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Heterotrophic nitrifying bacteria from activated sludge in DHS reactor for ammonium removal of natural rubber processing wastewater treatment

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Abstract. Two heterotrophic nitrifying bacterial strains, D2 and D7 were isolated from an activated sludge of sponges in a laboratory-scale downflow hanging sponge reactor. Both strains exhibited efficient ammonium removal ability over a wide range of ammonium loads. At the initial concentration of 100 mg/L, NH_4^+-N was completely degraded within 20 h by both strains. When the initial concentration was increased to 200 mg/L, the NH_4^+-N removal efficiency was 99.6 % within 20 h by the strain D2 and 61.3 % by the strain D7. In natural rubber processing wastewater, the ammonium removal efficiencies of strain D2 and D7 were 38 % and 99 % with the initial N-NH_4^+ concentration of 280 and 380 mg/L after 88 h, respectively. The 16S rRNA gene sequence of D2 and D7 showed the highest similarity to the *Pseudomonas aeruginosa* and *Glutamicibacter nicotianae*, respectively. This is the first report to demonstrate the ability to remove ammonium in NRPW by heterotrophic nitrifying bacteria isolated from activated sludge in DHS reactor.

Keywords: heterotrophic nitrifying bacterium, downflow hanging sponge, ammonium removal, natural rubber processing wastewater.

Classification numbers: 3.7.2.

1. INTRODUCTION

Viet Nam is one of the most natural rubber growing and exporting countries in the world. Despite the enormous profits of natural rubber latex production, its processing discharges large amounts of wastewater containing high concentrations of organic compounds, ammonium, and other contaminants. Natural rubber processing wastewater (NRPW) is commonly treated by anaerobic-aerobic systems, which have a high chemical oxygen demand (COD) removal efficiency (of 65 % to 95 %) [1]. However, the ammonium removal efficiency of these systems was limited, and their effluents did not meet the discharge regulations for NRPW in terms of NH_4^+-N concentration [1 - 5]. Downflow hanging sponge (DHS) reactor is a type of trickling filter reactor that uses sponges as media. It can achieve high oxidation efficiencies for both organic matter and ammonium of NRPW [6]. The microbial community structure of the DHS

sludge was investigated by using 16S rRNA gene sequencing analysis and both nitrifying and denitrifying bacteria were detected in the sponge carrier [2-5]. *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Chloroflexi* were the predominant phyla in DHS sludge [4]. However, no information for isolation on heterotrophic nitrifying bacteria from DHS sludge has been published yet.

Heterotrophic nitrification includes ammonia oxidation under aerobic condition by heterotrophic nitrifying bacteria using organic matter as an energy resource. These bacteria show rapid growth and are better adapted to wastewater and sludge than autotroph nitrifying bacteria [7]. To date, several heterotrophic nitrifying bacteria have been discovered and have attracted extensive attention and research due to their potential value of biological removal of nitrogen such as *Bacillus methylotrophicus* [8], *Pseudomonas stutzeri* [9 - 12], *P. guguanensis* [13], *Acinetobacter junii* [14], *Alcaligenes faecalis* [15], and *Glutamicibacter nicotianae* [16]. These strains have been used for high ammonium wastewater treatment such as petrochemical wastewater [13], synthetic ammonium wastewater [14], and domestic sewage [9].

As the heterotrophic nitrifying bacteria have potential application in wastewater treatment, screening for more efficient heterotrophic nitrifying bacteria and understanding the characteristics of these bacteria in detail are urgently needed to meet the demand for rich ammonium wastewater treatment. The aim of this work was to isolate heterotrophic nitrifying bacteria from DHS activated sludges and to determine the ammonium removal efficiency of NRPW using the isolates.

2. MATERIALS AND METHODS

2.1. Materials

DHS reactor (volume 40 L) employs polyurethane sponges as supporting material. The influent of the DHS reactor was fed through a liquid distributor on top of the DHS reactor, which subsequently trickled down toward the bottom part through the sponge medium. During the experimental period, the aggregation of suspended solids and microorganisms in the wastewater gradually formed biofilms on the fresh sponge. This amount of biomass is called activated sludge of sponges.

Activated sludge samples were collected from a laboratory-scale DHS reactor which was installed for ammonium removal of natural rubber wastewater treatment and used to isolate heterotrophic nitrifying bacteria.

The basal medium (BM) used for isolation of heterotrophic nitrifying bacteria consisted of the following components (gram per liter): 0.47 $(NH_4)_2SO_4$, 10.25 CH_3COONa, 0.2 K_2HPO_4, 0.12 NaCl, 0.05 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 0.01 $MnSO_4.4H_2O$ and pH 7.0 [17]. The nitrite medium (NM) used for nitrite conversion study contained 0.246 $NaNO_2$, 10.25 CH_3COONa, 0.2 K_2HPO_4, 0.12 NaCl, 0.05 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 0.01 $MnSO_4.4H_2O$ and pH 7.0 [17]. All chemicals were of analytical grade.

NRPW was obtained from the post treatment of anaerobic reactor effluent in a laboratory-scale which was applied to the influent of DHS reactor. NRPW was sterilized at 121 °C for 20 min and referred to as sterilized natural rubber wastewater (SNRW). The NRPW and SNRW contained 380, 280 mg/L of $N\text{-}NH_4^+$, respectively; and were used to determine the ammonium and nitrite removal efficiencies of the isolates.

2.2. Methods

2.2.1. Isolation of heterotrophic nitrifying bacteria

The sludge sample (10 mL) was transferred to sterile 0.9 % NaCl solution (90 mL) in a 250-mL Erlenmeyer flask and shaken at 150 rpm to obtain a homogeneous suspension. Gradient dilutions (10^{-2} - 10^{-5}) were performed, and 100 µL of the suspensions were spread onto plates coated with BM agar medium (2 % agar) and incubated at 30 ^{o}C in 24 h until visible colonies were formed. Single colonies were picked and streaked on fresh BM agar plates to obtain purified isolates. The isolates were picked and individually determined for ammonium and nitrite removal efficiencies. The strains with high ammonium and nitrite removal efficiencies were selected for further experiments.

2.2.2. Assessment of ammonium or nitrite removal efficiency

The isolates were inoculated into 10 mL of BM for 72 h at 30 ^{o}C on a rotary shaker at 150 rpm, and then centrifuged at 10000 rpm for 10 min to obtain the biomass. Each biomass was washed three times by sterile distilled water and inoculated into 25 mL of fresh BM or NM to give the final absorbance of each culture at 600 nm of 0.01. The cultures were inoculated in the same condition above. After each 24 h, 1 mL of broth culture in BM or NM was taken to determine ammonium or nitrite concentration. Based on that the ammonium or nitrite removal efficiency was calculated using the equation:

$$\text{Ammonium or nitrite removal efficiency (\%)} = \frac{M0-M1}{M0} \times 100 \quad (1)$$

where M0, M1 (in mg. L^{-1}) are the corresponding concentration of ammonium or nitrite at the initial time and the time of sampling, respectively.

2.2.3. Effect of initial seed density on ammonium removal efficiency

The selected strains were incubated into 25 mL of BM in the flask at 30 ^{o}C and shaken at 150 rpm. The initial seed density of culture was determined by the spectrophotometry at a wavelength of 600 nm (OD_{600}), which was changed from 0.01 to 1. After 12 h of incubation, 1 mL of the culture was taken to determine the ammonium concentration. Based on that the ammonium removal efficiency was calculated.

2.2.4. Effect of initial ammonium concentration on ammonium removal efficiency

The selected strains were incubated into 25 mL of BM in the flask with OD_{600} of 0.5 at 30 ^{o}C and shaken at 150 rpm. The ammonium concentration in BM varied from 100 to 500 mg/L. After each 8 h incubation, 1 mL of the culture was taken to determine the ammonium concentration. Based on that the ammonium removal efficiency was calculated.

2.2.5. Assessment of ammonium removal efficiency of strains in NRPW or SNRW

The selected strains were incubated into 25 mL of NRPW or SNRW in the flask with OD_{600} of 0.5 at 30 ^{o}C and shaken at 150 rpm. Each 8 h incubation, 1 mL of the culture was taken to determine the ammonium concentration. Based on that the ammonium removal efficiency was calculated.

2.2.6. Identification of selected strains using the 16S rRNA gene

The total genomic DNAs of the selected strains were prepared as described previously [18]. A partial 16S rRNA gene fragment (ca. 1400 bp) was amplified by PCR using the universal primers for bacteria [19]. Based on the 16S rRNA gene sequence, the phylogenetic position of the isolate was determined by comparing it with those in the Genbank database using the basic local alignment search tool (BLAST). The partial 16S rRNA nucleotide sequence of the selected strain has been deposited in the GenBank database. A phylogenetic analysis was performed with the MEGA 11 using the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value [20].

2.2.7. Analytical methods

The culture after incubation with isolates was centrifuged at 10000 rpm for 10 min and collected the supernatant for determination of ammonium and nitrite concentration. The ammonium concentration was measured using Nessler's reagent spectrophotometry [21]. The nitrite concentration was determined by the diazotization method (Hach nitrogen-nitrite kit, USA [22].

2.2.8. Statistical analysis

All experiments in this study were performed in triplicate, and the results were presented as means ± SD (standard deviation of means).

3. RESULTS AND DISCUSSION

3.1. Screening of heterotrophic nitrifying bacteria

Six distinct colonies that can utilize ammonium were obtained on the BM agar plates. They were incubated in BM and NM. The ammonium and nitrite removal efficiency by isolates were shown in Figure 1.

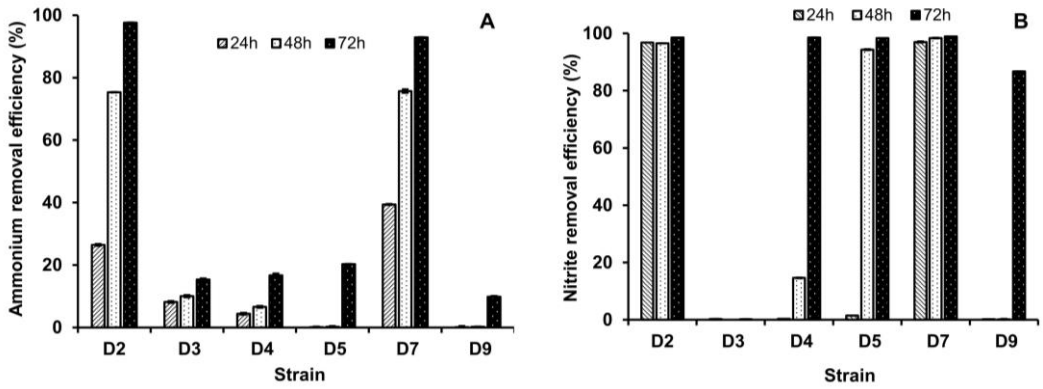

Figure 1. Ammonium (A) and nitrite (B) removal efficiency by isolates in BM and NM.

The concentration of ammonium and nitrite in BM and NM was 100 mg/L of $N\text{-}NH_4^+$ and 50 mg/L of $N\text{-}NO_2^-$, respectively. The ammonium removal efficiency of two strains D2 and D7 were higher than that of other strains. The highest efficiency was obtained in BM with strain D7

of 98 %, followed by strain D2 of 93 % after 72 h incubation (Figure 1A). There were five strains capable of removing nitrite of D2, D4, D5, D7, and D9 strains. The highest efficiency was obtained in NM with D2 and D7 strains were 97 % after 24 h (Fig.1B). Zhang *et al.* reported that the ammonium removal efficiency of the *B. methylotrophicus* L7 strain in heterotrophic nitrification medium was 74 % after 216 h with NH_4^+ concentration of 146.71 mg/L [8]. Motamed *et al.* showed that *Pseudomonas guguanensis* strain 4-n-1 had the ammonium removal efficiency of 93.2 % in basal salt medium after 24 h at 40 mg/L $(NH_4)_2SO_4$ concentration initial [13]. *P. stutzeri* YZN-001 strain was able to completely remove NO_2^- with concentrations of 171.40 mg/L, after 48 h [9]. It suggested that both strains D2 and D7 could effectively remove ammonium and can be used in further study.

3.2. Effect of initial inoculation on ammonium removal efficiency

The effect of the initial inoculation of the two strains in BM on the ammonium removal efficiency was shown in Figure 2.

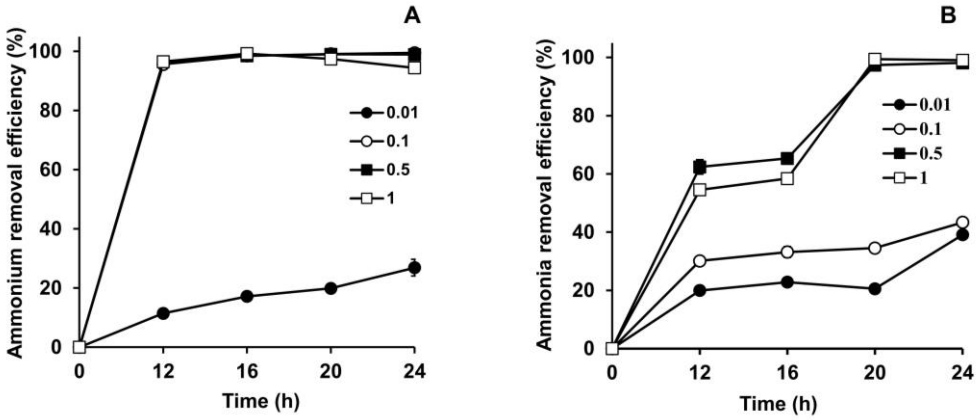

Figure 2. The effect of initial inoculation of strain D2 (A) and strain D7 (B) in BM on ammonium removal efficiency.

The ammonium removal efficiency depends on initial inoculation. There was a different ammonium removal efficiency among inoculation sizes. The ammonium removal efficiencies of the D2 and D7 strains were less than 40 % after 24 h with an OD_{600} of 0.01. The highest efficiency of strain D2 was 99 % in 16 h with $OD_{600} \geq 0.1$ (Fig. 2A). While the efficiencies of strain D7 after 24 h incubation with OD_{600} of 0.5 and 1 were 97 % and 99 %, respectively (Figure 2B). Therefore, the inoculation of both strains with OD_{600} of 0.5 was selected for the next experiments. Huang *et al.* reported that the optimum inoculation of strain Smobiisys501 with OD_{600} value was 0.53 after 48 h incubation, the ammonium removal efficiency reached 99.67 % [23].

3.3. Effect of ammonium concentration on ammonium removal efficiency

The NH_4^+-N removal curves of strains D2 and D7 under different NH_4^+-N loads is shown in Figure 3. At the initial concentration of 100 mg/L, NH_4^+-N was completely degraded within 20 h by both strains. When the initial concentration was increased to 200 mg/L, the NH_4^+-N removal efficiency was 99.6 % within 20 h by strain D2 and only 61.3 % by strain D7. The ammonium removal efficiency of strain D2 with the N-NH_4^+ concentration varied from 300 to 500 mg/L was 92, 76, and 72 %, while that by strain D7 was 67, 63, and 59 % after 88 h, respectively. These

results suggest that strain D2 has a higher ammonium removal potential than strain D7. Sun *et al.* showed that the ammonium removal efficiency of *P. stutzeri* T13 was 39.56 % after 18 h with the initial concentration of 224.68 mg/L [10]. The efficiency of *P. stutzeri* XL-2 was 69.86 % in 48 h with the initial concentration of 100 mg/L [11]. When the initial NH_4^+-N amounts were 200 and 500 mg/L, the removal rates of *P. stutzeri* GEP-01 were approximately 93 % within 30 h and 90.4 % within 72, respectively [12]. It seems that strains D2 and D7 have strong ammonium removal capacity and these strains can be applied for ammonium removal in rubber wastewater.

The ammonium removal efficiency of strains in natural rubber wastewater.

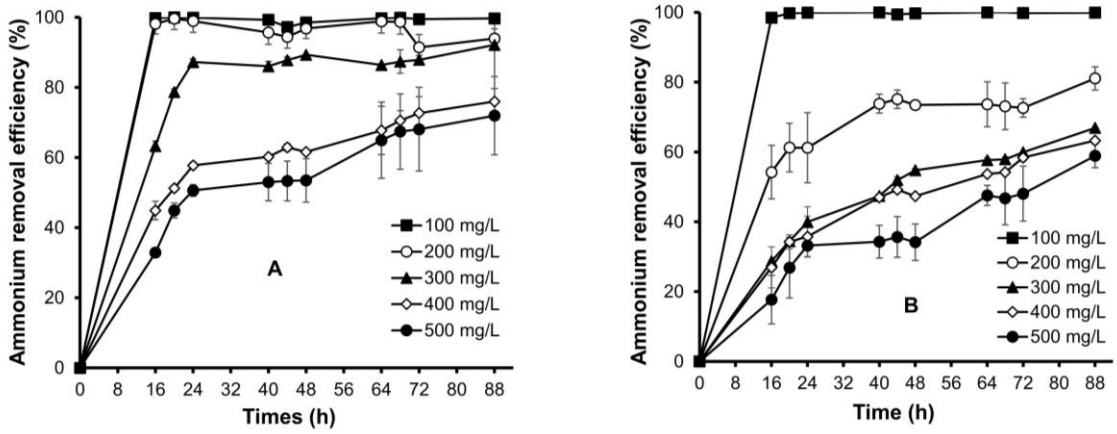

Figure 3. The effect of ammonium concentration in BM incubation with strain D2 (A) or strain D7 (B) on the ammonium removal efficiency.

In this study, the ammonium removal efficiencies of isolates were determined using NRPW and SNRW. The key difference between NRPW and SNRW is that the microbial consortium present in the NRPW have been destroyed by high temperature. Therefore, the ammonium removal ability of the isolates in SNRW was evaluated. Besides, the influence of the microbiota in the NRPW on the isolates can be predicted.

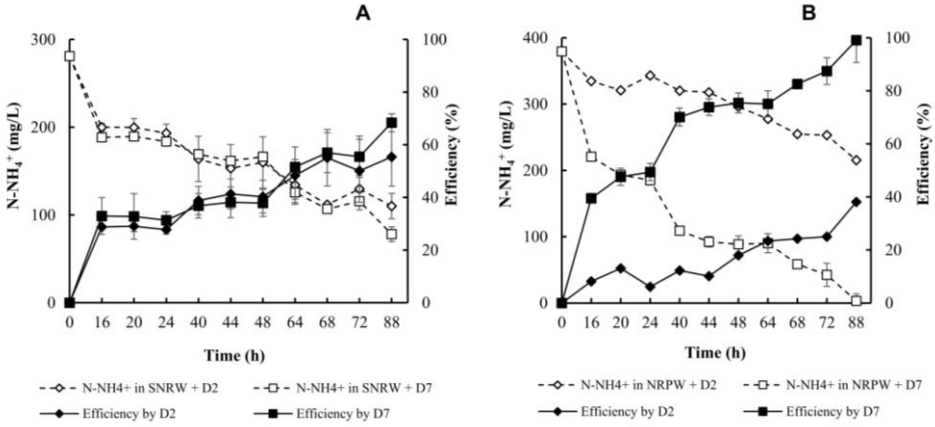

Figure 4. The ammonium concentration and removal efficiency of selected strains during incubation in sterilized natural rubber wastewater (A) or natural rubber processing wastewater (B).

Figure 4 shows that both strains D2 and D7 were able to remove ammonium in SNRW and NRPW with NH_4^+ concentrations of 280 and 380 mg/L, respectively. After 88 h, the NH_4^+ concentration remaining in the SNRW of strains D2 and D7 were 110.18 and 78.19 mg/L with an ammonium removal efficiency of 55 % and 68 %, respectively (Figure 4A). In NRPW, the efficiency of strains D2 and D7 were 38 % and 99 % while the NH_4^+ concentration remaining in the culture was 215.55 and 4.81 mg/L, respectively after 88 h (Figure 4B). Thus, the efficiency of ammonium removal in wastewater by strain D7 was higher than that by strain D2. Besides that, the ammonium removal efficiency of strain D7 in NRPW was higher than that of SNRW, suggesting that the microbial consortium presented in the NRPW could have positive effects on the ammonium removal process of strain D7. Zhang *et al*. reported that when *P. stutzeri* strain YZN-001 was added to the domestic sewage, the removal rate of ammonium can reach 85.91 % at 72 h [9]. *Alcaligenes faecalis* strain NR, isolated from activated sludge, exhibited the ability to remove 66.7 - 78.3 % of NH_4^+ using synthetic wastewater with 150 - 200 mg/L of NH_4^+ concentration [15].

3.3. Phylogenetic analysis using 16S RNA gene sequence

The 16S rRNA gene sequences of strains D2 and D7, approximately 1400 bp, were obtained via PCR amplification and sequencing. As shown in Figure 5, strain D2 and D7 shared up to 99.93 % similarity with *Pseudomonas aeruginosa* strain YL and 100 % similarity with *Glutamicibacter nicotianae* MSSRFPD36, respectively. The isolates were named *Pseudomonas aeruginosa* strain D2 and *Glutamicibacter nicotianae* strain D7 and were submitted to GenBank under the accession numbers ON 127900 and ON 129410, respectively.

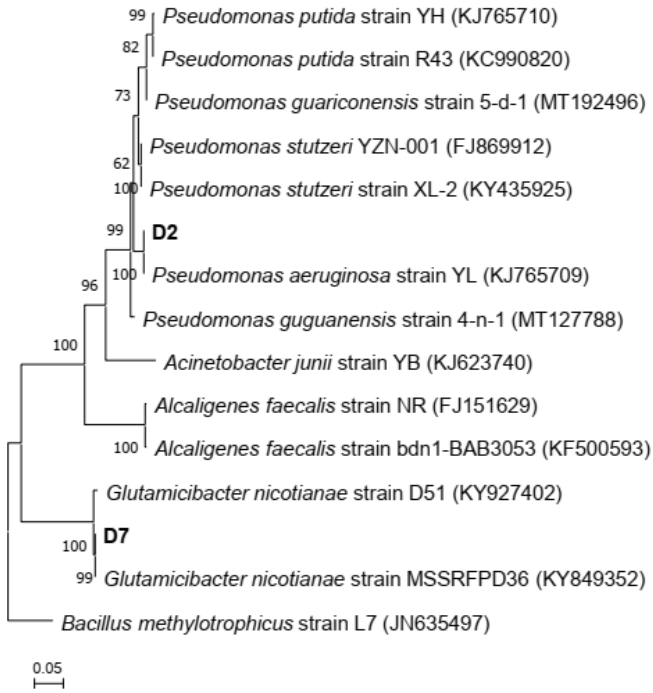

Figure 5. Phylogenetic tree of 16S rRNA gene sequences of strain D2 and strain D7 with the other bacteria. The tree was constructed by the applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and the scale corresponds to a genetic distance of 0.05 nucleotide substitutions per position.

The phylogenetic tree based on evolutionary distance (neighbor-joining) showed that the isolates were quite close to some members of *Pseudomonas* and *Glutamicibacter*, reported as the heterotrophic nitrifying bacteria (Figure 5).

A heterotrophic nitrifying bacterium, *G. nicotianae* strain D51 isolated from long-term flooded paddy fields, showed an ammonium removal efficiency of 98.70 % with an initial ammonium concentration of 53.17 mg/L after 48 h of incubation [16]. The 16S rRNA gene sequence of strain D7 showed 97.78 % similarity with that of *G. nicotianae* strain D51. To date, this is the first report of a member of *Glutamicibacter* having heterotrophic nitrification, while many strains of the genus *Pseudomonas* have been reported to be capable of heterotrophic ammonium removal such as *P. aeruginosa* strain YL isolated from the sludge of sequencing batch reactor [14], *P. guguanensis* strain 4-n-1 isolated from petrochemical wastewater [13], and *P. stutzeri* strain YZN-001 isolated from swine manure effluent [9]. The 16S rRNA gene sequence of strain D2 showed more than 96 % similarity with that of strain YL or strain 4n-1 or strain YZN-001.

4. CONCLUSIONS

In this study, two heterotrophic nitrifying bacteria, *P. aeruginosa* strain D2 and *G. nicotianae* strain D7, were isolated from the DHS activated sludge. They demonstrated the ammonium removal efficiency of natural rubber processing wastewater. However, it seems that the efficiency of strain D7 was higher than that of strain D2. Further studies are needed to investigate the aerobic denitrification of the isolates to find the potential candidates for application in the treatment of ammonium-rich wastewater as well as natural rubber processing wastewater.

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CRediT authorship contribution statement. Tran Minh Duc: Methodology, Formal analysis, Data Visualization, Writing original draft. Phan Thi Thanh Thuy: Formal analysis, Methodology. Nguyen Thi Huyen: Formal analysis, Methodology. Nguyen Lan Huong: Conceptualization, Supervision, Writing-Review and Editing, Funding acquisition.

Declaration of competing interest. The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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