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# IRON-REDUCING β- AND γ-PROTEOBACTERIA ISOLATED FROM LABORATORY-SCALED HETEROTROPHIC FEAMMOX BIOREACTOR

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SUMMARY

Ammonium removal from wastewater is a crucial step in wastewater treatment. Presently employed technologies based on nitrification/denitrification and partial nitritation/anammox principles require oxygen for the nitrification step, and are therefore still not yet fully satisfied with the application practice. In recent years, biological ammonium oxidation coupled with ferric iron reduction (feammox) has been proposed to be responsible for the nitrogen loss in different ecological habitats. Related to the wastewater aspect, the feammox principle has been discussed as an alternative approach for ammonium removal without dependency on oxygen. From a laboratory-scaled feammox bioreactor operated under neutral pH, two bacterial strains FN7 and FN9 were isolated by using the anaerobic Hungate technique. Comparative analyses of 16S rDNA sequences showed that these strains were most closely related to the β-proteobacterium *Aciclyphilus denitrificans* and the γ-proteobacterium *Pseudomonas stutzeri*, respectively. Although being phylogenetically apart, strains FN7 and FN9 shared several common physiological characteristics that are considered meaningful for the feammox process, i.e. (i) heteroptrophic ammonium oxidation, (ii) denitrification, and (iii) ferric iron reduction. These isolates are proposed to play certain roles in the studied feammox system, contributing to the ammonium removal under heterotrophic feammox condition. The 16S rDNA sequences of strains FN7 and FN9 were available in GenBank under the accession numbers LC474369 and MT568614, respectively.

**Keywords:** ammonium oxidation, ferric iron reduction, Feammox, beta-proteobacterium *Aciclyphilus denitrificans,* gamma-proteobacterium *Pseudomonas stutzeri*

## INTRODUCTION

Ammonium nitrogen removal is a central process of wastewater treatment. Untreated ammonium can lead to eutrophication in water bodies and other environmental effects on aquatic systems (Wu *et al.*, 2008). Generally, the removal of ammonium is achieved by two steps nitrification and denitrification, releasing molecular nitrogen as the final product (Grady *et al.*, 1999). This technological principle has been proven efficient and widespread adopted (EPA, 1993; Water, 2011). In the nitrification process, $NH_4^+$ is first oxidized to $NO_2^-$ by ammonium oxidizing bacteria (AOB), then to $NO_3^-$ by nitrite oxidizing bacteria (NOB). Both AOB and NOB are litho-, auto-trophs, have a high requirement for oxygen for the ammonium conversion (Grady *et al.*, 1999; Picioreanu *et al.*, 1997). In contrast, denitrification is carried out by nitrate reducing

bacteria (NRB) in the absence of oxygen, whereas organic carbon is required as the energy source (Bitton, 2005). Among all reactions, the ammonium oxidation is considered the rate-limiting step of the overall nitrification-denitrification process. The known AOB mainly belong to β-*Proteobacteria* (such as *Nitrosomonas, Nitrosospira*) and γ-*Proteobacteria* (such as *Nitrosococcus*) (Allison *et al.*, 1999; Purkhold *et al.*, 2000; Schmidt *et al.*, 2003; Wheaton *et al.*, 1994).

$NH_4^+$ oxidation using electron acceptors other than oxygen has been also reported. In the anaerobic ammonium oxidation (anammox), $NH_4^+$ is oxidized coupling with nitrite reduction by members of the Planctomycetes such as *Brocadia anammoxidans*, *Kuenenia stuttgartiensis*, *Scalindua brodae* (Jetten *et al.*, 2001; Lei *et al.*, 2008; Perxas, 2005; Strous *et al.*, 1999). Anammox microorganisms are characterized by slow growth rate and low biomass yield, the doubling time is 10.6 days, and accordingly, the estimated specific growth rate is 0.003/h (Strous *et al.*, 1998; Water, 2011).

In recent years, a novel anaerobic ammonium oxidation process coupled with ferric iron reduction (feammox), was documented in different ecological habitats such as forested riparian wetland, tropical forest soil (Clement *et al.*, 2005; Shrestha *et al.*, 2009), and is considered as a significant mechanism of nitrogen loss in wetland environments. Feammox process was also reported in laboratory-scaled biological reactors (Sawayama, 2006; Yang *et al.*, 2012). Depending on the environmental pH, $NH_4^+$ would be converted to $NO_2^-$, $NO_3^-$ or $N_2$ (Clement *et al.*, 2005; Huang and Jaffé, 2015; Sawayama, 2006; Yang *et al.*, 2012). Utilizing ferric iron as the electron acceptor for the $NH_4^+$ oxidation, feammox is proposed to be an alternative technology for $NH_4^+$ removal from wastewater.

To present, little is known about feammox microorganisms. Culture independent approach has led to several known genera such as *Exiguobacterium*, *Pseudomonas*, *Carnobacterium*, *Anaeromyxobacter*, *Geobacter* (Sawayama, 2006; Ding *et al.*, 2017). The first and only pure feammox strain A6 belonging to *Acidimicrobiaceae* was reported by Huang and Jaffe in 2015. This organism grows under acidic condition (at pH 5) and oxidizes $NH_4^+$ to $NO_2^-$ using $Fe^{3+}$ as the electron acceptor (Huang, Jaffé, 2015).

In this study, we describe iron-reducing bacterial strains that were isolated from a laboratory-scaled bioreactor operated under feammox principle at neutral pH. The strains belonged to the β- and γ-proteobacteria, possessed versatile metabolic lifestyles, i.e. aerobic ammonium oxidation, anaerobic $Fe^{3+}$, and $NO_3^-$ reduction with electrons from organic compounds. These newly isolated strains would play certain roles in the ammonium removal under heterotrophic feammox condition.

## MATERIALS AND METHODS

### Isolation of bacteria from the laboratory-scaled feammox system

The laboratory-scaled feammox system operated continuously using an influent with $NH_4^+$ concentration of 50 mg/L and $Fe^{3+}$ in the form of ferrihydrite at concentration of 30 mM. Organic carbon was provided (as sodium acetate) at the concentration of 70 mg/L. pH of the medium throughout the operation was maintained at 7 ± 0.2. For the isolation of bacteria, 1 g sludge sample was taken from the system, shake vigorously before performing a dilution series in anoxic 1× PBS buffer. Afterward, 0.1 mL of each dilution was inoculated in anoxic semi-liquid agar (1% w/v) FM medium with following composition: NaCl, 1; $MgCl_2.6H_2O$, 0.4; $CaCl_2.2H_2O$, 0.15; KCl, 0.5; $MgSO_4.7H_2O$, 0.25; $NH_4Cl$, 0.25; $KH_2PO_4$, 0.2; distilled $H_2O$, 1 L (Widdel and Bak, 1992). The medium was supplemented with $NH_4^+$ (50 mg/L) as electron donor and $Fe^{3+}$-citrate (30 mM) as electron acceptor, Na-acetate (1 mM) as carbon source. The agar tubes were incubated in

upside down position at 28 – 30°C for 2 – 3 weeks. Single bacteria colonies were picked by glass capillaries and transferred into airtight tubes containing fresh anoxic liquid FM medium supplemented with the same energy and carbon sources.

**Analyses of 16S rDNA**

The genomic DNA of bacterial isolates was extracted using the method described by Zhou *et al*. with slight modifications (Zhou *et al.*, 1996). Briefly, 2 mL of the liquid culture was added to 5.4 mL DNA extraction buffer (100 mM Tris pH 8; 100 mM EDTA pH 8; 120 mM of phosphate buffer $Na_2HPO_4/NaH_2PO_4$; 1,5 M NaCl; 1% CTAB) in a 15 mL falcon tube. Afterward, cells were treated with proteinase K (40 µL of 10 mg/mL) at 37°C for 30 min (under shaking horizontally at 225 rpm), then SDS (600 µL of 20%) at 55°C for 1 h. Extraction was performed by adding an equal volume of phenol:chloroform:isoamine alcohol (25:24:1, v/v), mixing and centrifugation at 5,000 rpm for 10 min at room temperature. Genomic DNA was precipitated using 0.6 volume of isopropanol, incubated at room temperature for 1 h, then collected by centrifugation at 12,000 rpm for 25 min at room temperature. Finally, the DNA was rinsed in 80% ethanol, air dried and dissolved in 50 µL of sterile MQ water.

The 16S rDNA gene was amplified using primers 27F (AGAGTTTGATCCTGG CTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Weisburg *et al.*, 1991). The reaction mixture (25 µL) contained 13.5 µL of PCR water, 2.5 µL of reaction buffer 10×, 2.5 µL of BSA (3 mg/mL), 2 µL of $MgCl_2$ (20 mM), 2 µL of dNTP(2.5 mM), 0.5 µL of each primer (50 pmol/µL), 2.5 U of *Taq* DNA polymerase (Promega) and 1 µL of template DNA (10 ng/µL).

Thermocyles for the PCR included denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 1.5 min and completed by a final extension at 72°C for 7 min. The PCR products were then analyzed by electrophoresis on 1% agarose gel. Prior to sequencing, the PCR products were purified by PCR-purification Kit (Bioneer). The sequencing was performed on an ABI 3110 Avant Appied Biosystems sequencer.

The 16S rDNA gene sequences were compared with related sequences available on the GenBank/DDBJ databases by using the BLAST Search tool. The alignment of sequences was performed with the 16S rDNA sequences by using CLUSTAL_X program. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). Topography of the constructed tree was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

**Physiological studies**

Studies on aerobic ammonium oxidation were performed in Erlenmeyer bottles containing FM medium supplemented with $NH_4^+$ (10 mM) as the electron donor and sodium acetate (1 mM) as carbon source. The culture bottles were incubated at 30°C with shaking at 120 rpm. The bacterial activity was assessed via the amount of $NH_4^+$ oxidized, comparing with sterile control.

Anaerobic growth of isolates under ferric iron reduction condition was examined in gas tight serum bottles containing FM medium supplemented with $Fe^{3+}$ (30 mM, in chelated form Fe-citrate or insoluble form ferrihydrite) as the electron acceptor. The ferrihydrite slurry was prepared according to Raterring (1999). As electron donor, $NH_4^+$ or sodium acetate (10 mM) was added. The bacterial activity was assessed via the amount of $Fe^{2+}$ generated in the medium as the product of $Fe^{3+}$ reduction, comparing with sterile control.

Similarly, the bacterial nitrate reduction was examined in anoxic FM medium supplemented with $NO_3^-$ (5 mM) as electron acceptor and sodium acetate (10 mM) as electron donor. The bacterial activity was assessed via the amount of $NO_3^-$ reduced in the medium.

**Chemical analyses**

Ammonium concentration was determined photometrically by using sodium nitroprusside

reagent with a calibration curve obtained from a serial dilution of $NH_4Cl$ solution (using previously dried $NH_4Cl$ crystals) in the range of 0.018–1.8 mg/L (DIN-38406-5, 1983).

Ferrous iron concentration was determined photometrically by using O-phenanthrolin reagent with a calibration curve obtained from a serial dilution of $FeSO_4$ solution in the range of 0.28–2.8 mg/L (DIN-38406-E1, 1983).

Nitrate concentration was determined by using NitraVer® 5 Nitrate Reagent Powder Pillows (Hach Instruments Inc., USA) with a calibration curve obtained from a serial dilution of $NaNO_3$ solution in the range of 0–30 mg/L. Using NitriVer® 3 Nitrite Reagent Powder Pillows (Hach Instruments Inc., USA) to determine nitrite concentration. The nitrite calibration curve obtained from a serial dilution of $NaNO_2$ solution in the range of 0–150 mg/L.

Unless otherwise stated, all measurements were performed in three replicates. The data were processed using Microsoft Excel software (for average and standard deviation functions) and graphed by SigmaPlot 14 software.

## RESULTS AND DISCUSSION

### Bacterial isolation and phylogenetic identification of the isolates

Isolation procedure carried out in anoxic semi-liquid agar tubes using $NH_4^+$ as the electron donor (10 mM), Fe-citrate as the electron acceptor (30 mM), and sodium acetate (1 mM) as the carbon source has resulted in two bacterial isolates, strains FN7 and FN9. Under phase-contrast microscope, strain FN7 was observed as actively moving short rods of $1 – 1.5 \times 0.4 – 0.5$ µm in size (Fig. 1A), whereas strain FN9 comprised of slowly moving short rods of $2.1 – 3.3 \times 0.6 – 0.7$ µm in size (Fig. 1B).

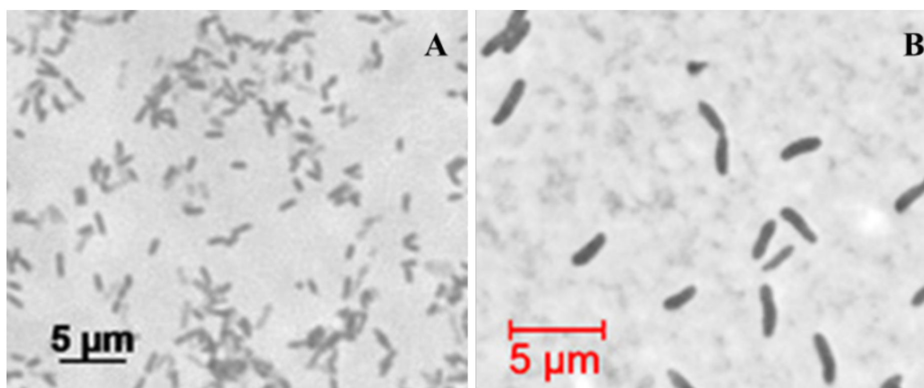



**Figure 1.** Cell morphology of the new isolates, strain FN7 (A) and strain FN9 (B) observed under phase-contrast microscope.

Comparative analyses of 16S rDNA sequences of the two isolates revealed that strain FN7 belonged to the genus *Alicycliphilus*, the most closely related species was *A. denitrificans* (99% sequence homology). Strain FN9 belonged to the genus *Pseudomonas*, the most closely related species was *P. stutzeri* (99% sequence homology). The 16S rDNA sequences of strains FN7 and FN9 were deposited at GenBank under the accession numbers LC474369 and MT568614, respectively. Phylogenetic positions of the new isolates are illustrated in phylogenetic trees reconstructed from the 16S rDNA sequences of theses strains and the related species available on GenBank (Fig. 2).

*Alicycliphilus denitrificans* was first isolated by Mechichi *et al.* in 2003 and was described as a cyclohexanol-degrading, nitrate-reducing β-proteobacterium (Mechichi *et al.*, 2003). The type strain K601$^T$ of this species was characterized with the capability of utilizing a broad range of organic substrates using oxygen

as the electron acceptor and growing by nitrate reduction while oxygen was not available. Later, genome of strain $K601^T$ together with genome of another *A. denitrificans* train BC were sequenced to get insight into physiology of the species (Oosterkamp *et al.*, 2013). The large number of mono- and di-oxygenase genes in the genomes suggests that *A. denitrificans* would have broader substrate range than known so far.

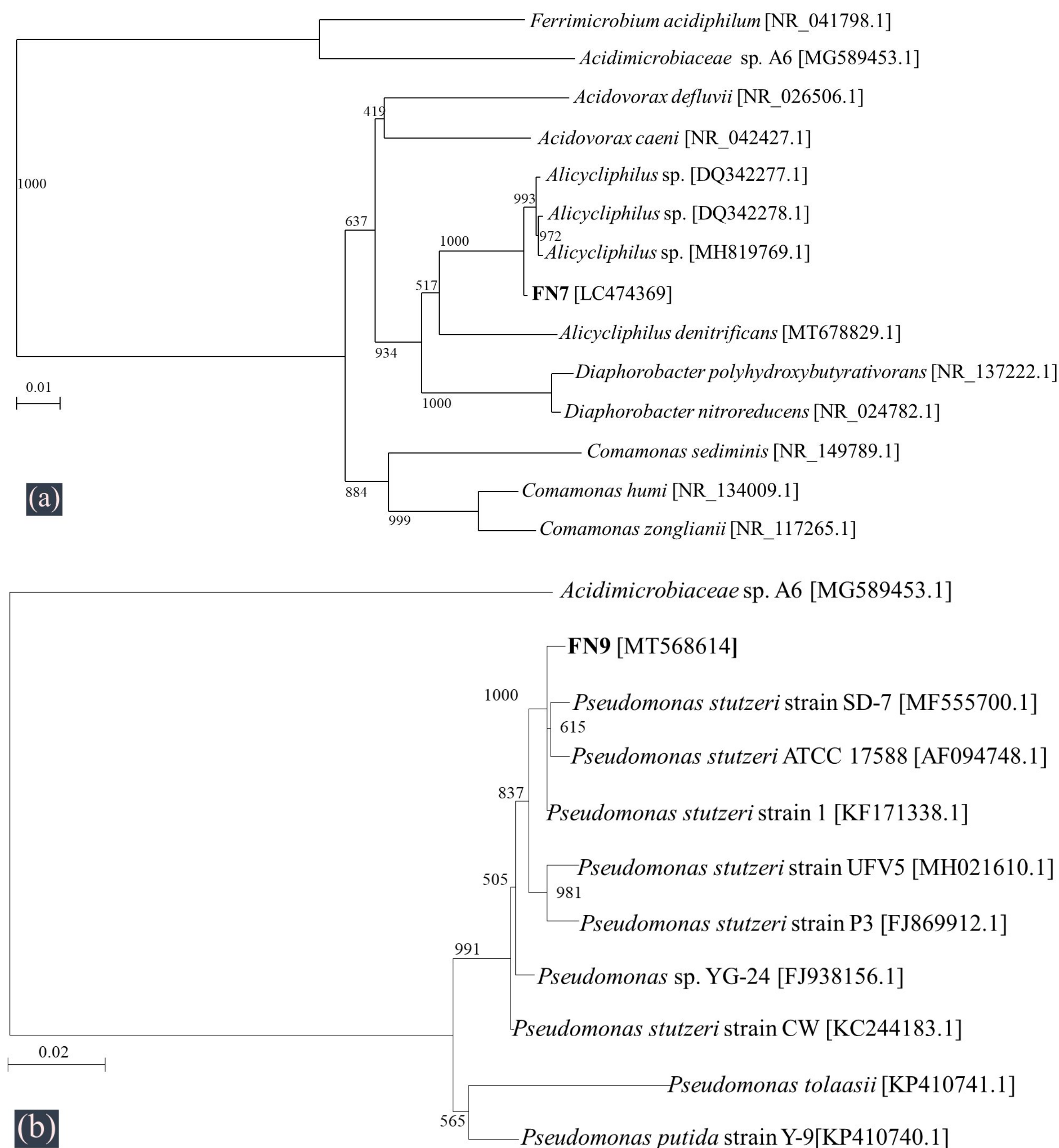



**Figure 2.** Phylogenetic trees showing positions of the new isolates strain FN7 (a) and strain FN9 (b) in relationship with relates species. The tree was reconstructed using neighbor-joining method and its topography was evaluated by bootstrap analysis with 1000 replicates. *Acidimicrobiaceae* sp. A6 was used as outgroup.

*Pseudomonas stutzeri* is a γ-proteobacterium capable of nitrate reduction and heterotrophic ammonium oxidation (Silva *et al.*, 2020). This bacterium thrives in many habitats, including

contaminated municipal and industrial effluents. *P. stutzeri* strain UFV5 was reported for efficient removal of ammonium up to 57% within 24 hours of incubation, much higher than that by the activated sludge (Silva *et al.*, 2020).

Since the new isolates strains FN7 and FN9 were obtained from a laboratory-scaled feammox system, it was of special interest to study their roles related to the conversion of nitrogen and iron species in the growth media.

**$NH_4^+$ oxidation**

Ammonium oxidation by the new isolates was examined under aerobic and anaerobic iron reduction conditions. The obtained results showed that both strains oxidized ammonium effectively in the presence of oxygen (Fig. 3), but not under anaerobic iron reduction condition (not shown).

The ammonium oxidation property was demonstrated for *A. denitrificans* and *P. stutzeri* strains (Klodowska *et al.*, 2018; Silva *et al.*, 2020), thus it is expected that the new isolates also possessed this characteristic. After 2 days, strain FN7 removed more than 90% ammonium in the medium, whereas a removal efficiency of 54.7% was recorded for strain FN9. In comparison with other heterotrophic ammonium oxidizing bacteria (hAOB), strains FN7 and FN9 were considered of high and moderate efficiency, respectively (Klodowska *et al.*, 2018; Silva *et al.*, 2020).

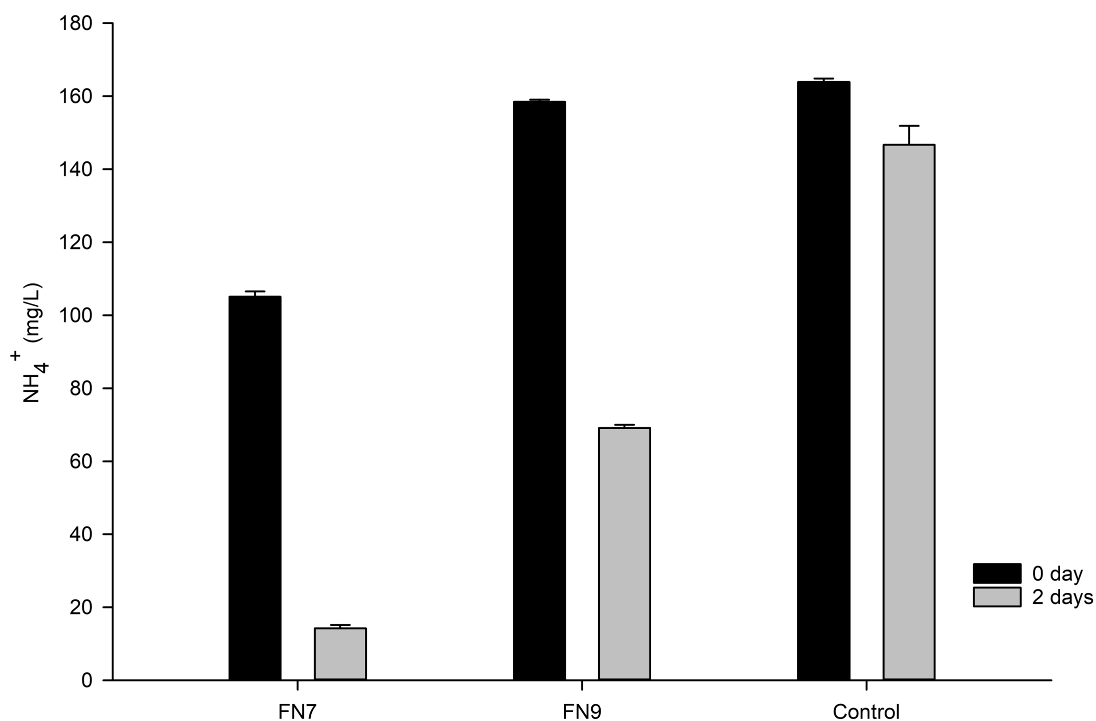



**Figure 3.** Ammonium oxidation by strain FN7 and strain FN9 under aerobic condition. Sodium acetate (1 mM) was supplied to the medium as the organic carbon source.

**$NO_3^-$ and $Fe^{3+}$ reduction**

In the absence of oxygen, strains FN7 and FN9 reduced nitrate while oxidized sodium acetate for ATP generation (Fig. 4). The ability of nitrate reduction was reported for *A. denitrificans* and *P. stutzeri*, who used to perform denitrification lifestyle in natural habitat when oxygen is limited (Klodowska *et al.*, 2018; Silva *et al.*, 2020). In this study, strain FN7 removed

almost all nitrate in the medium, whereas strain FN9 removed ~85% of nitrate in the medium.

It should be noted that the denitrification property of the new isolates together with their capability of heterotrophic ammonium oxidation would provide an efficient mechanism of $NH_4^+$ removal, alternative to the conventional autotrophic nitrification/denitrification principle.

Under the ferric iron reduction condition, both strains FN7 and FN9 reduced $Fe^{3+}$ actively, and generated $Fe^{2+}$ into the medium (Fig. 5). More importantly, both strains reduced $Fe^{3+}$ in the form of chelated-, soluble ferric-citrate (Fig. 5A) as well as in the form of insoluble ferrihydrite (Fig. 5B).

The capability of ferric iron reduction was reported previously for *Pseudomonas* spp. and some other genera of the γ-proteobacteria such as *Sewanella* spp., *Acidibacter* spp. (Falagán & Johnson, 2014; Nielsen *et al.*, 2002). On the other hand, *A. denitrificans* was reported for the anaerobic growth under nitrate and chlorate reduction condition (Atashgahi *et al.*, 2018; Weeklink *et al.*, 2008), but not under ferric iron reduction condition. Strains FN7 and FN9 reported in this study would be of interest for further study on biological conversion processes that took place in the feammox system operating at neutral pH.

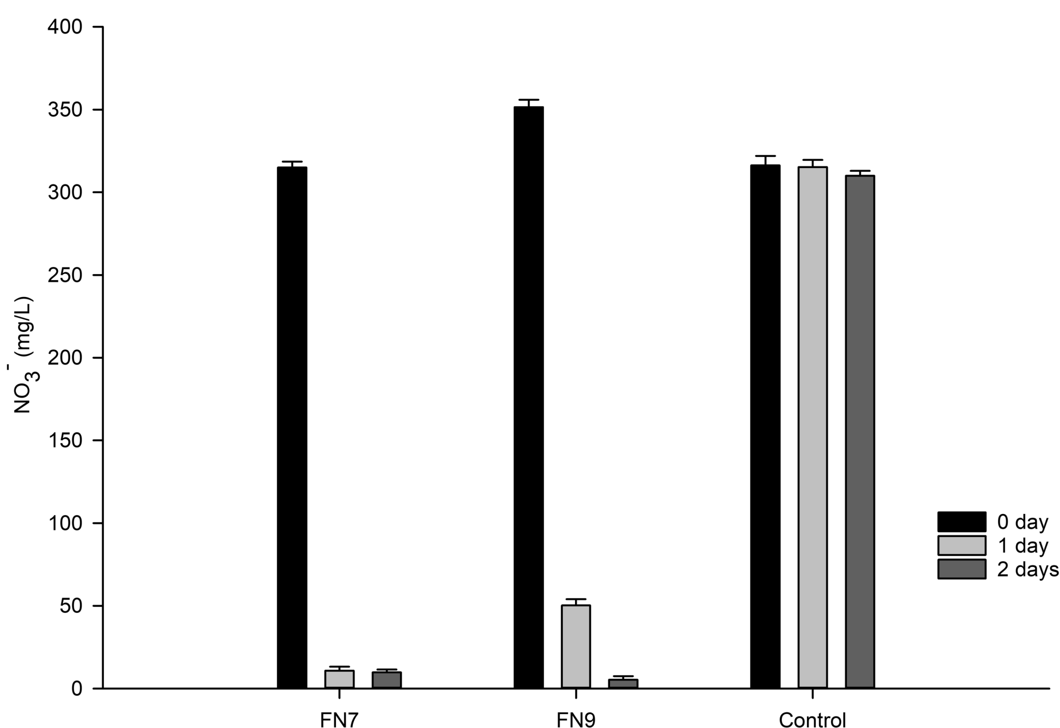
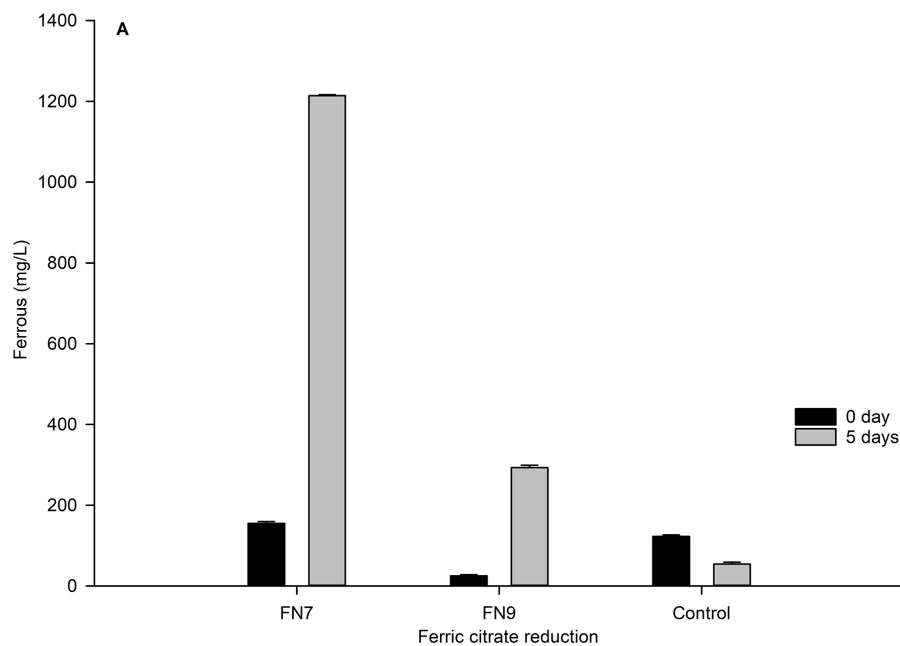



**Figure 4.** Nitrate reduction by strains FN7 and FN9 comparison to controls without bacteria.



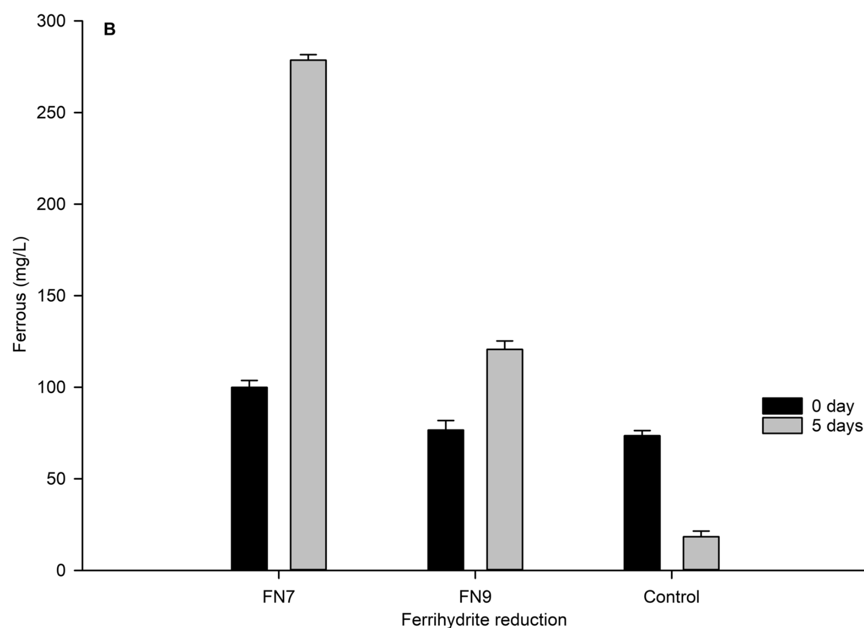




**Figure 5.** Ferric iron reduction by the new isolates, strain FN7 and strain FN9in comparison to controls without bacteria.

CONCLUSION

Two bacterial strains isolated from sludge of laboratory-scaled feammox system operated at neutral pH were designated as *Alicycliphilus denitrificans* FN7 and *Pseudomonas stutzeri* FN9. Despite being apart in phylogenetic affiliation, both strains shared several common

physiological characteristics, i.e. (i) heteroptrophic ammonium oxidation, (ii) denitrification, and (iii) ferric iron reduction. These isolates are proposed to play certain roles in the studied feammox system, contributing to ammonium removal under feammox condition. The 16S rDNA sequences of strains FN7 and FN9 were available in GenBank under the accession numbers LC474369 and MT568614, respectively.

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# VI KHUẨN KHỬ SẮT β- VÀ γ-PROTEOBACTERIA ĐƯỢC PHÂN LẬP TỪ BỂ PHẢN ỨNG SINH HỌC FEAMMOX DỊ DƯỠNG QUY MÔ PHÒNG THÍ NGHIỆM

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[3]*Trường Đại học Khoa học tự nhiên, Đại học Quốc gia Hà Nội*

TÓM TẮT

Loại ammonium là bước quan trọng của quá trình xử lý nước thải. Các công nghệ hiện nay dựa trên nguyên lý nitrate hóa/khử nitrate và nitrite hóa bán phần/anammox đều có nhu cầu sử dụng oxy ở bước nitrite hóa, do vậy chưa đáp ứng hoàn toàn yêu cầu xử lý trong thực tế. Trong vài năm gần đây, quá trình oxy hóa ammonium sinh học kết hợp với khử sắt (feammox) được đề xuất là đóng vai trò quan trọng trong việc thất thoát nitơ ở các hệ sinh thái ngập nước. Liên quan đến lĩnh vực nước thải, nguyên lý feammox cũng đang được quan tâm xem xét như một cách tiếp cận khác để loại ammonium mà hoàn toàn không phụ thuộc vào oxy. Từ hệ feammox quy mô phòng thí nghiệm vận hành ở pH trung tính, hai chủng vi khuẩn FN7 và FN9 đã được phân lập thông qua sử dụng kỹ thuật Hungate kỵ khí. Phân tích so sánh trình tự 16S rDNA dẫn đến kết luận hai chủng này có quan hệ gần gũi tương ứng với loài β-Proteobacterium *Aciclyphilus denitrificans* và loài γ-Proteobacterium *Pseudomonas stutzeri*. Mặc dù cách biệt về vị trí phân loại, hai chủng FN7 và FN9 chia sẻ một số đặc điểm sinh lý chung liên quan đến quá trình feammox, như (i) oxy hóa ammonium dị dưỡng, (ii) khử nitrate, và (iii) khử sắt. Các chủng này có thể đóng vai trò nhất định trong hệ feammox được nghiên cứu và đóng góp vào việc loại ammonium ở điều kiện feammox dị dưỡng. Trình tự 16S rDNA của hai chủng FN7 và FN9 được ký gửi tại GenBank với mã số tương ứng là LC474369 và MT568614.

**Từ khóa:** *oxy hóa ammonium, khử sắt*, *Feammox,* beta-proteobacterium *Aciclyphilus denitrificans,* gamma-proteobacterium *Pseudomonas stutzeri*