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(Research Paper)

Enhanced Nitrogen Removal by a Heterotrophic Nitrifying-Aerobic Denitrifying Bacterial Consortium with Potential for the Treatment of High-strength Ammonium Wastewater

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Abstract

Introduction: Nitrogen compounds are used in various industries and cause pollution of water and soil. As a result, the bioremediation of nitrogen compounds from the environment becomes an important issue. In this way, the use of microorganisms as a consortium has enhanced bioremediation. This study aimed to remove inorganic nitrogen forms by heterotrophic nitrifying-aerobic denitrifying (HN-AD) bacterial consortium and compare it to pure cultures.

Materials and Methods: HN-AD bacteria were isolated from soil and aquarium water and identified. After that, the bacterial consortium was prepared from two isolated bacteria and two strains from the microbiological culture collection of the University of Isfahan (Iran). The ability to remove ammonium, nitrite, and nitrate was assayed using spectrophotometry for both pure cultures and the consortium.

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Results: *Pseudomonas mendocina* AquaN and *Pseudomonas monteilii* Nht were isolated and identified for their efficient capabilities in HN-AD. The bacterial consortium including two isolated bacteria and *Acinetobacter calcoaceticus* SCC2, *Rhodococcus erythropolis* R1 was able to consume NH_4^+-N (85.6 mg.L^{-1}), NO_2^--N (23.1 mg.L^{-1}), and NO_3^--N (102.5 mg.L^{-1}) as sole nitrogen sources by removal efficiencies 98.4%, 90.5%, and 40.0%, respectively that were better than pure cultures performances. Specifically, the consortium was capable of removing 250 to 2045 mg.L^{-1} NH_4^+-N with up to 95% efficiency within 48h.

Discussion and Conclusion: The compatibility of the strains as a consortium was considerable and the bacterial consortium showed significant performances in nitrogen removal, especially the removal of 2045 mg.L^{-1} NH_4^+-N. These findings indicated the bacterial consortium could be a promising candidate for the treatment of high-strength ammonium wastewater.

Keywords: *Acinetobacter*, Consortium, Heterotrophic nitrification, High-strength ammonium removal, *Pseudomonas*, *Rhodococcus.*

Introduction

Nitrogen compounds are used in a variety of industrial and agricultural processes. Due to their wide use, they are frequently found in soil, air, wastewater, and aquatic environments (1). Inorganic nitrogen is present in ammonium, nitrite, and nitrate forms. Ammonium lowers the quality of drinking water, and makes a serious danger to human health because of eutrophication, is toxic to aquaculture, and changes the structure of microbial communities (2, 3). High nitrate concentrations can cause methemoglobinemia or blue baby syndrome in newborns. In addition, nitrate is reduced to nitrite and carcinogenic chemicals nitrosamines in the digestive system, which cause several forms of cancer, including gastric cancer (4, 5).

The concentration of ammonium nitrogen is 1-10 mg.L^{-1} in the soil without fertilizer, 50 mg.L^{-1} in areas contaminated with agricultural fertilizers and 1000 mg.L^{-1} in industrial wastewater (6). In sum, due to the concentration of ammonium in soil, and all the existing problems need to be paid attention to nitrogen removal in wastewater.

Nitrogen forms in the water and wastewater can be removed by physicochemical and biological methods. Physicochemical treatments include reverse osmosis, ion exchange, electrodialysis, and activated carbon technology systems (7). Biological methods can perform in the conventional way that autotrophic bacteria are involved. Conventionally, ammonium is oxidized to nitrite and nitrate under aerobic conditions by autotrophic nitrifying bacteria. Nitrate could be transformed to nitrogen gaseous by heterotrophic nitrate-reducing bacteria under anaerobic conditions (8). This method is time-consuming and small variations in temperature, pH, and substrate concentration affect the activity and growth of autotrophic nitrifiers (9, 10).

During the past few years, a new method, heterotrophic nitrification-aerobic denitrification (HN-AD) has been studied. HN-AD is done by heterotrophic bacteria such as *Pseudomonas* (8), *Acinetobacter* (11), *Alcaligene*s (12), *Klebsiella* (13), and *Bacillus* (14). In this manner, ammonium can be converted into gaseous nitrogen under aerobic conditions in one reactor (12). HN-AD bacteria generally remove nitrogen by

using carbon sources like sodium citrate, sodium acetate, glucose, and sodium succinate (9). In comparison to the conventional method, HN-AD exhibited a fast-growing rate, simultaneous ammonium and nitrate or nitrite removal, less contamination, and cost-effectiveness (15). There are different biochemical pathways of HN-AD in bacteria (16).

Most studies about HN-AD were done in pure culture of bacteria; less attention has been paid to co-culture or consortium of HN-AD bacteria. The application of microbial consortium is beneficial over pure culture because synergistic interactions between different strains can provide the use of complex carbon sources, increase each other's growth by cross-feeding, managing their environment, and enhancing productivity (17). The consortium of HN-AD bacteria with various nitrogen removal pathways may be more efficient than one pure culture in complex environments (7).

This study focused on the HN-AD consortium with high nitrogen removal efficiency. The specific goals of the present study were 1) isolation of HN-AD bacteria, 2) assay strains interactions, 3) determination of the cell growth and nitrogen removal characteristics of pure cultures and bacterial consortium, and 4) assay high-strength ammonium removal by HN-AD bacterial consortium.

Materials and Methods

Bacterial strains: The HN-AD bacteria were isolated from the soil of the garden area at the University of Isfahan and the aquarium freshwater of the aquaculture center in Isfahan, Iran. In addition, two strains from the microbiological culture collection of the University of Isfahan (Iran) *Rhodococcus erythropolis* R1 (GU570564.1) (18) and *Acinetobacter calcoaceticus* SCC2 (KU685396.1) (19) were used in this research.

Media and Chemicals: Modified heterotrophic nitrification medium (HNM) contained ($g.L^{-1}$ of distilled water): $(NH_4)_2SO_4$ 0.33, sodium succinate 2.8, KH_2PO_4 0.5, Na_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.2, NaCl 3.0, trace element solution 2.0 mL, and pH 7 ± 0.2 (26). Nitrite denitrification medium (DM-1) and nitrate denitrification medium (DM-2) are based on HNM but DM-1 consisted of $NaNO_2$ (0.05 $g.L^{-1}$) and DM-2 contained KNO_3 (0.722 $g.L^{-1}$) instead of $(NH_4)_2SO_4$ as a nitrogen source. The trace element solution contains ($g.L^{-1}$ of distilled water) EDTA 57.1, $ZnSO_4$ 3.9, $CaCl_2$ 7, $FeSO_4.4H_2O$ 5, $(NH_4)_6Mo_7O_{24}.4H_2O$ 1.1, $CoCl_2.6H_2O$ 1.6, and $CuSO_4.5H_2O$ 1.6 (20). The Luria Bertani (LB) broth contained ($g.L^{-1}$) peptone 10.0, yeast extract 5.0, and NaCl 10.0. Solid media were prepared from mentioned media with an addition of 1.5% agar. The media were sterilized by autoclave for 15 min at 121˚C. Nessler's reagents were purchased as Merck nessler's reagents 109011 and 109028 from Merck Germany. Griess's reagent was purchased as Griess's reagent 03553 from Sigma-Aldrich USA. All the chemicals used were of analytical grade.

Isolation of HN-AD Bacteria: To enrich heterotrophic nitrification-aerobic denitrification bacteria, at first, the soil sample (10 g) was added to 100 mL Ringer's solution and then shaken at room temperature. Secondly, 5 mL of soil solution was mixed with 95 mL HNM and incubated at 30˚C on a rotary shaker at 120 rpm (modified (21)). Aquarium water samples (50 mL) were added to 250 mL conical flasks containing 50 mL sterile liquid HNM and incubated at 30˚C on a rotary shaker at 120 rpm. After three days, 5 mL of the cell suspension from each culture was transferred to 95 mL fresh HNM and incubated at 30˚C and 120 rpm for three days. The enriched bacterial cultures were gradient-diluted, and 0.1 mL of each diluted

medium was spread on solidified HNM medium and incubated at 30˚C for two days under aerobic conditions. Each separate colony was isolated and purified. The nitrification ability of the isolates was determined in HNM broth and the isolates with the maximum nitrification ability were selected (8).

Molecular and Biochemical Identification: The best two isolates were identified by morphological, biochemical, and molecular methods. Gram staining and biochemical tests include catalase, SH_2 production, indole, anaerobic nitrate reduction, V-P test, methyl red test, urease, oxidase, hydrolysis of starch, O/F test, using citrate, production of acid from glucose, fructose, lactose, and mannitol (22).

16S rRNA genes of the selected isolates were amplified by PCR for molecular identification. The DNA was extracted from bacterial suspension by a DNA extraction kit (Cinnagen Co., Iran). The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used (23). The PCR program was 35 cycles and steps were carried out as follows: denaturation at 95˚C for 5 min, 94˚C for 45 seconds, annealing at 55˚C for 1 min, extension at 72˚C for 1 min; and final extension at 72˚C for 7 min. The PCR products were analyzed by electrophoresis on the 1% agarose gel and sequenced by the Sanger method (Model ABI 3730 XL, FAZA Biotech Co., Iran). To compare sequences with other microorganisms, sequence alignment was applied by using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The 16S rRNA gene sequences were submitted to the GenBank database. A neighbor-joining tree was created using the MEGA 6.0 program.

Inoculum Preparation and Culture Conditions: To prepare the inoculum, a single pure colony of each strain was transferred to 20 mL of fresh media including HNM, DM-1, and DM-2 as pre-cultures. All media were incubated at 30°C with shaking at 120 rpm to cells reached the middle of the logarithmic phase and the cell concentration became equal to 0.5 McFarland. Afterward, 5 % (v/v) of inoculum was transferred to an Erlenmeyer flask containing 95 ml of HNM, DM-1, and DM-2 broth media. To make consortium inoculation, inoculum solutions of strains were mixed in equal volumes and the total inoculum size reached 5% (v/v).

Heterotrophic Nitrification/Aerobic Denitrification Capability of Pure Cultures: To assess the capabilities of ammonium, nitrite, and nitrate removal, 5% (v/v) of each pure culture suspension was inoculated into HNM (100 $mg.L^{-1}$ of NH_4^+-N), DM-1 (10 $mg.L^{-1}$ NO_2^--N), and DM-2 (110 $mg.L^{-1}$ of NO_3^--N), respectively. The flasks were incubated at 30˚C and 120 rpm under aerobic conditions. A medium without inoculation was used as a control for each examination. The samples were periodically taken to determine the concentration of NH_4^+-N, NO_2^--N, NO_3^-N, and optical density at 600 nm (OD_{600}). After 24 h incubation, total nitrogen (TN) was measured in all media.

Bacterial Consortium Design

Consortium Pre-Tests: To find out the interactions between the strains, two tests were done. The first test was the Bilayer culture that one strain was cultivated as a circular form on the nutrient agar plate center, and then the second strain was cultivated by the pour plate method on the top layer (24). The cross streak method was used as the second test that one strain was cultivated as a linear form on the center of HNM agar and the other strains were cultivated vertically by 1 cm space from the line of the culture (modified (25)).

Assessment of Nitrogen Removal Capability of Bacterial Consortium

Assessment of Nitrogen Removal Capability of Bacterial Consortium in the Growth Phase: To determine the performance of nitrogen removal by the bacterial consortium in the growth phase, strains were inoculated to HNM, DM-1, and DM-2. Samples were taken regularly to determine NH_4^+-N, NO_2^--N, NO_3^-N, and OD_{600}. Media without inoculation were used as controls.

Assessment of Ammonium Removal by Resting Cells of Bacterial Consortium: For the preparation of consortium resting cells, 1% (v/v) inoculum of 0.5 McFarland concentration of each strain was inoculated to 50 ml of LB Broth in 250 ml Erlenmeyer flasks and incubated at 30˚C and 120 rpm agitation. At the end of the exponential growth phase, cells were harvested by centrifugation (Z 300 k, Hermle Labortechnik. Germany) at 6000 rpm and 14˚C for 6 min. The pellets were washed twice with sodium phosphate buffer (pH 7.2). Afterward, prepared resting cells of all strains were resuspended in 100 mL of HNM. The OD_{600} of the resting cell suspension was adjusted to 2.3. After incubation at 30˚C and 120 rpm, samples regularly were taken at 2 h intervals to evaluate NH_4^+-N, NO_2^--N, and NO_3^--N. Total nitrogen (TN) was measured at the end of the incubation period.

High-strength Ammonium Removal Performance by Bacterial Consortium: To measure the high-strength ammonium removal efficiency of the bacterial consortium, the initial NH_4^+-N concentration was 250, 425, 575, 740, 990, 1150, 1650, and 2045 $mg.L^{-1}$. All the experiments were carried out with 5 % (v/v) inoculation, and then the flasks were incubated at 30℃ and 120 rpm agitation. Nitrification rate and efficiency were calculated after 24 and 48 h incubation. The nitrification capability of each strain in the medium contained 2045 $mg.L^{-1}$ of NH_4^+-N was also assayed for comparison with the consortium.

Analytical Methods: To measure some parameters, the samples were centrifuged (Sigma 1-14, Sigma Laborzentrifugen, Germany) at 11000 rpm for 4 min and filtered if required. Levels of total nitrogen and NO_3^--N were measured according to the standard methods (26); briefly, total nitrogen was assayed based on the persulphate digestion method. NO_3^--N was calculated by the absorbance at 220 nm and subtracting two times the absorbance at 275 nm. NH_4^+-N was determined by Nessler's reagent spectrophotometry at a wavelength of 420 nm (27). NO_2^--N was determined by Griess's reagent spectrophotometry at a wavelength of 540 nm (28). The pH was measured by a pH meter (TS-Technology, Iran). The cell growth was determined by spectrophotometry OD_{600} (PD-303, Apel, Japan).

Statistical Analysis: The data were presented as means ± standard deviation (SD) of three replicates. Nitrogen removal rate (N.R.R.) and nitrogen removal efficiency (N.R.E.) were calculated using the following formulas, respectively:

$N.R.R. = (C_0 - C_t)/t.$

$N.R.E. (\%) = 100 \times (C_0 - C_t) / C_t$

C_0 is the initial concentration ($mg.L^{-1}$) of nitrogen, C_t ($mg.L^{-1}$) is the final concentration at a given time, and t is the time (h) that the concentration of nitrogen becomes constant (29).

The statistical analysis was performed by one-way ANOVA with Duncan's test ($P < 0.05$) in SPSS Statistics 22.0 software. Graphical works were carried out by Microsoft Excel 2016.

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Results

Isolation and Identification of Isolates: After enrichment and screening, two strains were selected based on heterotrophic nitrification and aerobic denitrification performances. Nht isolate was a Gram-negative bacterium with rod-shaped, its colony on nutrient agar was cream in color, rounded with a regular edge, convex, and sticky. AquaN isolate was a Gram-negative rod-shaped bacterium and its colonies on nutrient agar were light pink, rounded with a regular edge, convex, and opaque. Both the Nht and AquaN isolates showed negative results for the production of SH_2, indole, anaerobic nitrate reduction, V-P test, methyl red test, and acid production from fructose, lactose, and mannitol. Both the Nht and AquaN isolates showed positive results for catalase, oxidase, urease, motility, and using citrate. The Nht isolate was positive in the acid production from glucose and it was negative for the O/F test. The AquaN isolate showed negative results for the production of acid from glucose and O^+/F^-.

The amplified 16S rRNA gene of Nht and AquaN isolates were shown in Figure 1. The BLAST results indicated that the Nht isolate was closely related to *Pseudomonas monteilii*, *P. taiwanensis*, *P. putida*, and *P. mosselii* with 99.71% similarity. Finally, the Nht isolate was identified as *Pseudomonas monteilii* by biochemical tests (Table 1). Also, the AquaN isolate was closely related to *Pseudomonas mendocina*, with a similarity of 99.29%. The partial 16S rRNA gene sequence data of Nht and AquaN strains have been deposited in "GenBank" with the accession numbers MN167837.1 and MN435149.1, respectively. The constructed phylogenetic trees of Nht and AquaN strains based on the 16S rRNA gene sequence of the isolates and some other phylogenetically related strains by the neighbor-joining method were shown in Figure 1. The topology of trees, supported by high bootstrap values, clearly showed that strain AquaN is within the *Pseudomonas mendocina*, and strain Nht is most probably similar to *Pseudomonas monteilii.*

The Nitrogen Removal Performance of Pure Cultures

Pure Cultures Nitrification Performances: The ammonium removal capability and the growth of four strains include *Pseudomonas monteilii* Nht, *Acinetobacter calcoaceticus* SCC2, *Pseudomonas mendocina* AquaN, and *Rhodococcus erythropolis* R1 were depicted in Figure 2. NH_4^+-N removal by the Nht strain was started after the lag phase (6 h) and completely removed at 9 h, but the cell growth was continued up to 18 h (Fig. 2a). Nitrite and nitrate were not produced by the Nht strain in this process and total nitrogen was reduced by 47.7%. The strain SCC2 removed NH_4^+-N during the log phase of cell growth after the short lag phase (3 h), and complete ammonium removal occurred at 9 h but cell growth continued to 21 h (Fig, 2b). No nitrite was produced during nitrification and just 5 $mg.L^{-1}$ nitrate was produced and total nitrogen was removed by 31.2% in this process. The AquaN strain removed NH_4^+-N in the log phase after 6 h start-up time (Fig, 2c). This strain completely degraded ammonium after 12 h, while cell growth continued up to 15 h. During this process, the AquaN strain did not produce nitrite and just 5 $mg.L^{-1}$ nitrate was produced at 15 h and then decreased. Total nitrogen was reduced by the AquaN strain by 30.7%. R1 strain was a slow-growing bacterium and its growth phase started after 6 h and removed ammonium from 6 h to 36 h and cell growth was stopped by ammonium ending (Fig, 2d). Nitrite was not produced during nitrification and just 4 $mg.L^{-1}$ nitrate was produced. No total nitrogen depletion was observed in the R1 strain after 24 h.

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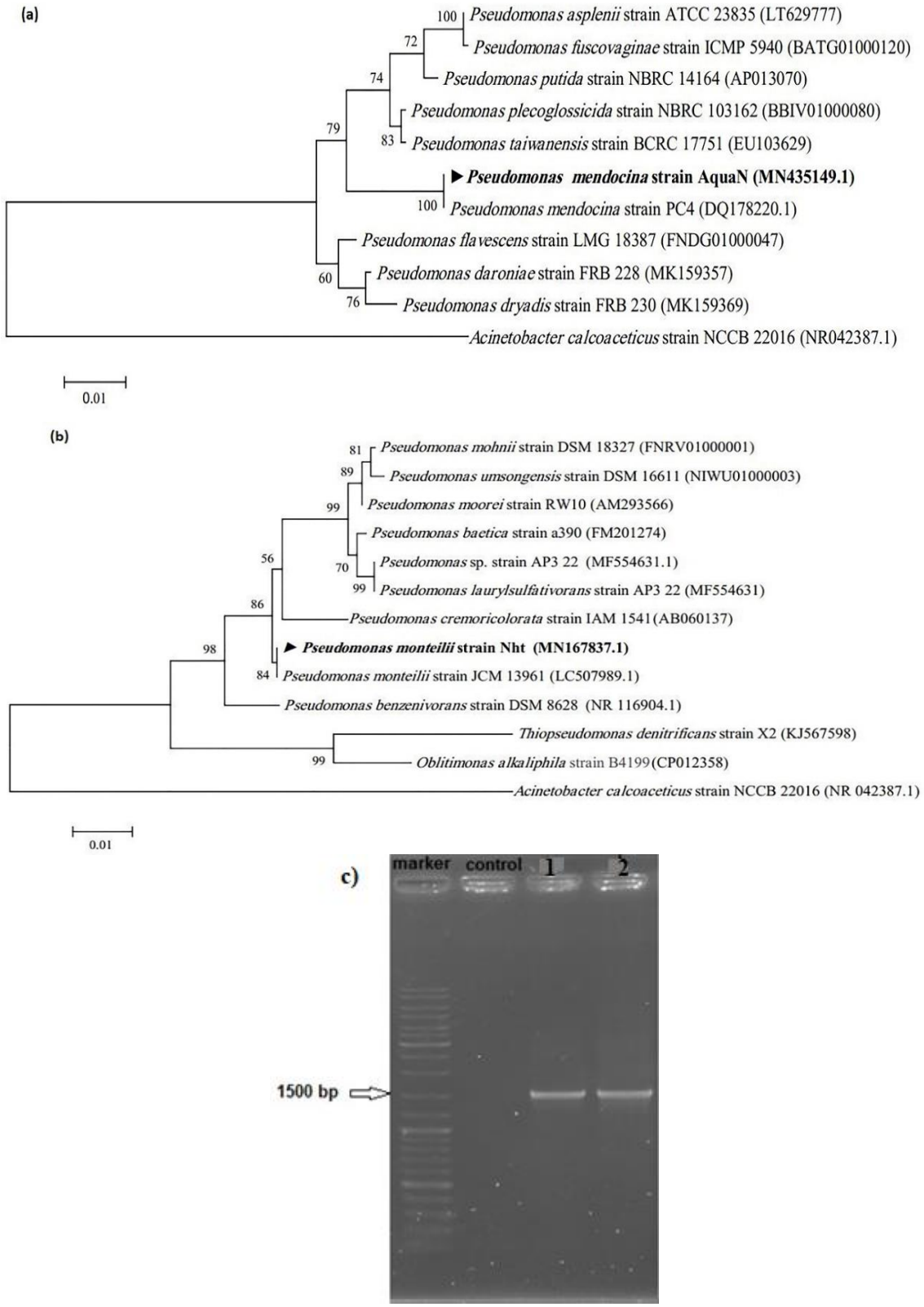

Fig. 1- The phylogenetic tree derived from the neighbor-joining analysis of partial 16S rRNA gene sequences. Bootstrap values (%) are based on 1000 replicates. Bar 0.01 substitutions per nucleotide position. (a,b). PCR amplification results for 16S rRNA genes (M: 10kb DNA Ladder; 1: *Pseudomonas mendocina* strain AquaN; 2: *Pseudomonas monteilii* strain Nht) (c).

Table 1- Differential Biochemical Characteristics of the Nht Isolate from Related *Pseudomonas* Type Strains

Bacterium	Growth on 6% NaCl	Mannitol utilizing	Growth at 45℃	Growth at 4℃
Isolate Nht	+	-	-	-
Pseudomonas monteilii	+	-	-	-
Pseudomonas taiwanensis	+	-	+	+
Pseudomonas mosselii	+	+	-	-
Pseudomonas putida	-	-	-	+
Pseudomonas plecoglossicida	-	-	-	-

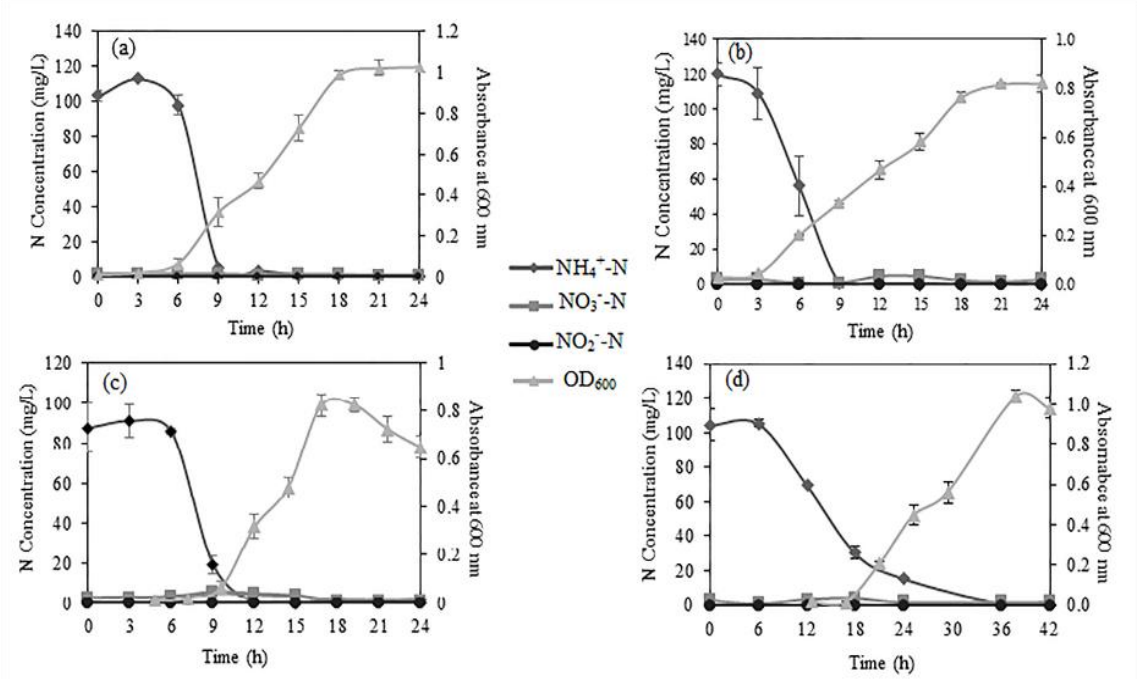

Fig. 2- Ammonium removal characteristics of pure cultures in HNM at 30 ˚C. The inoculum size was 5% v/v. a: *P. monteilii* Nht, b: *A. calcoaceticus* SCC2, c: *P. mendocina* AquaN d: *R. erythropolis* R1. Error bars indicate the standard deviation of the mean for three replicates. Figures characterize NH_4^+-N (diamond), NO_2^--N (circle), NO_3^--N (square), and OD_{600} (triangle)

Aerobic Denitrification Performance by Pure Cultures: Aerobic denitrification characteristics of Nht, SCC2, AquaN, and R1 strains were investigated in DM-1 and DM-2 for nitrite (Fig. 3) and nitrate (Fig. 4), respectively. The maximum NO_2^--N concentration removal by the strains was obtained at 10 $mg.L^{-1}$. Nht, SCC2, and AquaN strains removed NO_2^--N at the growth phase for 9 h. The R1 strain started nitrite removal in the stationary phase after 12 h incubation. In all strains, when nitrite started to decrease, nitrate increased. There was a small amount of nitrate at the beginning of the tests which was probably due to the chemical oxidation of nitrite (30).

Strains had shown different behaviors to remove nitrate. NHt, SCC2, and AquaN strains grew in DM-2 and their OD_{600} reached 0.6 after 24 h. Due to the slow growth of the R1 strain, nitrate removal lasted up to 72 hours and its OD_{600} reached 0.44. SCC2, R1, and AquaN showed increasing NO_2^--N concentration at the same time as NO_3^--N reduction. In the performances by NHt, SCC2, and AquaN strains, after nitrate decreasing at 24 h, a step of nitrate enhancement was observed simultaneously with the stationary phase. This may be due to the disruption of the cells and the release of nitrate inside them. The Nht strain did not produce NO_2^--N during denitrification.

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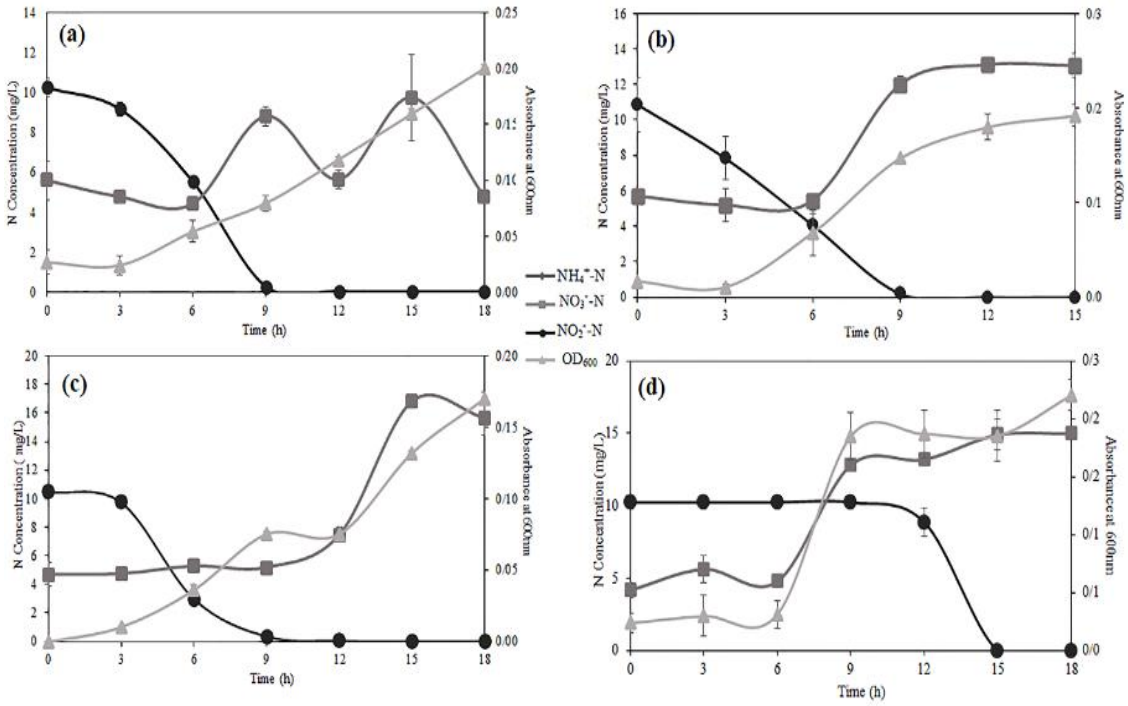

Fig. 3- Nitrite removal characteristics of pure cultures in DM-1 at 30 ˚C. The inoculum size was 5% v/v. a: *P. monteilii* Nht, b: *A. calcoaceticus* SCC2, c: *P. mendocina* AquaN d: *R. erythropolis* R1. Error bars indicate the standard deviation of the mean for three replicates. Figures characterize NO_2^--N (circle), NO_3^--N (square), and OD_{600} (triangle)

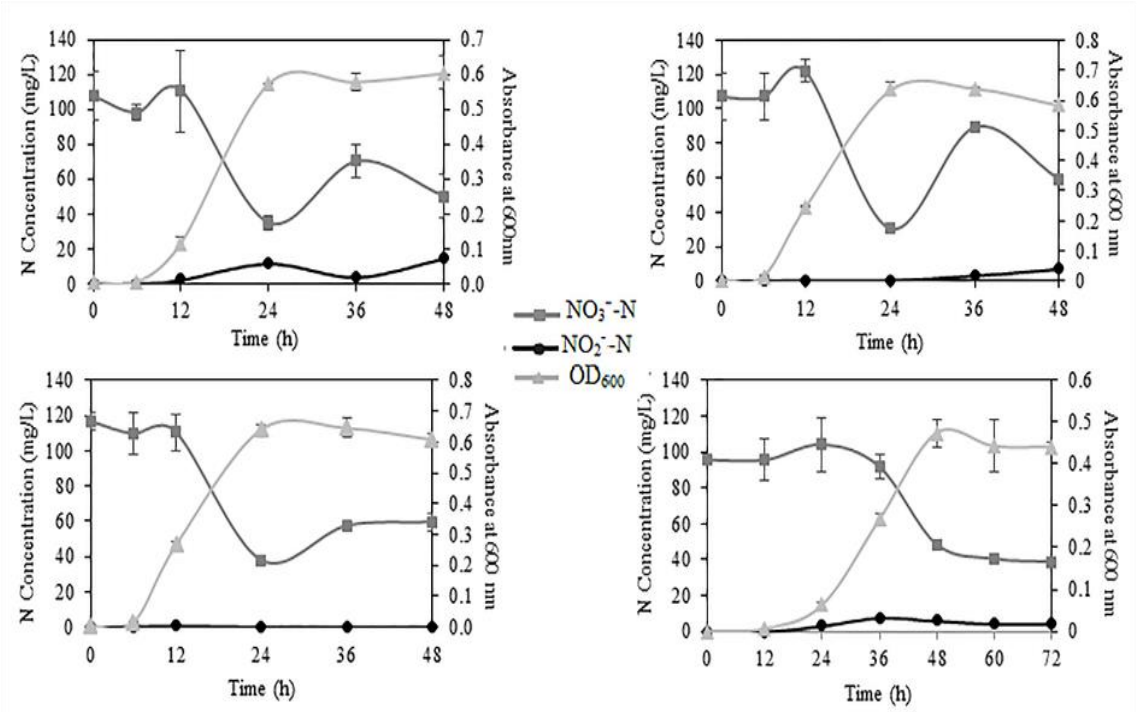

Fig. 4- Nitrate removal characteristics of pure cultures in DM-2 at 30 ˚C. The inoculum size was 5% v/v. a: *P. monteilii* Nht, b: *A. calcoaceticus* SCC2, c: *P. mendocina* AquaN d: *R. erythropolis* R1. Error bars indicate the standard deviation of the mean for three replicates. Figures characterize NO_3^--N (square), NO_2^--N (circle), and OD_{600} (triangle)

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Nitrogen Removal Performance by Bacterial Consortium: In the beginning, two interaction tests were done. In the bilayer test, the effect of one strain on another was assayed on nutrient agar which showed no inhibition zone for all strains. Also, an inhibition effect was not observed by the cross-streak method on HNM. The bacterial consortium was made of *A. calcoaceticus* SCC2, *R. erythropolis* R1, *P. mendocina* AquaN, and *P. monteilii* Nht. NH_4^+-N was removed as 85.3 $mg.L^{-1}$ by the bacterial consortium during 9 h, while at this time, nitrate and nitrite were not produced (Fig. 5a). The average ammonium removal rate and efficiency of the bacterial consortium and pure cultures were shown in Table 2.

The aerobic denitrification characteristics of the bacterial consortium were assayed in DM-1 and DM-2 as depicted in Figures 5b and c. In contrast to pure cultures, the bacterial consortium was able to degrade 23 $mg.L^{-1}$ NO_2^--N and began nitrite removal as soon as it was inoculated and the growth was stopped when nitrite was completely consumed. During nitrite removal, NO_3^--N concentration increased within 12 h and the final concentration of nitrate was 22.5 $mg.L^{-1}$.

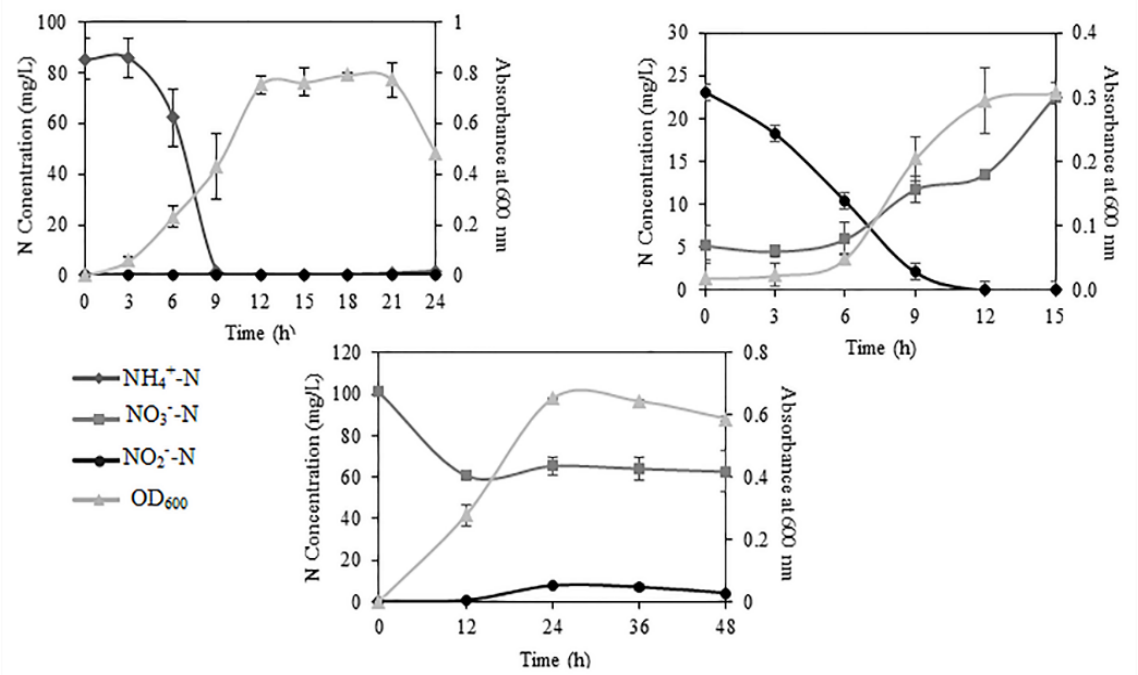

Fig. 5- Nitrogen removal characteristics by the bacterial consortium at 30 ˚C. The inoculum size was 5% v/v. a: Ammonium removal characteristics by growing cells in HNM, b: Nitrite removal characteristics in DM-1, c: Nitrate removal characteristics DM-2. Error bars indicate the standard deviation of the mean for three replicates. Figures characterize NH_4^+-N (diamond), NO_2^--N (circle), NO_3^--N (square), and OD_{600} (triangle)

Table 2- NH_4^+-N, NO_2^--N, and NO_3^--N Removal Efficiency and Rate by *A. calcoaceticus* SCC2, *P. monteilii* Nht, *P. mendocina* AquaN, *R. erythropolis* R1 and Their Consortium after 9 h, 9 h, and 36 h Incubation

Strain	NH_4^+-N removal efficiency (%)	NH_4^+-N removal rate ($mg.L^{-1}.h^{-1}$)	NO_2^--N removal efficiency (%)	NO_2^--N removal rate ($mg.L^{-1}.h^{-1}$)	NO_3^--N removal efficiency (%)	NO_3^--N removal rate ($mg.L^{-1}.h^{-1}$)
A. calcoaceticus SCC2	99.3 ± 0.20 [a]	13.23 ± 0.74 [a]	98.0 ± 0.45 [a]	1.38 ± 0.38 [b]	21.2 ± 2.6 [c]	0.67 ± 0.12 [d]
P. monteilii Nht,	95.0 ± 0.05 [a]	10.92 ± 0.43 [b]	97.2 ± 2.10 [a]	1.1 ±0.03 [c]	50.9 ± 3.52 [a]	0.84 ± 0.13 [c]
P. mendocina AquaN	82.3 ± 1.30 [b]	7.62 ± 1.80[c]	96.8 ± 0.20 [a]	1.1 ± 0.00 [c]	47.4 ± 6.27 [a]	1.76 ± 0.20 [a]
R. erythropolis R1	19.3 ± 3.75 [c]	1.86 ± 1.01 [d]	0.0 [c]	0.00 [d]	3.8 ± 1.17 [d]	0.1 ± 0.03 [b]
Bacterial consortium	98.3 ± 0.36 [a]	9.35 ± 0.85 [bc]	90.5 ± 2.20 [b]	2.42 ± 0.44[a]	36.7 ± 7.11 [b]	1.03 ± 0.21 [b]

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The bacterial consortium reduced 102.5 mg.L^{-1} of NO_3^--N during the first 12 hours and then the nitrate concentration remained constant. Just after nitrate-reducing, nitrite was increased to 4.0 mg.L^{-1}. The Aerobic denitrification removal efficiency and rate in the presence of nitrite and nitrate for pure cultures and consortium are shown in Table 3.

The resting cells of the bacterial consortium removed ammonium within 12 h time as shown in Figure 6. Nitrite and nitrate were not detected in the resting cell supernatants. Total nitrogen analysis detected 65.9% nitrogen removal during the resting cells assay.

High-strength Ammonium Removal by the Consortium: The high-strength NH_4^+-N removal efficiency of the bacterial consortium under different NH_4^+-N loads was shown in Table 4. Consortium removed completely 249 mg.L^{-1} NH_4^+-N within 24 h, but subsequent higher concentrations were degraded by more than 50% efficiency at 24 h and approximately 100% efficiency after 48 h. These results showed consortium can completely degrade 2045 mg.L^{-1} of NH_4^+-N during 48 h. The NH_4^+-N removal performance at the concentration of 2045 mg.L^{-1} by bacterial consortium showed a statistically significant difference compared to pure cultures (Fig. 7).

Table 3- Removal Efficiency of High Strength Ammonium after 24 h and 48 h Incubation by Bacterial Consortium

NH_4^+-N Concentration (mg.L^{-1})	NH_4^+-N removal efficiency (%) after 24 h	NH_4^+-N removal efficiency (%) after 48 h
249	100 ± 0.00	100 ± 0.00
426	63.1 ± 11.24	99.7 ± 0.00
575	54.3 ± 11.87	99.2 ± 0.21
743	65.4 ± 2.12	99.5 ± 0.14
990	53.1 ± 4.31	99.8 ± 0.07
1155	57.2 ± 5.51	99.8 ± 0.14
1648	N.D*	99.9 ± 0.00
2045	N.D	99.9 ± 0.00

* Not Detected

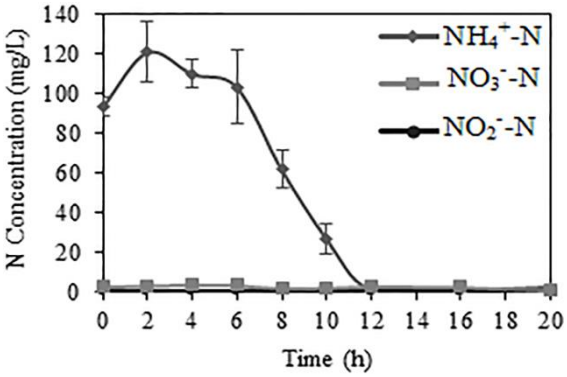

Fig. 6- Ammonium removal characteristics by resting cells of the bacterial consortium at 30˚ C in HNM. a: Ammonium removal characteristics, b: Total nitrogen removal characteristics. Error bars indicate the standard deviation of the mean for three replicates. Figure characterizes NH_4^+-N (diamond), NO_2^--N (circle), and NO_3^--N (square)

Table 4- Removal Efficiency of High Strength Ammonium (2045 mg.L^{-1}) after 48 h Incubation by Pure Cultures and Bacterial Consortium

Strain	NH4+-N removal efficiency (%)
A. calcoaceticus SCC2	47.1 ± 2.3a
P. monteilii Nht,	77.6 ± 1.2b
P. mendocina AquaN	79.6 ± 0.2b
R. erythropolis R1	74.7 ± 2.4c
Bacterial consortium	99.9 ± 0.0d

Data in the same column with different alphabetical superscripts differ significantly by one-way ANOVA with Duncan's test ($p<0.05$)

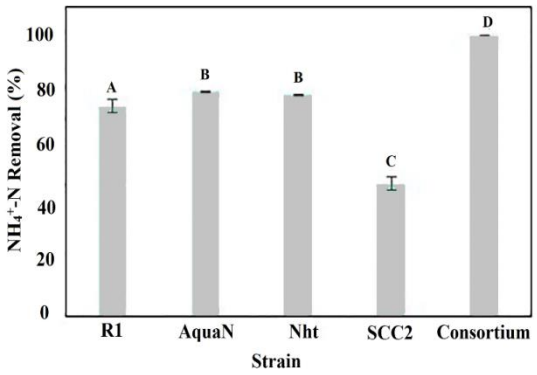

Fig. 7- Characteristics of high strength NH_4^+-N (2045 mg.L^{-1}) removal of the bacterial consortium and pure cultures at 30 ˚C after 48 h incubation. The inoculum size was 5% v/v. Error bars indicate the standard deviation of the mean for three replicates. Different alphabetical superscripts indicate significant differences by one-way ANOVA with Duncan's test ($p < 0.05$)

Discussion and Conclusion

Currently, heterotrophic nitrification-aerobic denitrification bacteria attract researchers' attention because they are able to degrade ammonium, nitrite, and nitrate in aerobic conditions and are suitable to use in the bioremediation of water and wastewater (31).

In this study, the HN-AD bacteria were selected and their consortium was designed. Two strains *Pseudomonas monteilii* Nht (99.71% similarity) and *Pseudomonas mendocina* AquaN (99.71% similarity) were isolated from soil and aquarium water, respectively. The bacterial consortium consisted of *A. calcoaceticus* SCC2, *R. erythropolis* R1, *P. mendocina* AquaN, and *P. monteilii* Nht. The interaction experiments did not show any interference between the strains which this result indicated strains were suitable for consortium preparation. All strains have well performance in the HN-AD process. The single strains and consortium were able to remove ammonium, nitrite, and nitrate. *P. mendocina* AquaN, *A. calcoaceticus* SCC2, and *P. monteilii* Nht performed well in ammonium removal by up to 98.5% efficiency within 24 h; however, the performances of other strains like *Alcaligenes* sp. TB and *Enterobacter cloacae* CF-S27 were slower as well as removed ammonium by 99.42% and 81% efficiency during 44 h and 36 h, respectively (13, 20). *R. erythropolis* R1 was a slow-growing bacterium and removed 85.0% of ammonium during 24 h incubation. During denitrification in DM-1, nitrite converted to nitrate but nitrate was not removed. Also, the result of denitrification in DM-2 showed pure cultures consumed a part of nitrate. Similar results were also reported in previous studies such as *Aeromonas* sp. HN-02 and *Arthrobacter nicotianae* D51 removed 33.32% and 65% of nitrate in aerobic conditions, respectively (32, 33). According to Stein (34), aerobic denitrifying bacteria reduced nitrate and oxygen simultaneously to maximize respiration, and due to this process nitrate was not completely removed in denitrification tests (35). It can be concluded that the strains have little ability in nitrate denitrification and the nitrate reduction was either assimilated by the cells or consumed in competitive denitrification with oxygen. Many studies have indicated that HN-AD bacteria have different complex nitrogen metabolic pathways, which transform inorganic-N into organic or gaseous nitrogen by partial nitrification, denitrification, assimilation, and dissimilation (16). The pure cultures did not produce any nitrite and nitrate during the nitrification process because of their HN-AD pathways. Based on the studies of the HN-AD genes, the HN-AD pathway can be as follows: ammonium could convert to nitrite and then to nitrate. Afterward, nitrate rapidly entered aerobic denitrification processes and was removed. In some cases, nitrite was used directly in aerobic denitrification (13, 33). In some cases, ammonium was converted to nitrogen gaseous without being converted to nitrite and nitrate (36).

Remarkably, microbial communications stabilize their cooperative behaviors and improve enzyme activity and biomass yield (8, 37, 38). Therefore, bacteria in the consortium can complete each nitrogen metabolic pathway and accelerate activities. In this research, the bacterial consortium also showed a significantly higher NH_4^+-N removal efficiency (98.3%) than their pure cultures at the end of 9 h cultivation, nitrite and nitrate were not produced at this time which is similar to the pure culture's performances. These results investigated enhancement enzyme function in the consortium state. In Angar, Kebbouche-Gana (37), the consortium of *Alcaligenes* sp. S84S3 and *Proteus* sp. S19

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removed 65% of the ammonium as high as 272.72 mg.L^{-1} NH_4^+-N during 69 h incubation under optimal conditions. In another study, the consortium of *Pseudomonas fulva* K3, *Pseudomonas mosselii* K17, and *Enterobacter hormaechei* A16 removed 99.62% of the ammonium as high as 100 mg.L^{-1} NH_4^+-N during 12 h incubation that was significantly better than pure cultures removal function (39).

Ammonium removal analysis of consortium resting cells showed increasing in NH_4^+-N concentration at first 2 h due to the activation of proteolytic enzymes at the end of the logarithmic growth phase and the release of the dead bacterial cells into the medium (32, 40). At the end of the resting cell test, 65.9% of total nitrogen was removed from the medium; this value was expected to be converted to gaseous. This means that the bacterial consortium had nitrification ability after passing the growth phase and in a high-density condition. The important result of this study is a higher tolerance of consortium to ammonium and a higher biodegradation rate than previous results reported by other co-cultures. The consortium tolerated and grew in a medium with 250 to 2045 mg.L^{-1} NH_4^+-N concentration and degraded up to 95% of NH_4^+-N during 48 h, with significantly high degradation efficiency than the pure culture of bacteria. However, the high-strength ammonium degradation reported earlier. *Pseudomonas stutzeri* GEP-01 removed 1000 mg.L^{-1} NH_4^+-N by 78.9% efficiency within 72 h (41). In addition, *Alcaligenes faecalis* TF-1 tolerated 1963.94 mg.L^{-1} NH_4^+-N and removed by 65% efficiency after 144 h (36).

The bacterial consortium had a denitrification function in the presence of 23 mg.L-1 NO_2^-N. This concentration was higher than the tolerable concentration for the pure cultures. The bacterial consortium removed 40% of NO_3^--N in DM-2 at first 12 h. This result was in contrast to pure culture performances because NO_3^--N concentration in the consortium test did not fluctuate and remained stable as well as pure cultures started to consume nitrate after 12 h.

In this study, HN-AD strains including *P. mendocina* AquaN and *P. monteilii* Nht were isolated and designed for the consortium with *A. calcoaceticus* SCC2, and *R. erythropolis* R1. These strains could utilize ammonium, nitrate, and nitrite as the sole nitrogen sources. The bacterial consortium performed better HN-AD function than single strains. In addition, the consortium exhibited high tolerance capacity on 2045 mg.L^{-1} NH_4^+-N and was able to remove such concentrated ammonium completely within 48 h. Thus, the co-culture and interactions between HN-AD strains also contribute to high-efficiency ammonium removal.

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Declaration of interest statement

The authors declare that they have no conflict of interest.

Data availability statement

All data generated or analyzed during this study are included in this published article. "Sequence" data that support the part of the findings of this study have been deposited in "GenBank" with the accession codes MN167837.1 and MN435149.1.

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