Evaluating the performance of two denitrifying bacteria and their synergistic relationship in remediating nitrate-polluted wastewater

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ABSTRACT

This study compared the performance of two denitrifying bacteria and investigated their cooperative relationship to enhance heterotrophic denitrification to remediate nitrate-polluted wastewater. The selected denitrifying bacteria were *Pseudomonas nitritireducens* (H6) and *Klebsiella* sp. (D5). The results showed that sodium acetate was the best carbon source for strain H6, resulting in removal efficiency of 98.7% with negligible nitrite accumulation in 36 h. Sodium citrate was more suitable for strain D5, resulting in removal efficiency of 94.4% in 36 h; however, nitrite accumulation was significant, with a maximum concentration of 22.24 mg NO_2^-–N/L. These findings indicate that strain H6 exhibited excellent performance because of its high nitrate removal efficiency and low nitrite accumulation during denitrification. The strain D5 could achieve effective denitrification, but also caused nitrite accumulation. Additionally, at 15°C, the nitrate removal efficiency was only 32.3% (H6) and 8.5% (D5), respectively, but increased significantly when the temperature was raised to 25°C and 30°C, which increased the removal efficiency above 95%. Moreover, when strains H6 and D5 were inoculated together, the nitrate removal efficiencies were above 98%, and nitrite accumulated was negligible. These findings indicate that synergy exists between strains H6 and D5 during denitrification, which can improve the nitrate removal efficiency, thus avoiding nitrite accumulation in wastewater treatment for nitrogen removal.

Keywords: Denitrification; Wastewater; Carbon sources; Temperature; Sodium citrate; Sodium acetate

1. Introduction

Currently, nitrate pollution in water bodies has become increasingly significant because of a variety of domestic, agricultural, and industrial practices [1–5]. High nitrate concentrations cause eutrophication, which deteriorates the water quality and causes the death of fish and other aquatic organisms [6]. In addition, long-term consumption of nitrate-polluted water may cause serious illnesses, such as blue baby syndrome [7,8]. Consequently, nitrate removal from nitrate-polluted water has become an area of active research. Previous studies demonstrated that denitrification is one of the most effective methods for complete nitrate removal because it is efficient, has a moderate cost, and is environmentally feasible [9,10]. In this process, heterotrophic denitrifying bacteria reduce nitrate to N_2 using a carbon source as an electron donor [11–13]. Several factors affect heterotrophic denitrification, including organic

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carbon, denitrifying bacteria, nitrate concentrations, dissolved oxygen (DO), and temperature [14–16]. Previous studies on heterotrophic denitrification have focused on carbon sources, temperature, and the denitrifying bacterial community [17,18], while the synergy between two denitrifying bacteria has rarely been investigated. Several denitrifying bacteria occur naturally [19]. Liao et al. [20] found that the dominant group during denitrification of high-nitrate wastewater was Proteobacteria (84.53%), followed by Firmicutes (13.24%). Yoshie et al. [21] observed that γ-Proteobacteria plays a crucial role in the denitrification of saline wastewater. However, β-Proteobacteria is a dominant bacterial population in freshwater environments, such as rivers and lakes [22,23]. Overall, the results of studies conducted to date indicated that the optimal environments differed for different denitrifying bacteria and selecting suitable denitrifying bacteria can enhance nitrate removal efficiency of different denitrification systems. Moreover, electron competition or synergistic relationships among different denitrifying bacteria have significant effects on nitrate removal [24]. Accordingly, investigating the cooperating mechanisms among different denitrifying bacteria are important in improving denitrification efficiency.

The most critical limiting factor for denitrification is the carbon source, and easily degradable organic carbon can promote denitrification [25,26]. Therefore, additional carbon sources are necessary to enhance denitrification efficiency, especially for certain types of wastewaters with relatively low C/N ratios. Moreover, some denitrifying bacteria can use a variety of carbon sources, while others can use only a few [27,28]. Consequently, carbon sources considerably influence the bacterial communities in denitrification systems [29]. Indeed, certain studies have found glucose to be the most suitable carbon source for denitrification by *Enterobacter cloacae* HNR and *Providencia rettgeri* YL; however, glucose was not conducive to the growth of the strain *Psychrobacter* sp. S1-1 [30,31]. Consequently, it is necessary to select the most suitable carbon source for denitrifying bacteria to improve the denitrification efficiency.

Notably, the optimum temperature range for denitrifying bacteria is 25°C–35°C [32]. Sirivedhin and Gray [33] found that the denitrification rate decreased below 15°C and completely ceased below 5°C. Zhang et al. also observed incomplete denitrification when the temperature was reduced to 16°C ± 2°C [34]. However, some denitrifying bacteria may tolerate low temperatures or grow at high temperatures [14,35]. Previous studies have shown that the *Pseudomonas tolaasii* strain Y-11 can reduce nitrite and total nitrogen at 15°C [36]. Hence, it is crucial to study the effects of temperatures, because the optimum temperatures vary for different denitrifying bacteria.

In this study, two denitrifying bacteria were selected to evaluate their denitrification performance, and the capacity of different carbon sources to promote denitrification and the effects of temperature on denitrification were investigated. Subsequently, the denitrifying bacteria were inoculated into a denitrification system to study their synergistic relationship during nitrate reduction.

The specific objectives of this study were to (a) assess the denitrification performance of two isolated denitrifying bacteria supported by different carbon sources, (b) assess the effects of temperature on the denitrification performance of different denitrifying bacteria; and (c) study the relationship among different denitrifying bacteria during denitrification.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were analytical reagent grade. Potassium sodium tartrate, sodium citrate, and sodium acetate were selected as potential carbon sources.

2.2. Synthetic wastewater

In this study, $NaNO_3$ was weighed and added to distilled water to prepare the synthetic wastewater with a nitrate concentration of 120.01 mg NO_3^-–N/L and 105.00 mg NO_3^-–N/L. K_2HPO_4 was added as a nutrient at an N/P ratio of 20. Trace elements were also added to promote denitrification. The pH of the freshly prepared wastewater was between 7.0–8.0.

2.3. Denitrifying bacteria

The selected denitrifying bacteria were *Pseudomonas nitritireducens* (H6) and *Klebsiella* sp. (D5), which were isolated from sludge and paddy soil, respectively, and identified by the China Center for Type Culture Collection. Before use, the strains H6 and D5 were cultured to their exponential growth phase.

2.4. Denitrification experiments

2.4.1. Evaluation of denitrification by strains H6 and D5 using different carbon sources

In this experiment, sodium citrate, potassium sodium tartrate, and sodium acetate were added separately to synthetic wastewater containing 120.01 mg NO_3^-–N/L to obtain a C/N ratio of 8. Next, 300 mL of synthetic wastewater was added to separate 500 mL Erlenmeyer flasks, and the flasks were sterilized in an autoclave (121°C, 0.1 MPa) for 15 min. After cooling, 5 mL of the bacterial suspension of strains H6 or D5 was added to the Erlenmeyer flasks. There were six groups of experiments, and each group contained three parallel experiments.

The DO concentration in the wastewater was reduced to <2.0 mg/L by purging the flasks for 20 min with high purity nitrogen. Next, the flasks were sealed and incubated at 30°C on a rotary shaker at 150 rpm for 84 h, during which the supernatants were periodically collected from each flask and analyzed for pH, DO, NO_3^-–N, and NO_2^-–N as described below.

2.4.2. Denitrification by strains H6 and D5 under different temperatures

The effect of different temperatures on denitrification was investigated for strains H6 and D5. The nitrate concentration in the synthetic wastewater was 105.00 mg NO_3^-–N/L. The sole carbon source was sodium acetate, with a C/N ratio of 5. Six 500 mL Erlenmeyer flasks were filled with 300 mL

of synthetic wastewater and sterilized using an autoclave, after which 5 mL of bacterial strain H6 was inoculated into three Erlenmeyer flasks and 5 mL of strain D5 was inoculated into the other three Erlenmeyer flasks. Each group consisted of three parallel experiments. The flasks were purged with nitrogen and sealed, and were incubated at 15°C, 25°C, or 30°C on a rotary shaker at 150 rpm for 96 h, and periodically analyzed for pH, DO, NO_3^-–N, and NO_2^-–N as described below.

2.4.3. Cooperating relationship between strains H6 and D5

The competition or systematic action of different denitrifying bacteria may have a significant effect on denitrification efficiency. Sodium citrate and sodium acetate were added separately to wastewater containing 120.01 mg NO_3^-–N/L to obtain a final C/N ratio of 8. Next, 300 mL of these wastewaters were added to two separate 500 mL Erlenmeyer flasks and sterilized. Subsequently, 5 mL of mixed bacterial suspension (2.5 mL H6 and 2.5 mL D5) was inoculated into each flask to study the synergistic relationship between the strains. The flasks were purged and sealed as described above, and incubated at 30°C on a rotary shaker at 150 rpm for 84 h. Three parallel experiments were conducted for each group. During the experiment, pH, DO, NO_3^-–N, and NO_2^-–N were periodically analyzed as described below.

2.5. Analytical techniques

The pH was determined using a pH meter (UB-7, Denver Instrument, USA). Dissolved oxygen was measured using a Eutech Instruments DO 110 meter (Eutech Instruments, Singapore). NO_3^-–N and NO_2^-–N were measured using a UV-2550 spectrophotometer (Shimadzu, Japan), according to water and wastewater monitoring analysis method [37].

Each sample was analyzed in triplicate after filtering using a 0.45 µm Whatman filter paper. The arithmetic average concentration was used as the final concentration.

3. Results and discussion

3.1. Denitrification performance of strains H6 and D5 using different carbon sources

3.1.1. Environmental parameters

Denitrification can be affected by environmental parameters, including pH and DO. In this study, the pH fluctuated between 7.0 and 7.6 during the experiments, and the DO concentrations were less than 2.0 mg/L. These findings suggest that anoxic conditions favored the growth and reproduction of denitrifying bacteria in each flask.

3.1.2. Nitrate removal

The nitrate concentrations in the flasks inoculated with the strain H6 are shown in Fig. 1A. The evolution of nitrate in the three flasks with different carbon sources differed significantly during the experiments. The nitrate concentration decreased from 120.01 mg NO_3^-–N/L to 100.56 mg NO_3^-–N/L in 48 h when potassium sodium tartrate was used as a carbon source, indicating a removal

efficiency of only 16.2%. Thereafter, the nitrate concentrations continued to decrease gradually, with the concentrations reducing to 48.00 mg NO_3^-–N/L, resulting in nitrate removal efficiencies of 60.0%. The strain H6 could not completely remove nitrate when potassium sodium tartrate was used as a carbon source, which resulted in high nitrate concentrations in the supernatants. Sodium citrate as a carbon source decreased nitrate concentrations from 120.01 mg NO_3^-–N/L to 81.69 mg NO_3^-–N/L in 48 h, and to 12.43 mg NO_3^-–N/L in 84 h, resulting in nitrate removal efficiencies of 31.9% and 89.6%, respectively. These findings indicate that nitrate removal efficiencies using sodium citrate were significantly higher than those using potassium sodium tartrate as a carbon source. Unlike potassium sodium tartrate and sodium citrate, in the presence of sodium acetate, the nitrate concentrations decreased rapidly to 1.57 mg NO_3^-–N/L in 36 h, with a removal efficiency of 98.7%, and remained relatively constant thereafter. These findings indicate that the strain H6 could completely and rapidly reduce nitrogen with sodium acetate as a carbon source. Several studies have found that microorganisms readily use acetate, which is an effective substrate for denitrification [38,39]. Kozub and Liehr used sodium acetate as the carbon source and reported that the denitrification rates measured in a laboratory experiment were higher than the background denitrification rates [25].

When D5 was used as the denitrifying bacteria, nitrate concentrations in the three flasks exhibited different behaviors (Fig. 2A). Specifically, nitrate removal efficiency was nevertheless low in all experiments when potassium sodium tartrate was used as the carbon source, with a nitrate removal efficiency of only 12.7% at the end of the experiments. Therefore, potassium sodium tartrate is not an effective carbon source for strains H6 or D5. However, D5 showed higher activity when sodium citrate was used as a carbon source, with nitrate concentrations in the supernatants decreasing to 6.73 mg NO_3^-–N/L in 36 h, with a removal efficiency of 94.4%. This was higher than that observed for strain H6 supported by sodium citrate. Therefore, when sodium citrate was used as a carbon source, the activity of strain D5 was higher than that of strain H6, resulting in the rapid removal of nitrate. In contrast, when sodium acetate was added as a carbon source, the nitrate removal efficiency by strain D5 was 81.1% within 36 h, which was lower than that of strain H6. The concentrations subsequently decreased to 2.51 mg NO_3^-–N/L at the end of the experiments, with a nitrate removal efficiency of 97.9%.

These results indicate that strains H6 and D5 can use sodium acetate and sodium citrate, respectively, as electron donors during denitrification, where sodium citrate was the best carbon source for strain D5 and sodium acetate was more suitable for strain H6. Overall, these findings indicate that the carbon source significantly influences the denitrification rate, which is concurrent with the findings of several previous studies [26,40].

3.1.3. Accumulation of nitrite

Denitrification is a dissimilative pathway, and the denitrification process involves the reduction of nitrate to nitrite by nitrate reductase, then to nitrogen dioxide by

nitrite reductase, and finally to dinitrogen [33,41]. Nitrite is an intermediate byproduct of denitrification. It is more toxic to human health than nitrate, and therefore undesirable in nitrate reduction. The World Health Organization has set limits of 11.3 mg NO_3^-–N/L and 0.9 mg NO_2^-–N/L [42]. Nitrite accumulation is influenced by pH, organic carbon sources, phosphate concentration, temperature and the denitrifying bacteria themselves [41,43–46]. As shown in Fig. 1B, the nitrite accumulation in the three flasks with different carbon sources evidently differed when strain H6 was used. Nitrite was not accumulated in any of the experiments when potassium sodium tartrate and sodium acetate were used as carbon sources. This was likely because H6 could not completely utilize potassium sodium tartrate during nitrate reduction, and only trace amount of nitrate was completely reduced to N_2; accordingly, no nitrite was accumulated. In contrast, there was no nitrite accumulation during denitrification supported by sodium acetate, even though strain H6 could remove nitrate completely. These findings indicate high nitrite reductase activity in strain H6; hence, nitrite was reduced rapidly during denitrification. Contrary to the use of sodium acetate and potassium sodium tartrate as carbon sources, nitrite was accumulated during denitrification by H6 using sodium citrate as the carbon source. The nitrite concentration in the experiments was 7.23 mg NO_2^-–N/L in 24 h, and reached 8.86 mg NO_2^-–N/L at 60 h, which subsequently decreased to below 0.9 mg NO_2^-–N/L by 72 h. It can be inferred that carbon sources significantly affect nitrite accumulation. The nitrite reductase activity was higher than the nitrate reductase activity in strain H6 when sodium acetate was used as the carbon source, which reduced the nitrite produced during the denitrification process with time; therefore, nitrite was not accumulated in the system. However, activity of nitrate reductase was higher than nitrite reductase in strain H6 when sodium citrate was added as the carbon source, resulting in a higher nitrate reduction rate than that of nitrite. As a result, nitrite accumulation was evident during denitrification. This phenomenon indicates that although strain H6 could use sodium citrate as a carbon source, it resulted in significant nitrite accumulation during denitrification. Accordingly, it is important to select an appropriate carbon source that enhances both the nitrate and nitrite removal rates during denitrification. Rocher et al. [41] also reported that carbon sources have a discernible influence on nitrite accumulation during denitrification.

Similar to H6, nitrite was not accumulated when potassium sodium tartrate was used as the carbon source for denitrification by strain D5. When combined with nitrate removal, incomplete denitrification occurred when potassium sodium tartrate was used as the carbon source; therefore, nitrate could not be reduced effectively. Consequently, nitrite accumulation was not observed in the experiments. However, there was significant nitrite accumulation during denitrification when sodium citrate and sodium acetate were used as carbon sources (Fig. 2B). Specifically, the nitrite concentration at 12 h reached 14.58 mg NO_2^-–N/L when sodium citrate was added as the carbon source, which gradually increased to 22.24 mg NO_2^-–N/L. Similarly, the nitrite concentration increased to 18.08 mg NO_2^-–N/L at 24 h when sodium acetate was added as the carbon source, and was maintained at a high concentration throughout the experiments. This phenomenon demonstrates that nitrite was accumulated significantly during denitrification by strain D5 than by strain H6, when sodium citrate and sodium acetate were used. This indicates that the nitrate reductase activity of strain D5 was much higher than that of nitrite reductase; therefore, strain D5 is a denitrifying bacterium that easily induces nitrite accumulation. Payne observed that certain heterotrophic denitrifying bacteria only contain nitrate reductase and could therefore only reduce nitrate to nitrite, while others contain all the enzymes required for denitrification [47].

Based on the above analysis, the carbon source is a critical factor influencing denitrification. Moreover, different denitrifying bacteria use different carbon sources and have distinct species specificity [48]. Potassium sodium tartrate could not support complete denitrification and was not an effective carbon source for strains H6 and D5. The strains H6 and D5 used both sodium acetate and sodium citrate, but sodium acetate was the best carbon source for strain H6, enabling complete denitrification with no nitrite accumulation. For strain D5, sodium citrate showed the best performance in stimulating denitrification; however, this strain showed significant nitrite accumulation during denitrification, regardless of sodium citrate or sodium acetate as the carbon source. In contrast to strain D5, strain H6 did not accumulate nitrite throughout the experiments. Overall, these findings indicate that strain H6 has excellent denitrification performance and can be used in remediating nitrate-polluted water.
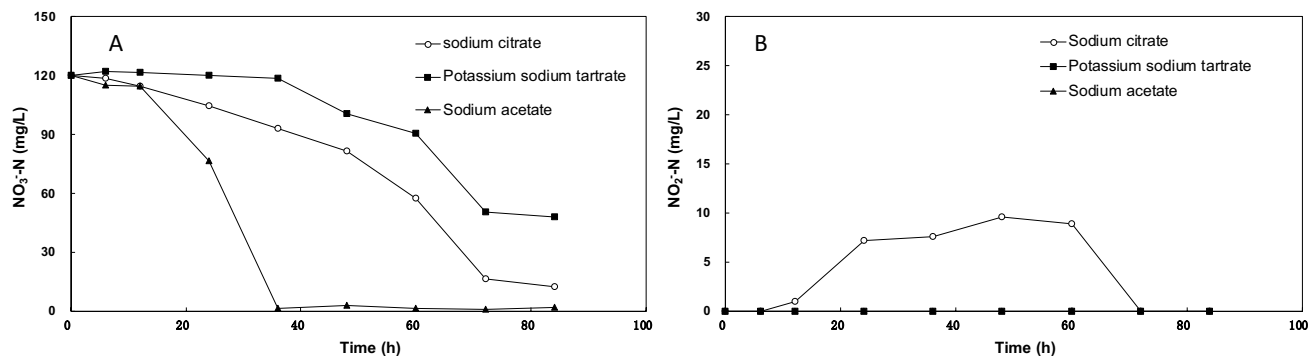

Fig. 1. Concentrations of (A) nitrate and (B) nitrite for all carbon sources with strain H6 as the denitrifying bacteria. The C/N ratio was 8.

3.2. Performance of strains H6 and D5 under different temperatures

3.2.1. Nitrate removal

Temperature has a significant effect on biological denitrification. In this study, the denitrification performance of strains H6 and D5 was evaluated at 15°C, 25°C, and 30°C.

Fig. 3 presents the results of denitrification experiments using strain H6. As shown in Fig. 3A, the nitrate concentrations gradually decreased from the initial level of 105.00 NO_3^-–N/L to 70.75 mg NO_3^-–N/L over the course of the experiment at temperature 15°C, indicating the nitrate removal efficiency of only 32.3%. These findings suggested that nitrate cannot be completely removed by strain H6 at 15°C, which was similar to the results of previous studies [33,34]. When the temperature was increased to 25°C and 30°C, the nitrate removal trend was similar. Specifically, the nitrate concentrations decreased gradually during the first 12 h, after which they decreased rapidly to 64.60 mg NO_3^-–N/L (25°C) and 16.36 mg NO_3^-–N/L (30°C) at 36 h, with nitrate removal efficiencies of 38.5% and 84.4%, respectively, and demonstrated rapid nitrate removal at 30°C. After 36 h, the concentrations continued to decrease and the nitrate removal efficiencies were above 95% at 25°C or 30°C. Collectively, these findings indicate that nitrate can be completely removed by strain H6 at 25°C and 30°C.

In the flasks inoculated with strain D5, the effects of temperature on nitrate removal were similar to those of strain H6. When the temperature was 15°C, the nitrate removal efficiency was only 8.5%, which increased significantly at 25°C and 30°C (Fig. 4A). The removal efficiency was greater than 95% at the end of the experiment.

3.2.2. Nitrite accumulation

Nitrite was either absent or observed in trace amounts in the experiments with strain H6 at 15°C, 25°C, and 30°C (Fig. 3B), indicating that this strain was an excellent denitrifying bacterium that did not accumulate nitrite during denitrification. However, when strain D5 was used, there was substantial nitrite accumulation during the experiment at 25°C and 30°C, with nitrite concentrations of 88.14 mg NO_2^-–N/L (25°C) and 76.70 mg NO_2^-–N/L (30°C), respectively, observed at the end of the experiments (Fig. 4B), which further verified that strain D5 was a denitrifying bacterium that accumulated nitrite during denitrification.

Based on the aforementioned results, the denitrification activities of strains H6 and D5 were low at 15°C, with incomplete nitrate removal. However, the activity of these two denitrifying bacteria evidently improved at 25°C and 30°C, with nitrate removal efficiencies above 95%. Sirivedhin and Gray [33] also found that the denitrification rate increased when the temperature was increased from 4°C to 25°C.
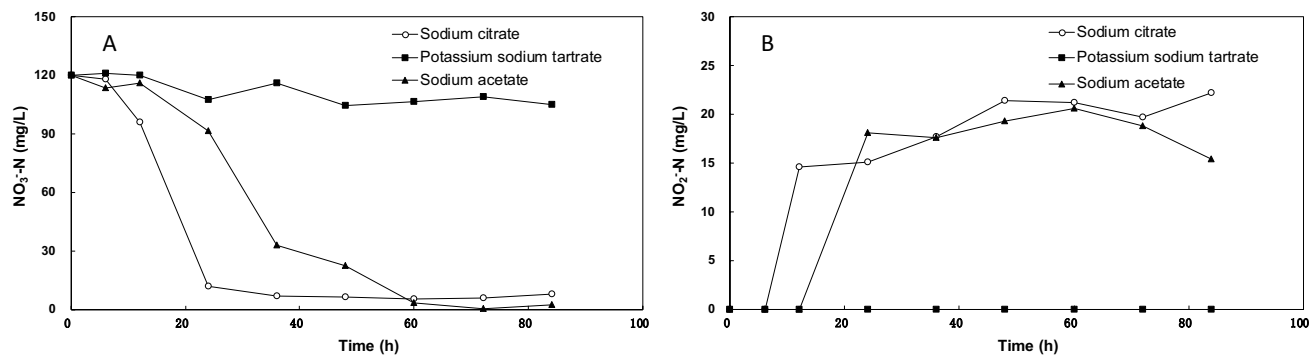

Fig. 2. Concentrations of (A) nitrate and (B) nitrite for all carbon sources with strain D5 as the denitrifying bacteria. The C/N ratio was 8.
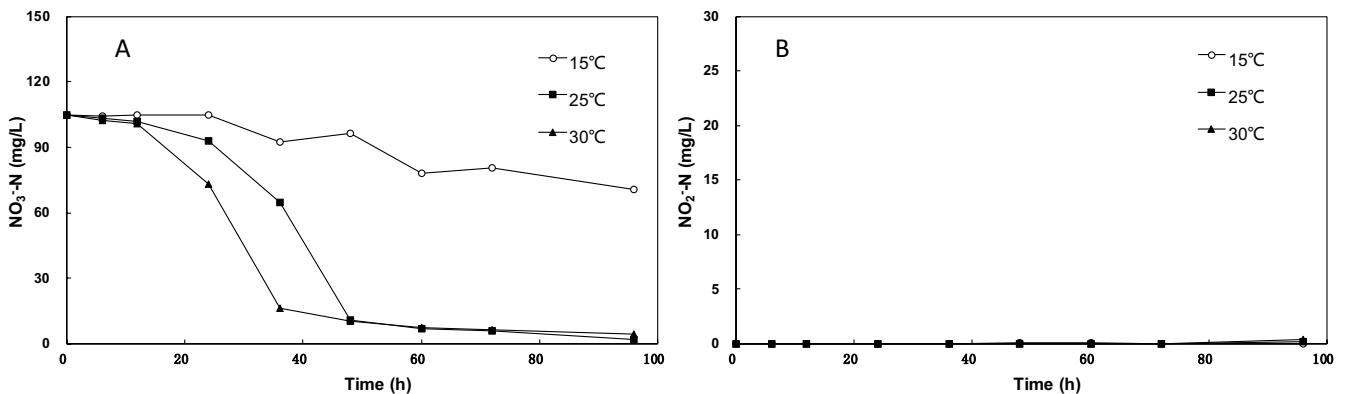

Fig. 3. Concentrations of (A) nitrate and (B) nitrite at 15°C, 25°C, and 30°C with strain H6 as the denitrifying bacteria. Carbon source: sodium acetate, C/N ratio: 5.
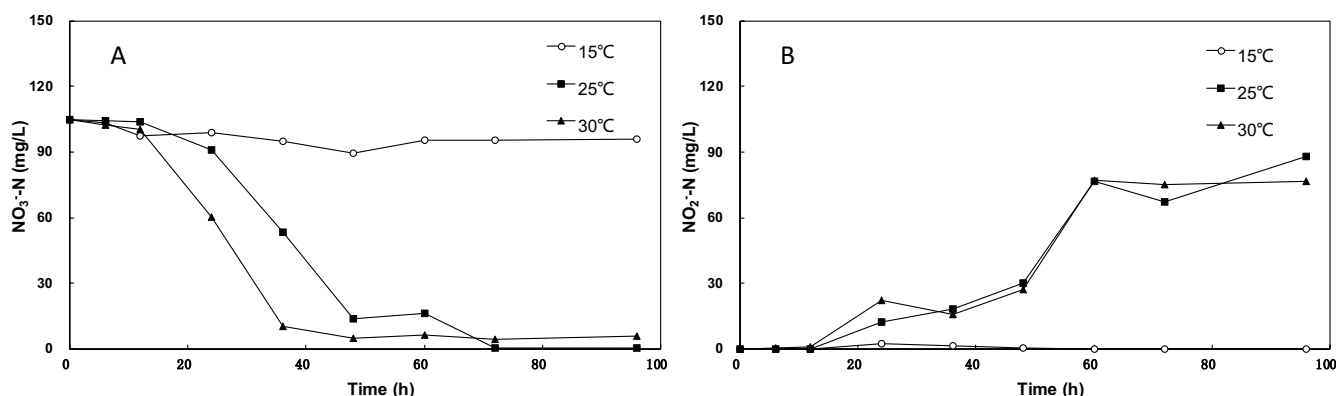
Fig. 4. Concentrations of (A) nitrate and (B) nitrite at 15°C, 25°C, and 30°C with strain D5 as the denitrifying bacteria. Carbon source: sodium acetate, C/N ratio: 5.

3.3. Cooperating relationship between the strains H6 and D5 during denitrification

Denitrification often involves synergistic effects of a variety of bacteria. Competition for electron donors and cooperation exists when different denitrifying bacteria are present in the same environment, and these factors impact nitrate removal. The relationship between the strains H6 and D5 during denitrification was investigated by inoculating the two strains together.

As shown in Fig. 5A, the nitrate concentration rapidly decreased to 10.77 and 3.88 mg NO_3^-–N/L in 36 h with sodium citrate and sodium acetate as carbon sources, with nitrate removal efficiencies of 91.0% and 96.8%, respectively. Thereafter, the nitrate concentrations decreased gradually, resulting in nitrate removal efficiencies of above 98% at the end of the experiments. This reduction was greater than that observed when H6 was used individually with sodium citrate as the carbon source (31.9% reduction in 48 h and 89.6% reduction; Fig. 1A). Similarly, the nitrate removal efficiency was only 81.1% in 36 h when strain D5 was used with sodium acetate as the carbon source (Fig. 2A). These results indicate that to overcome the carbon-source-based limitations of denitrification observed using individual strains, both bacteria can be used in denitrification.

Nitrite accumulation was also observed in the synergy experiment (Fig. 5B). When sodium citrate was used as the

carbon source, nitrite accumulated significantly during the experiment, with a maximum nitrite concentration of 21.56 mg NO_2^-–N/L at 48 h, which rapidly decreased thereafter, until no nitrite was detected. The results presented above indicate that the best carbon source for strain D5 was sodium citrate. In addition, denitrification by strain D5 led to nitrite accumulation due to large amount of nitrite generated during the experiment. Payne also reported that when a certain factor restrained denitrifying bacteria containing all reductases, but had negligible effect on denitrifying bacteria containing only nitrate reductase, nitrite accumulation occurred [47]. In the synergy experiment conducted in the present study, the nitrite accumulated by strain D5 was gradually reduced by strain H6; hence, no net accumulation of nitrite was observed at the end of the experiment. Additionally, nitrite accumulation was slightly observed throughout the experiments when sodium acetate was used as the carbon source. The above results indicated a high nitrite reductase activity in strain H6, which easily degraded the nitrite produced during denitrification. In addition, sodium acetate was the best carbon source for strain H6. Consequently, the activity of strain H6 was higher than that of strain D5 for sodium acetate as a carbon source; therefore, even though large amount of nitrite was produced by strain D5 during denitrification, strain H6 could reduce it rapidly, and nitrite was not accumulated in the entire experiment. This indicates
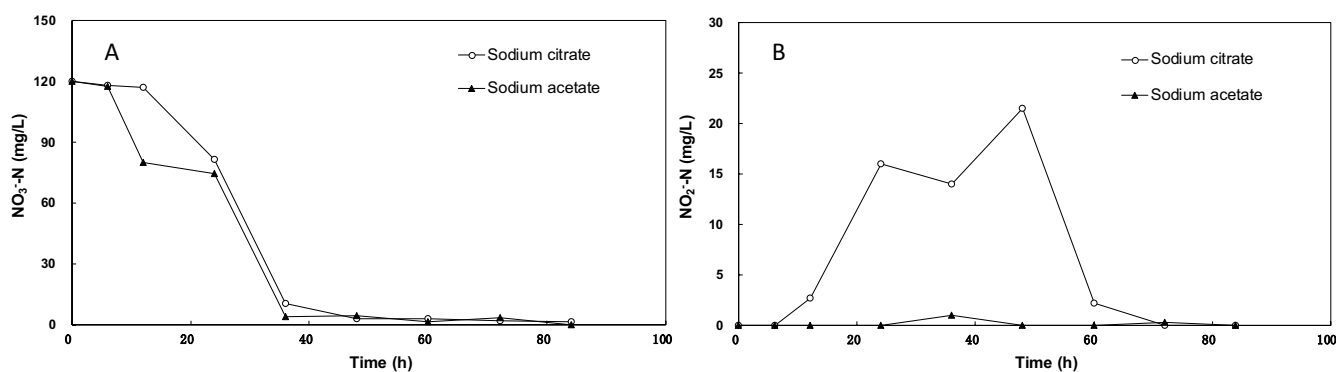

Fig. 5. Concentrations of (A) nitrate and (B) nitrite in supernatants inoculated with strains H6 and D5 as the denitrifying bacteria. Sodium citrate and sodium acetate were used as carbon sources. The C/N ratio was 8.

that the growth of dominant bacteria in a denitrification system can be promoted by selecting an appropriate carbon source, which can prevent the accumulation of intermediate products.

Based on the above analysis, H6 and D5 have positive synergy with each other. The nitrite produced by strain D5 in the denitrification process could be degraded by H6, thus avoiding the final accumulation of nitrite. Moreover, when the added carbon source was not the most suitable for one strain, it was the most suitable for another strain. The denitrification efficiency of the entire system can still be maintained at an optimum level. Consequently, the synergistic effects of a variety of bacteria can expand the selection range of carbon sources while improving nitrate removal efficiency to reduce nitrite accumulation.

4. Conclusions

This study demonstrated the performance of two denitrifying bacteria and their cooperating mechanisms during denitrification. When compared with strain D5, strain H6 exhibited improved denitrification because of its high nitrate removal efficiency and low nitrite accumulation. Additionally, sodium citrate was the best carbon source for strain D5, while sodium acetate was the most suitable carbon source for strain H6. Moreover, the strains H6 and D5 could not remove nitrate at a temperature of 15°C, while the nitrate removal efficiency was above 95% when the temperature was increased to 25°C and 30°C. Synergy also existed between strains H6 and D5, with nitrate efficiency exceeding 98%, and nitrite was undetected when the strains were inoculated into the denitrification system.

Acknowledgements

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