0.45 μm GF/C filter paper before ammonium, nitrite, and nitrate concentrations were analyzed. Ammonium concentration was analyzed using an ion selective electrode (NH500, WTW, Germany) in accordance with APHA standard procedures [8]. Nitrite and nitrate concentrations were analyzed by colorimetric and UV spectrophotometric methods, respectively [8].

2.5. Investigation of an Internal Structure of Gel Matrix by Scanning Electron Microscope (SEM)

Samples of gel beads were harvested from the reactor on day 148 to investigate the internal structures of gel matrix by SEM technique. Briefly, after rinsing with a phosphate buffer saline (PBS) solution, the samples were fixed with 50% ethanol solution in 0.1 M phosphate buffered-saline (PBS), and dehydrated by immersing the samples sequentially for 10 minutes in 50%, 75% 95%, and 100% ethanol solutions. After that, each bead samples were chopped into 4-5 small pieces and further coated with gold under vacuum condition. Each piece of cut samples was observed under SEM (JEOL, JSM-5410LV, Tokyo, Japan).

2.6. Analysis for Relative Abundance of Microorganisms

Gel beads were collected from the reactor on day 148 and dissolved completely in DI water at 70°C for 5 min. Genomic DNA was extracted using the FastDNA® SPIN Kit for Soil (Qbiogene, USA). The primers 515F (5′ GTGYCAGCMGCCGCGGTAA 3′) [9] and 806R (5′ GGACTACHVGGGTWTCTAAT 3′) [10] were used for PCR amplification of the extracted DNA. The condition for PCR amplification was 3 min at 95°C followed by 23 cycles of 30 s at 95°C, 30 s at 53°C, and 30 s at 72°C and a final extension of 5 min at 72°C. The PCR mixture was prepared by *Taq* polymerase (Thermo Scientific, USA) and was carried out in a thermal cycler (Biorad Laboratories, USA). The PCR product was purified by a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The purified PCR product was sent to the Omics Sciences and Bioinformatics Center, Chulalongkorn University, Thailand, for Illumina library construction and data analysis. In brief, index was attached using 2X KAPA hot-start ready mix and 5 μl of each Nextera XT index primer in 50 μl reaction. The attached index product was then purified by AMPure XP beads (Beckman Coulter, USA), pooled and diluted to 4 pM. After an Illumina MiSeq was performed, quality of the sequencing reads was checked by FASTQC [11], then assembling overlapping paired end reads by PEAR [12]. FASTX-Toolkit [13] was used to filter assembled reads that at least 90% of bases had a quality score of less than 30 and were shorter than 400 bp. Chimeras was removed using the UCHIME method [14] as implemented in VSEARCH 1.1.1 [15] using –uchime_ref option against chimera-free Gold RDP database. The *pick_open_reference_otus.py* command in QIIME 1.9.0 was employed to generate operational taxonomic unit (OTU). SortMeRNA was used for the reference picking, and SUMACLUST [16] was used to *de novo* cluster the subsampled failure reads. Subsequently, the OTU sequences were taxonomically assigned to the Green gene database. In addition, OTUs that have less than 0.1% reads were excluded.

2.7. NOB Community Analysis

The gel beads collected on day 148 were analyzed for NOB community. The amplification of 635-based pair fragment of *Nitrobacter* 16S rRNA gene and 350-based pair fragment of the 16S rRNA gene of *Nitrospira* were carried out with the primer sets P338f (5' ACTCCTACGGGAGGCAGCAG 3') and NIT3 (5' CCTGTGCTCCATGCTCCG 3') [17, 18] and the primer sets EUB338f (5 ′

ACTCCTACGGGAGGCAGC3′) and Ntspa0685r (5′ CGGGAATTCCGCGCTC 3′) [19], respectively. The PCR mixture was prepared using Takara polymerase (Takara Bio Inc., Japan). The PCR reaction was conducted using a thermal cycler (Biorad Laboratories, USA) for 5 min at 95°C, followed by 25-30 cycles for 1.5 min at 95°C, 30 s at 65°C, and 1 min at 72°C. The PCR product was purified by gel electrophoresis method using NucleoSpin Extract II Kit (Clontech Laboratories Inc., USA). The purified PCR product was cloned using the pGEM-T Easy vector system (Promega, USA). For each library, 25 clones were randomly selected for Sanger sequencing at Macrogen Inc. An arrangement of OTUs of the analyzed sequences was determined at 99% cut off using CD-HIT [20]. The representative sequences of *Nitrobacter* and *Nitrospira* were separately aligned and phylogenetic trees of the two microorganisms were generated using MEGA7 program [21] and ARB program package [22], respectively.

3. Results and Discussion

3.1. Reactor Performance

Figure 1 shows nitrogen concentrations in the entrapped cell-based reactor during the 165 days of operation. At the start-up period (day 1 to day 88), ammonia removal efficiency was 46±13% and nitrite accumulation was 59±21%. After reaching the partial nitrification period (day 88 to day 165), ammonia removal increased to 65±12% while the accumulation of nitrite was slightly higher up to 66±23%. The effluent has a ratio of ammonia to nitrite of 1:1.15 which is a near perfect influent for ANAMMOX (ammonia:nitrite of 1:1.32) [23].

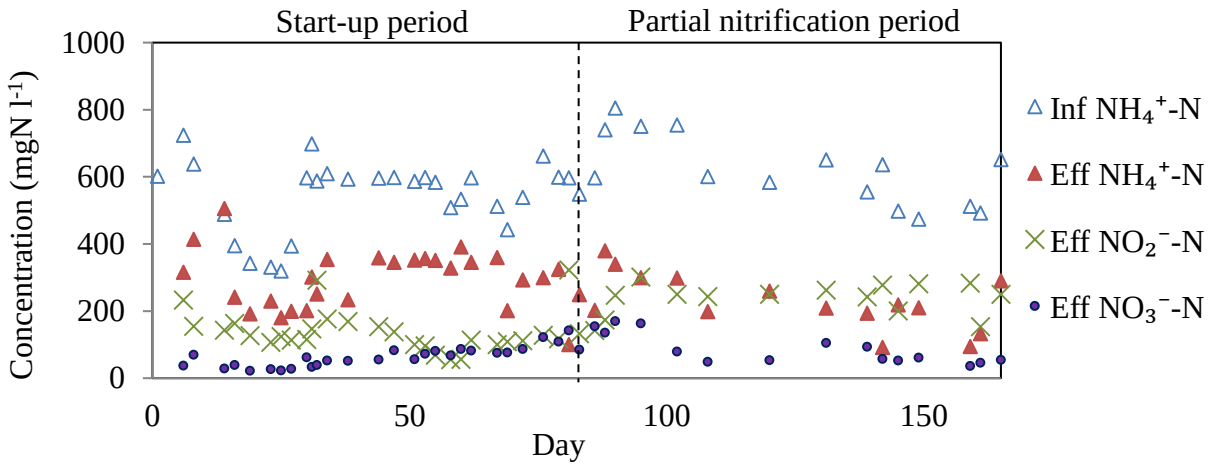

Fig. 1. Performance an entrapped cell-based partial nitrification reactor.

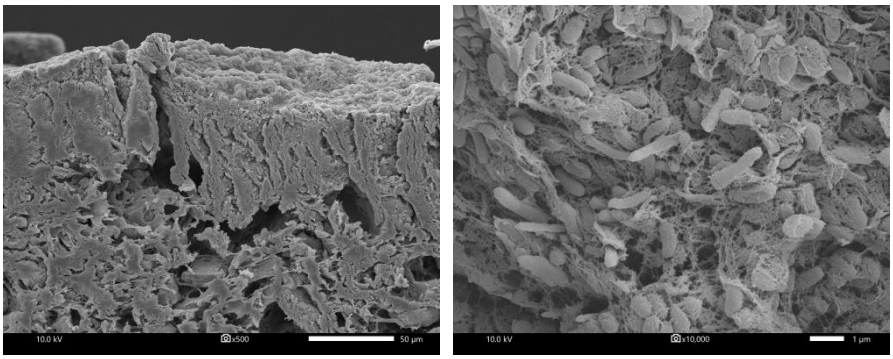

Fig. 2. SEM images showing an internal structure of gel matrix collected from reactor at the end of partial nitrification period (day 148). Left image shows the outer zone of gel matrix with magnification 500X and the right image shows the core of gel matrix with magnification 10,000X.

Oxygen-limited environment within the PPVA gel matrix has been recently proven as a key decisive factor controlling the achievement of partial nitrification in an entrapped cell-based reactor [5]. Since cell entrapment with PPVA gel (as clearly shown in Fig. 2) blocks the penetration of oxygen inside the gel matrix (i.e. < 100 μm from the surface of gel matrix) leading to substantially reduced oxygen available for most of NOB [5]. Free ammonia (FA) concentrations in the reactor (4-18 mg l^{-1}) were higher than a range of 0.1-1.0 mg l^{-1} which began to inhibit the NOB activity in a suspended cell system [24]. However, FA inhibition should not be the key factor that promotes partial nitrification in this study because forming dense bacterial aggregates like biofilm and entrapped cell system help reducing the level of toxicant that actually exposed to microbes.

While maintaining partial nitrification was being processed, a small portion of nitrate (79±44 mg l^{-1} as 5-17.6% of influent ammonia) always appeared in the effluent throughout the long-term operation. This implied that the oxygen-limited condition in the gel matrix was not able to suppress all nitrite-oxidizing activities. This leads to the question of how some NOB are still able to maintain their cells within the oxygen-limited environment of the gel matrix.

3.2. Relative Abundance of Microorganisms

Gel beads collected near the end of reactor operation (day 148) were analyzed to determine the relative abundance of microorganisms within the gel matrix at the phylum and family levels (Fig. 3). Proteobacteria (49.24 %) and Bacteroidetes (29.27 %) were the highest relatively abundant phylum in the gel matrix, while *Cytophagaceae* was the highest relatively abundant family (23.04%). Some members of *Cytophagaceae* have been claimed for their ability to denitrify [25, 26]. Approximately 11±17% of the influent ammonia was loss during operation of the reactor that is probably contributed by denitrification at the expense of organic compounds derived from endogenous decay as an energy source. AOB belonging to the family *Nitrosomonadaceae* were observed in the gel matrix at a relatively high abundance (18.21%). Unexpectedly, a small amount (1.18%) of ammonia-oxidizing archaea (AOA) belonging to the *Nitrososphaeraceae* were also found. The results suggest that AOB were the main ammonia oxidizers in the reactor and AOA may partially contribute to the oxidation of ammonia to nitrite. *Comamonadaceae* was observed at 15.89% of the relative abundance. Some members of *Comamonadaceae* was reported to denitrify [27]. Some loss of ammonia that may take place by denitrification may support this group of microorganisms in the reactor. The 4.44% of the analyzed sequences belonged to some members of the family *Bradyrhizobiaceae* to which NOB genus *Nitrobacter* belongs. Family *Nitrospiraceae*, to which NOB genus *Nitrospira* belongs, was not detected in the gel matrix using Illumina MiSeq sequencing.

3.3. NOB Community Structure

Because Illumina MiSeq sequencing did not provide a direct link to NOB community in the gel matrix, clone libraries were constructed from the PCR-amplified products obtained using the specific primers targeting 16S rRNA gene fragments of *Nitrobacter* and *Nitrospira*. Out of the 25 clones analyzed for 16S rRNA gene fragments of *Nitrobacter*, 19 clones were identified as *Nitrobacter*, while the others were found to be non-*Nitrobacter*, as analyzed by BLAST [28]. Figure 4 shows the phylogenetic tree of *Nitrobacter* 16S rRNA gene sequences. For 16S rRNA gene fragments of *Nitrospira*, 24 out of the 25 clones analyzed were identified as *Nitrospira*. The phylogenetic tree of *Nitrospira* 16S rRNA gene sequences is shown in Fig. 5. The analyzed *Nitrospira* sequences fell within *Nitrospira* lineage I and II.

DOI:10.4186/ej.2019.23.2.1

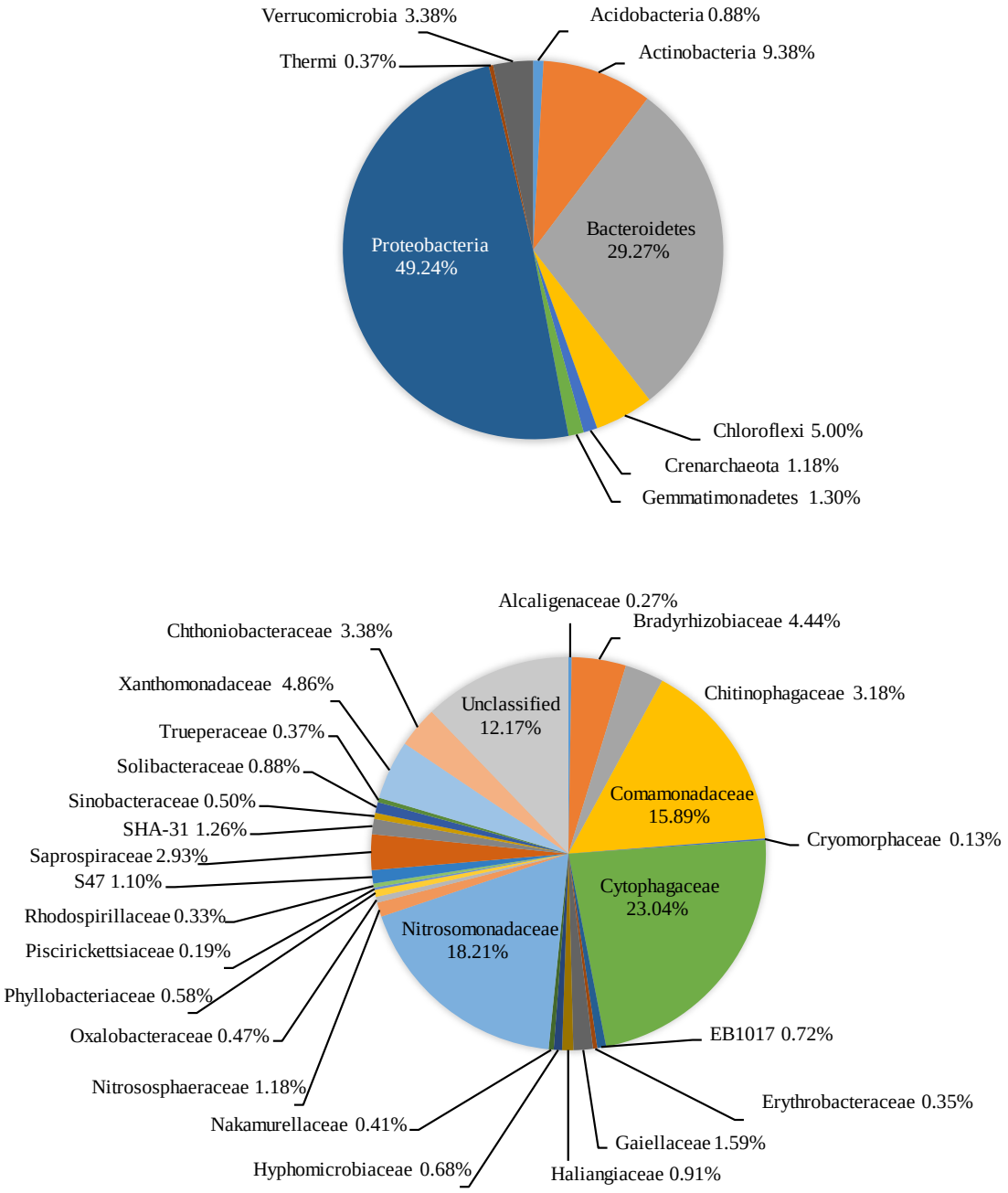

Fig. 3. Relative abundance of microorganisms at the phylum (up) and family (down) levels in the gel matrix of an entrapped cell-based partial nitrification reactor.

NOB genus *Nitrobacter* and *Nitrospira* are different in substrate affinities but were commonly found in wastewater treatment systems [29, 30]. *Nitrobacter* has lower nitrite and oxygen affinities than *Nitrospira* [31, 32]. The nitrite half-saturation coefficient (K_S) of pure cultures of *Nitrobacter* was 0.69-7.6 mg-N l^{-1}, while pure cultures of *Nitrospira* showed lower values of 0.13-0.38 mg-N l^{-1} [31]. The oxygen half-saturation coefficient (K_O) of mixed cultures of *Nitrobacter* was 0.43-5.31 mg l^{-1} [3, 33, 34] while the value of K_O of *Nitrospira* was 0.33-0.54 mg l^{-1} [33, 35]. The difference of K_S and K_O between *Nitrobacter* and *Nitrospira* allows them to be dominant in distinct environments. *Nitrobacter* often dominates a substrate (nitrite and oxygen)-concentrated environment, while *Nitrospira* often prefers a dilute, substrate-deficient environment [32, 36, 37]. In addition, differences in substrate affinity among some *Nitrospira* lineages, which have been often found in wastewater treatment systems [29] as lineage I and II, have been reported in a previous study [38].

Park and Noguera (2008) [38] found a partial shift toward *Nitrospira* lineage II along operating chemostat at high DO (8.5 mg l^{-1}) while the low DO (< 0.24 mg l^{-1}) chemostat was always dominated by *Nitrospira* lineage I. According to the results, they suggested that *Nitrospira* lineage I and II probably have different affinity to oxygen.

In the current study, *Nitrobacter* and *Nitrospira* lineage I and II were found coexisting in the entrapped cell-based reactor even though each of them was reported to have a unique affinity to substrates. Cell entrapment with PPVA gel creates substrate concentration gradients along the depth toward the core of the gel matrix. Substrate gradient-like environment in gel matrix allows various physiological groups of NOB to keep finding their niches and surviving in the entrapped cell-based reactor.

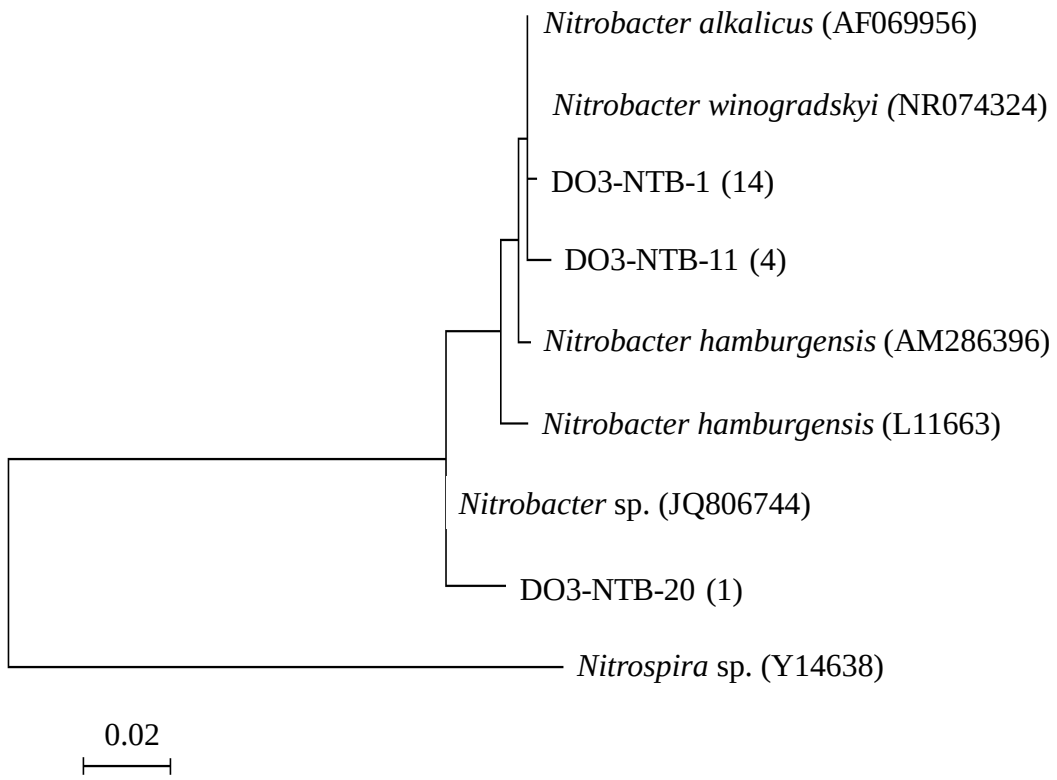

Fig. 4. Phylogenetic tree calculated based on 489 bp of the 16S rRNA gene sequences of *Nitrobacter* using the maximum likelihood method**.** The numbers in parentheses of the analyzed sequences represent numbers of sequences in the same OTUs.

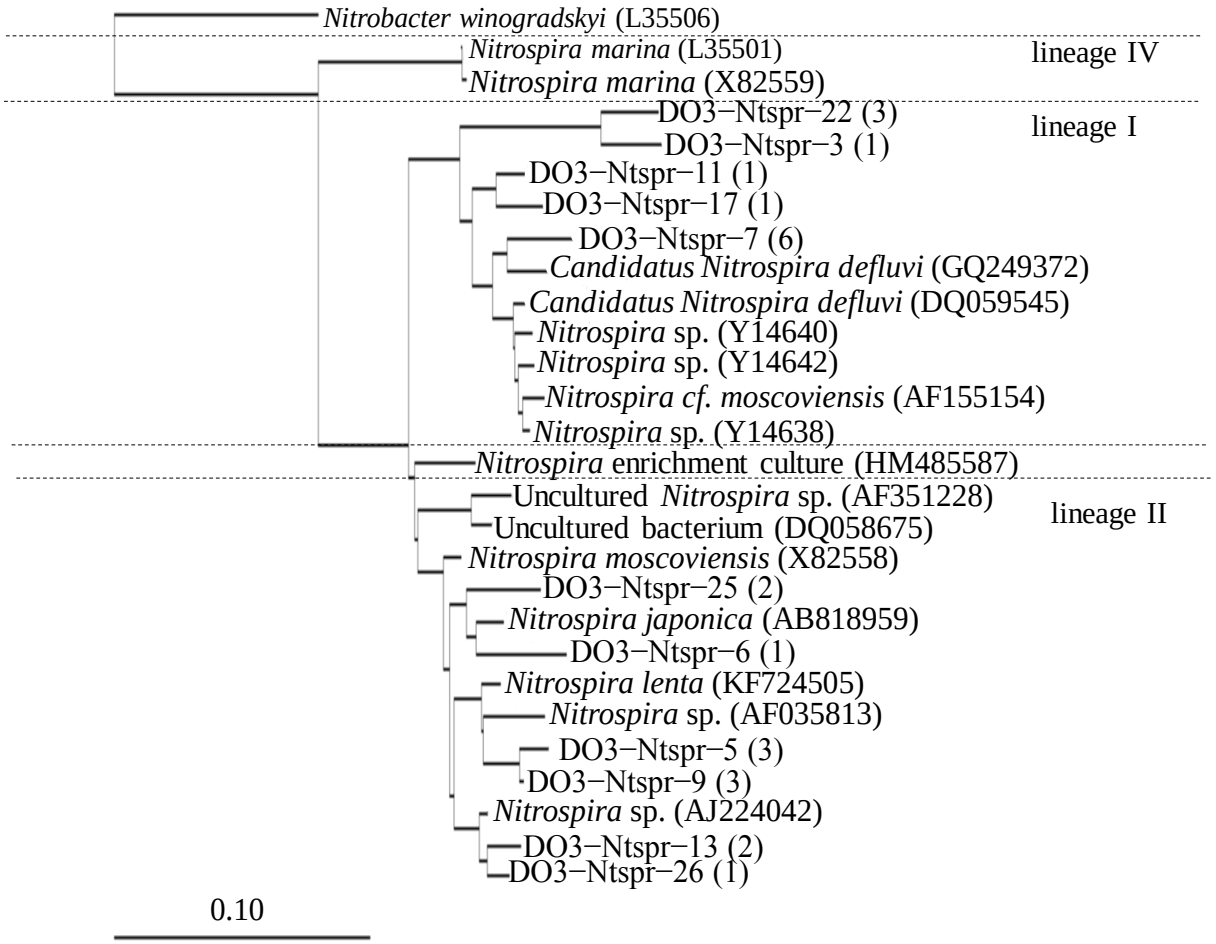

Fig. 5. Phylogenetic tree calculated based on the 16S rRNA gene sequences of *Nitrospira*. The trees were constructed by adding approximately 236-bp sequences using the parsimony method into the tree prior constructed with >1400-bp sequences of reference *Nitrospira* using the neighbor-joining method. The numbers in parentheses of the analyzed sequences represent numbers of sequences in the same OTUs.

3.4. Implication on Maintaining the Stability of Partial Nitrification

Cell entrapment creates an oxygen-limited environment where promptly suppress most of all NOB activity within the gel matrix. However, the formation of oxygen concentration gradient allows NOB particularly with having high oxygen affinity (i.e. *Nitrospira* lineage I and II) to keep growing and continuously producing nitrate in the entrapped cell-based reactor. Although the K-strategist like *Nitrospira* is inherently less growth competitive and obviously less abundance than *Nitrobacter* in the nitrite-accumulating reactor, their persistence may probably threaten a stability of entrapped cell-based partial nitrifying reactor. Therefore, an additional control strategy like promoting FA inhibition in reactor is needed to use in combination with cell entrapment when the long-term stability of partial nitrification is strictly required.

4. Conclusion

Cell entrapment with PPVA was applied to promote partial nitrification for ammonia-rich wastewater treatment. High partial nitrification (66% of nitrite accumulation in average) was reached indicating that low oxygen environment created within the gel matrix resulting in lowering the NOB activity. Nevertheless, small portion of nitrate (5-17.6% of the influent ammonia) still appeared along the whole operational period. NOB with different substrate affinities including *Nitrobacter*, *Nitrospira* lineage I and II were co-existed within the gel matrix. Substrate gradient-like microenvironment within the gel matrix is suggested as major cause of this coexistence and growth of NOB with diverse physiology in entrapped cell-based reactor.

This study recommends promoting free ammonia inhibition in reactor, instead of applying only a control strategy like low oxygen environment in gel matrix, for maintaining the stability of long-term partial nitrification.

Acknowledgment

This work was supported by the National Nanotechnology Center (NANOTEC), NSTDA, Ministry of Science and Technology, Thailand, through its program of Research Network NANOTEC (RNN). The authors also thank the Office of Higher Education Commission (OHEC) and the S&T Postgraduate Education and Research Development Office (PERDO) for the partial financial support of the Research Program.

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