The Alkali-Tolerant Bacterium of Bacillus thuringiensis EM-A1 Can Effectively Perform Heterotrophic Nitrification and Aerobic Denitrification

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Removal of nitrogen from hydroxylamine could effectively improve the wastewater treatment efficiency. In this work, Bacillus thuringiensis EM-A1 was obtained from a biogas digester with hydroxylamine as the only nitrogen source. Hydroxylamine (100%) and total nitrogen (71.86%) were efficiently removed under the following conditions: 30°C, sucrose as carbon source, carbon to nitrogen ratio 40, rotation speed 0 rpm, pH 9.58, and inoculant concentration of 0.58 × 10⁸ colony-forming units. Ammonium was completely consumed by strain EM-A1, and 8.32 ± 0.08 mg/L of nitrate was produced during the ammonium removal process. During aerobic denitrification, the removal efficiencies of NO₂⁻-N and NO₃⁻-N by strain EM-A1 were 100 and 76.67%, respectively. There were about 29.34 ± 0.18%, 26.71 ± 0.36%, and 23.72 ± 0.88% initial total nitrogen lost as nitrogenous gas when NH₄⁺, NO₃⁻, and NO₂⁻ were separately used as the sole nitrogen source. The specific activities of ammonia monooxygenase, hydroxylamine oxidoreductase, nitrate reductase, and nitrite oxidoreductase were successfully detected as 0.37, 0.88, 0.45, and 0.70 U/mg protein, respectively. These results indicated that B. thuringiensis EM-A1 is a promising candidate for bioremediation of inorganic nitrogen from wastewater.

Keywords: hydroxylamine removal, heterotrophic nitrification, aerobic denitrification, hydroxylamine oxidoreductase, nitrogen balance

INTRODUCTION

In recent years, human activities have resulted in surface water and groundwater pollution by nitrogen (N) compounds in many areas of China. The death of aquatic animals caused by the eutrophication of water bodies is a typical example of the destruction of the nitrogen cycle through human activities (Heil et al., 2016; Soler-Jofra et al., 2021). The increasing emission of N compounds into the environment has become a serious threat to human health. Accumulation of large amounts of inorganic N could affect the reproduction and metabolic activities of humans and animals (Jiang, 2000). High N pollution has become an urgent problem for humans, animals, and nature (Jiang, 2000; Zhang et al., 2008; Wu, 2018). The efficient and effective removal of inorganic N from wastewater has become an important research field. Recently, N removal by classical biological nitrification–denitrification has been widely used as one of the most effective and economical processes for wastewater treatment.
Forty percent of surface water does not meet international drinking water standards, and the ammonium nitrogen (NH$_4^+$-N) is the main N-containing pollutant (Feng et al., 2012). Additionally, the nitrite (NO$_2^-$-N) could be generated from NH$_4^+$-N oxidation process. When NO$_2^-$-N is accumulated in the body, it may affect the delivery of oxygen through the blood and cause death by hypoxia (Zhang et al., 2013). Compared with NH$_4^+$-N and NO$_2^-$-N, the nitrate (NO$_3^-$-N), as another pollutant in wastewater, is more stable and difficult to biodegrade at normal temperature (Su et al., 2016). Therefore, the isolation of bacteria that can efficiently remove NH$_4^+$-N, NO$_2^-$-N, and NO$_3^-$-N is very important to purify the N pollution wastewater.

Previous studies showed that hydroxylamine (NH$_2$OH) is an inevitable intermediate in the process of heterotrophic nitrification (Chen and Ni, 2011; Chen and Ni, 2012; Huang et al., 2013). Excessive NH$_2$OH accumulation would lower the total nitrogen (TN) removal efficiency and might also result in bacterial death in wastewater (Ouyang et al., 2020; Xing et al., 2020). For instance, 0.2, 1.0, and 5.0 mg/L of NH$_2$OH strongly inhibited the activities of nitrite-oxidizing bacteria, *Nitrobacter*, and *Nitrobroch agilis*, respectively (Soler-Jofra et al., 2021). Unfortunately, the NH$_2$OH could accumulate to 4.3 mg/L from ammonium oxidation in partial nitration granular airlift, which could result in an increase of ammonium accumulation in a continuous partial nitration airlift reactor (Poot et al., 2016; Soler-Jofra et al., 2021). Therefore, exploiting the bacteria that can effectively remove NH$_2$OH is important to improve the wastewater treatment efficiency. However, the studies on nitrifying or denitrifying bacteria that can effectively remove NH$_2$OH are rarely reported (Zhao et al., 2020b; Chen et al., 2014; Liu et al., 2019; Wang and He, 2020), because it is difficult to provide electrons to denitrifying bacteria through NH$_2$OH nitrification (Jetten et al., 1997). In addition, the majority of heterotrophic nitrification and aerobic denitrification bacteria are unsuitable to purify the alkaline wastewaters, such as papermaking, chemical, textile, food, petroleum, chloro-alkali, and beverage industries (Jain et al., 2014; Xia et al., 2020).

In this study, the strain of EM-A1 was isolated with the sole NH$_2$OH source, which was identified as *Bacillus thuringiensis*. The inorganic nitrogen removal efficiencies and intermediate products accumulation were conducted by a series of shake flask tests. The cell growth and NH$_2$OH removal characteristics with the critical influence factors, such as inoculum size, carbon (C) source, C to N ratio (C/N), pH, rotation speeds, and temperature, were carefully investigated. The pathways of heterotrophic nitrification and aerobic denitrification were analyzed by nitrogen balance. Furthermore, the specific activities of ammonia monoxygenase, hydroxylamine oxidoreductase, nitrate reductase, and nitrite oxidoreductase involved in the removal of NH$_4^+$-N, NH$_2$OH, NO$_2^-$-N, and NO$_3^-$-N were detected. The results observed from the above experiments may explain why strain EM-A1 could be used as an alternative microbial resource for the removal of multiple inorganic nitrogen forms, especially for NH$_2$OH, from the wastewater treatment system.

**MATERIALS AND METHODS**

**Materials**

The biogas residue sample was collected from biogas digester, Guiyang country, Guizhou province, China.

**Media**

The enrichment medium (g/L) consisted of 0.124 NH$_2$OH.HCl, 3.6763 sodium citrate, 0.04 MgSO$_4$, 1.5 KH$_2$PO$_4$, 0.009 Fe$_2$(SO$_4$)$_3$, 0.014 CaCl$_2$, and 3.5 K$_2$HPO$_4$. The pH was adjusted to 7.2.

The screening medium for NH$_2$OH (g/L) consisted of 0.0992 NH$_2$OH.HCl, 3.6763 sodium citrate, 3.5 K$_2$HPO$_4$, 1.5 KH$_2$PO$_4$, 0.04 MgSO$_4$, 0.009 Fe$_2$(SO$_4$)$_3$, and 0.014 CaCl$_2$. The pH was adjusted to 7.2.

The bromothymol blue (BTB) solid medium (pH 7.0) contained (g/L): 8.5 sodium succinate, 1 NaNO$_3$, 0.2 CaCl$_2$·7H$_2$O, 1 MgSO$_4$·7H$_2$O, 1 KH$_2$PO$_4$, 0.5 FeCl$_2$·6H$_2$O, BTB reagent 1 ml [1.5% in ethanol], and 20 agar (He et al., 2018).

LB medium (pH 7.2) was prepared according to standard procedures (Chen et al., 2011).

**Bacterial Isolation and Identification**

The sample from biogas digester (1 ml) was inoculated into enrichment medium and cultured for 4 days (20°C, 150 rpm), and the operation was repeated three times. The enriched culture (25, 35, and 45 µl) was spread on BTB agar plates with a glass rod. After inoculation (20°C, 48 h), bacteria with blue circular colonies were selected and cultured on fresh BTB plates. After 3–5 rounds of purification, the purified nitrifying bacteria were inoculated on LB solid medium (20°C, 48 h) and stored at 4°C for later experiments. All operations were performed under aseptic conditions.

The isolated single colonies were inoculated on BTB medium and the colony morphology was observed on the LB medium. The scanning electron microscopy (SU8100; Hitachi, Japan) is used for strain morphology, and gram staining was examined under optical microscopy (Olympus BX53-DIC; Olympus, Japan). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers 27F (5′-AGAGTTTGACCTGGCTTAG-3′) and 1492R (5′-GGTTACCTTGTGTTAATCCT-3′) (Guo et al., 2013). The 16S rRNA gene sequencing was performed by General Biol (Anhui, China). Obtained alignment was performed by the database of BLAST. The phylogenetic tree was constructed by MEGA 6.0.

**Nitrification and Denitrification Ability of Strain EM-A1**

To evaluate the nitrification and denitrification capacity of strain EM-A1, three N compounds (NH$_4^+$, NO$_2^-$, and NO$_3^-$) were used...
as sole N source in BM instead of NH$_2$OH. For each N compound, five species of sodium citrate were selected as the C sources. The OD$_{600}$ of the inoculant was adjusted to 0.25 $\times$ 10$^8$ CFU in 250 ml Erlenmeyer flasks, and then incubated aerobically at 20°C, 150 rpm for 48 h.

**Evaluation of NH$_2$OH and Total N Removal Capacity**

The effects of different culture conditions (inoculant concentration, dissolved oxygen (DO), temperature, C/N ratio, C source, and pH) on NH$_2$OH and TN removal by strain EM-A1 were evaluated. The inoculant concentration was evaluated by the optical density at 600 nm (OD$_{600}$). Controls were prepared containing screening medium without bacteria inoculation. After 91 h inoculation, cultures were taken to measure the cell density and various inorganic N contents (including NH$_4^+$-N, NO$_2^-$-N, NO$_3^-$-N, NH$_2$OH, and TN). Single-factor-effect tests on the N removal capacity of strain EM-A1 were tested as follows: temperature (5, 10, 15, 20, 25, and 30°C), pH (6.52, 6.92, 7.45, 7.98, 8.56, and 9.58), rotation speed (0, 50, 100, 150, and 200 rpm), C source (sodium citrate, sodium potassium tartrate, sodium carbonate, sucrose, and glucose), C/N ratio (5, 10, 15, 20, 25, 30, 35, 40, and 45), and inoculum concentration (0.35 $\times$ 10$^8$, 0.42 $\times$ 10$^8$, 0.5 $\times$ 10$^8$, 0.58 $\times$ 10$^8$, and 0.68 $\times$ 10$^8$ colony-forming units (CFUs)). Strain EM-A1 was inoculated at 20°C with rotation speed (150 rpm) for 91 h. All experiments were carried out at 20°C except for the temperature treatments. The removal efficiencies of NH$_2$OH and TN were calculated to determine the optimal conditions for nitrification by strain EM-A1.

The formula of nitrogen removal efficiency was (L1−L2)/L1 $\times$ 100%, where L1 and L2 represent the initial and final concentrations of NH$_2$OH (or TN) in the medium at 91 h, respectively.

**Nitrogen Balance Analysis**

To explore the nitrogen balance during inorganic nitrogen removal process, the strain EM-A1 was harvested after cultivation and centrifuged at 8,000 $\times$ g for 5 min. To ensure the inclusion of intracellular total nitrogen in the final total nitrogen, the Scientz-IID ultrasonic cell disruption system was used to break bacterial cells. The calculations of lost N as nitrogen gas were determined as described previously (Chen et al., 2021). And all the nitrogen balances of inorganic nitrogen were performed under the condition of C/N ratio of 15.

**Enzyme Activities**

After cultivation, the cells of strain EM-A1 were harvested with sole NH$_4^+$-N, NH$_2$OH, NO$_2^-$-N, or NO$_3^-$-N and centrifuged at 6,000 $\times$ g for 5 min. The total protein was extracted using a protein extraction kit (CWBIO), and the protein concentration was determined using the BCA protein assay kit (Beijing, China). The ammonia monooxygenase (AMO) was detected by Welai Company (Guiyang, China). The HAO activity trial included NH$_2$OH, and enzyme extract. The disappearance of hydroxylamine from the reaction mixture was used to demonstrate the HAO (Chen et al., 2021). For evaluation of the activity of nitrate reductase (NR), the reaction mixture (20 ml) contained Tris-HCl buffer (pH = 7.4), 0.02 mmol/l NADH, enzyme extract and nitrate. The disappearance of nitrate from the reaction mixture was used to estimate the activity of NR. The nitrite oxidoreductase (NIR) activity was measured by nitrite reductase kit (COMIN).

The specific activity (U/mg) was defined as the amount of enzyme that catalyzed the transformation of 1 pmol of the substrate per minute by the amount of protein in mg. The nonenzyme extract addition treatment was used as the blank controls. All assays were displayed in triplicate.

**Analytical Methods**

Bacterial growth was determined by OD$_{600}$. The concentrations of TN, NO$_3^-$-N, NO$_2^-$-N, and NH$_4^+$-N were measured by alkaline potassium persulfate digestion-ultraviolet spectrophotometry, ultraviolet spectrophotometry, N-(1-naphthalene)-diaminoethane photometry, and indophenol blue method, respectively (Guo et al., 2013). NH$_2$OH was analyzed by indirect spectrophotometry (Frear and Burrell, 1955). The pH meter (DDS-307A, China) was used to detect pH. All experiments were tested in triplicate.

**RESULTS AND DISCUSSION**

**Identification of Strain EM-A1**

Strain EM-A1 showed white circular, opaque, and smooth with dry surfaces on the LB plates (Figure 1A), gram positive (Figure 1B), and had rod-shaped cells (Figure 1C). BLAST alignment analysis exhibited that strain EM-A1 had the highest similarity (99%) with the B. thuringiensis. A phylogenetic tree was constructed and revealed that strain EM-A1 belongs to B. thuringiensis based on its phylogenetic relationship (Figure 2). Based on morphological analysis, strain EM-A1 was provisionally assigned to B. thuringiensis (GenBank accession MW551565.1).

**Analysis of N Removal Characteristics of Strain EM-A1**

To determine whether the bacteria have the ability of nitrification, NH$_4^+$-N (20 mg/L) was selected as the N source in the BM. After 48 h incubation, NH$_4^+$-N was completely removed from the BM, with corresponding TN removal of 56.31% (Figure 3). These results showed that strain EM-A1 could effectively perform heterotrophic nitrification to remove NH$_4^+$-N. Meanwhile, no NO$_2^-$-N generation was observed during the NH$_4^+$-N oxidation process, while high NO$_3^-$-N concentration (8.32 ± 0.08 mg/L) was accumulated. The transformation of NH$_4^+$-N to NO$_3^-$-N indicated that the strain EM-A1 was capable of heterotrophic nitrification. The NO$_3^-$-N accumulation from NH$_4^+$-N oxidation was inconsistent with some studies, in which NH$_4^+$-N was first converted into NO$_2^-$-N and then into NO$_3^-$-N by heterotrophic nitrifying bacteria such as Pseudomonas sp. HXF1 (Hu et al., 2021) and Pseudomonas gugganensis strain 4-n-1 (Motamedi and Jafari, 2020). In addition, the low TN removal efficiency by strain
EM-A1 was possibly because of NO$_3^-$-N accumulation (Liu et al., 2015).

Subsequently, the denitrification capacity of strain EM-A1 was measured by using NO$_2^-$-N or NO$_3^-$-N as the sole N source. When NO$_2^-$-N was selected as the only N source in BM, the NO$_2^-$-N was completely removed within 48 h, and no NH$_4^+$-N was observed, but NO$_3^-$-N (2.65 $\pm$ 0.52 mg/L) was observed during the process of NO$_2^-$-N transformation. These results were inconsistent with several reported strains, such as Pseudomonas mendocina IHB602 (Hong et al., 2020), Pseudomonas taiwanensis J488 (He et al., 2020), Streptomyces mediolani EM-B2 (He et al., 2021), and Acinetobacter sp. ND7 (Xia et al., 2020), in which no accumulation of NO$_3^-$-N was observed during the NO$_2^-$-N reduction process. When NO$_3^-$-N was selected as only N source, the NO$_3^-$-N removal efficiency was 76.67% after 48 h cultivation, and the residual concentration of NO$_3^-$-N was 5.74 $\pm$ 0.73 mg/L. Meanwhile, NO$_2^-$-N accumulation was hardly detected during NO$_3^-$-N removal, which contradicted the theory that
NO$_2^-$-N accumulation is inevitable during biological treatment of wastewater (Zou et al., 2014; Zhao et al., 2018; Lang et al., 2020). Overall, these results showed that strain EM-A1 is a heterotrophic nitrifying-aerobic denitrifying bacterium that can efficiently remove low concentrations of NH$_4^+$-N, NO$_2^-$-N, and NO$_3^-$-N within 48 h.

Effects of Different Factors on NH$_2$OH and TN Removal Capacity

Effect of Inoculum Concentration on NH$_2$OH and TN Removal

The inoculum concentration is an important factor in biological N removal. Low inoculum concentrations will fail to achieve high N removal, while excessive inoculum concentrations might lead to bacterial death because of competition for nutrients (Ye et al., 2016). The NH$_2$OH and TN removal efficiencies increased with increasing inoculum concentration (Figure 4A). When the inoculum concentration increased from $0.35 \times 10^8$ to $0.42 \times 10^8$ CFU, the removal efficiencies of NH$_2$OH and TN increased from 71.14 and 6.67% to 84.19 and 11.76%, respectively. When the inoculum concentration increased to $0.58 \times 10^8$ CFU, the NH$_2$OH and TN concentration decreased to $2.6 \pm 0.68$ mg/L and $18.02 \pm 0.53$ mg/L. The removal efficiencies of NH$_2$OH and TN showed an increasing trend and reached the maximum values of 87.93 and 28.04%, respectively. Meanwhile, the accumulation of nitrite was only $0.92 \pm 0.04$ mg/L, which was different from the heterotrophic nitrifying bacteria Pseudomonas putida Y-9, which transformed the whole NH$_2$OH to NO$_2^-$ (Huang et al., 2019). A different relationship was observed between inoculum concentration and bacterial denitrification for high inoculum concentration. When the inoculum concentration was increased from $0.58 \times 10^8$ CFU to $0.68 \times 10^8$ CFU, the TN removal efficiency decreased significantly to 14% ($p < 0.05$). Therefore, to maintain the effective removal of NH$_2$OH and
TN, the inoculum concentration of strain EM-A1 needs to be maintained at $0.58 \times 10^8$ CFU.

**Effect of C Source on NH$_2$OH and TN Removal**

A C source is necessary for cell growth and metabolism of microorganisms, and different C sources directly affect the N removal ability of heterotrophic nitrifying bacteria (Ren et al., 2014). In this experiment, the effects of five C sources on the removal of NH$_2$OH and TN by strain EM-A1 were studied. The bacterium could use a wide range of C sources, but only sodium carbonate and sucrose significantly promoted NH$_2$OH removal (Figure 4B). When in a medium with sodium carbonate as the sole carbon source, whether inoculated or not, the removal efficiencies of hydroxylamine and total nitrogen were 100%, so we can infer that the removal of nitrogen is not related with bacteria. In addition to sodium carbonate, the NH$_2$OH (83.1%) and TN (55.32%) removal efficiencies were highest when sucrose was used as the C source. After inoculation of 91 h, the levels of NH$_2$OH and TN kept about 4.55 ± 0.88 mg/L and 12.36 ± 1.68 mg/L, respectively. There was little accumulation of NH$_4^+$-N (5.72 ± 0.22 mg/L), NO$_2^-$-N (0.05 ± 0.1 mg/L), and NO$_3^-$-N (1.64 ± 0.02 mg/L) observed during NH$_2$OH oxidation with growth on sucrose. Therefore, sucrose was selected as the optimal C source for EM-A1. This result was consistent with that of Bacillus licheniformis FP6 (Nie et al., 2013), but was different from those for Bacillus methylotrophicus strain L7 (Zhang et al., 2012), Pseudomonas aeruginosa P-1 (Wei et al., 2021), Pseudomonas stutzeri GEP-01 (Gao et al., 2020), for which the optimal C sources were sodium succinate, glucose, and sodium citrate, respectively. These experimental results showed that carbohydrate was more beneficial to N removal than organic substances for strain EM-A1.

**NH$_2$OH and TN Removal at Different Temperature**

Temperature is not only an important factor for maintaining enzyme activity but also has a great impact on various metabolic processes in cells (Huang, 2009). Previous reports showed that low temperature would reduce or even completely inhibit the growth, activity, and function of relevant ammonia-oxidizing bacteria, and eventually lead to failure of the nitrification process (Taylor and Bishop, 1989). In this research, the capacity of strain EM-A1 to remove NH$_2$OH and TN at different temperatures was investigated. The N removal of strain EM-A1 occurred over a wide temperature range (from 5°C to 30°C; Figure 5A). Increasing the temperature significantly enhanced the NH$_2$OH and TN removal efficiencies. The NH$_2$OH removal efficiency showed a gentle upward trend (from 19.25 to 46.04%) when the temperature was changed from 5°C to 20°C, which was consistent with the theory that low temperature is not conducive to bacterial nitrification (He et al., 2018). When temperature rose from 20°C to 30°C, the NH$_2$OH and TN removal was continuously enhanced, and the maximum NH$_2$OH (93.44%) and TN (67.18%) removal efficiencies were observed at 30°C. Meanwhile, the low concentrations of NH$_2$OH (1.43 ± 0.03 mg/L) and TN (7.73 ± 0.18 mg/L) were detected at the end of cultivation. When the temperature was increased from 5°C to 30°C, the concentrations of inorganic N (ammonium, nitrite, and nitrate) were increased from 2.54 ± 0.21 mg/L to 6.29 ± 0.3 mg/L, which might be because the higher temperature was more conducive to NH$_2$OH conversion by strain EM-A1. The NH$_2$OH and TN removal efficiency dropped sharply if the temperature exceeded 30°C, whether inoculated with strain EM-A1 or not. This phenomenon exhibited that high NH$_2$OH removal efficiency at 35°C was
connected with its instability at high temperature, rather than inoculation of strain EM-A1. Above all, the optimum temperature for strain EM-A1 is 30°C, which was consistent with several reports that the optimum temperature is 30–35°C (Shammas, 1986; Hammer and Knight, 1994).

**NH$_2$OH and TN Removal Under Different C/N Ratios**

Sufficient C is necessary for microbial growth (Yan et al., 2002). To explore the optimal C/N ratio for strain EM-A1, C/N ratios (15, 20, 25, 30, 35, 40, and 45) were tested. As shown in Figure 5B, the C/N ratio obviously affected the N removal capacity of strain EM-A1 ($p < 0.05$). When the C/N ratio was 15, the NH$_2$OH (3.1%) and TN removal efficiencies (15.61%) were lowest, which might be because there was insufficient C for heterotrophic growth (Wan et al., 2017). When the C/N ratio was changed from 20 to 35, the NH$_2$OH and TN removal efficiencies have expanded from 4.51 and 23.84% to 65.49 and 99.78%, respectively ($p < 0.05$). This showed that increasing the C/N ratio significantly improved the N removal ability of strain EM-A1. The removal efficiencies of NH$_2$OH and TN were increased to the maximum of 100 and 73.91%, respectively, when the C/N ratio was 40. Only 6.94 ± 0.12 mg/L of TN was left in the medium. There was about C inorganic N (NH$_4^+$-N, NO$_2^-$-N, and NO$_3^-$-N) accumulation during the NH$_2$OH oxidation process, which indicated that NH$_2$OH was mainly transformed to gaseous N (Liu et al., 2019; He et al., 2020). The result was consistent with the theory that heterotrophic nitrification bacteria usually require high concentrations of organic C for nitrification (Li et al., 2021; Zhang et al., 2021). However, this does not mean that the higher C/N ratios are more favorable for the nitrification ability of bacteria. When C/N ratio rose to 45, the removal efficiencies of NH$_2$OH and TN decreased to 55.32 and 83.10%, respectively. Therefore, the optimum C/N ratio for strain EM-A1 was 40, which indicated that strain EM-A1 was more suitable for wastewater with high C content.

**Effect of pH on Removal of Hydroxylamine and Total Nitrogen**

According to previous reports, pH affects the absorption of nutrients and emission of membrane for bacteria by affecting cell membrane potential and emission of metabolites (Miao, 2011). The pH range suitable for nitrification by nitrifying bacteria is 7.9–9.0, and nitrification can be inhibited at pH values lower than 6.0 (Wang et al., 2003). Consequently, it is essential to explore the influence of pH on heterotrophic nitrification. As shown in Figure 6A, the removal efficiencies of NH$_2$OH and TN increased with increasing pH. When the pH was 6.52, strain EM-A1 had the lowest removal efficiencies of NH$_2$OH and TN, which were 25.31 and 0.1%, respectively. When the pH rose from 6.52 to 8.56, the removal efficiencies of NH$_2$OH and TN significantly improved to 47.84 and 23.72%, respectively. These results showed that higher pH was more beneficial for NH$_2$OH and TN removal by strain EM-A1. When the pH increased from 8.56 to 9.33, the NH$_2$OH and TN removal efficiencies increased rapidly, and maximum NH$_2$OH and TN removal efficiencies (100 and 71.86%) were observed when the pH was 9.33. Only 5.79 ± 0.63 mg/L of TN was not removed. However, after 91 h incubation, the NO$_2^-$-N accumulation peaked a maximum value of 2.22 ± 0.08 mg/L, and the concentration of NH$_4^+$-N was lowest (2.08 ± 0.08 mg/L) at pH 9.33. These aforementioned results suggested that a strong alkaline environment was more conducive to nitrification by strain EM-A1, which is inconsistent with previous studies, where the optimal pH of nitrification was 7.85 and 8–9 (Hans, 1994; Jiang et al., 2007; Chen et al., 2015). Strain EM-A1 is an alkali-resisting bacterium.

**The NH$_2$OH and TN Removal at Different DO**

To explore the influence of DO on the N removal ability of strain EM-A1, different shaker rotation speeds (0, 50, 100, 150, and 200 rpm) were measured. The N removal ability of strain
EM-A1 was decreased with the increasing rotation speeds (Figure 6B).

For instance, when the rotation speed was stationary, the lowest NH$_2$OH and TN removal efficiencies were achieved of 41.16 and 14.83%, respectively. The inorganic nitrogen of NO$_2^-\text{-N}$ (0.80 ± 0.04 mg/L), NO$_3^-\text{-N}$ (0.39 ± 0.36 mg/L), and NH$_4^+\text{-N}$ (6.73 ± 0.015 mg/L) were accumulated. The relative high level of NH$_2$OH oxidation at 0 rpm was inconsistent with a previous report (Liu and Wang, 2013). As the further increase of the speed was from 50 to 100 rpm, the NH$_2$OH removal efficiency increased from 48.99 to 59.07%. The highest NH$_2$OH and TN removal efficiencies of 73.42 and 48.2% were obtained when the speed climbed to 150 rpm. In such condition, the residual of NH$_2$OH and TN was 7 ± 0.57 mg/L and 14.58 ± 0.47 mg/L, respectively. This further confirmed that high DO condition was more conductive to strain EM-A1 to perform heterotrophic nitrification and cell growth, which was also reported by several studies (Wang et al., 2007; Lei et al., 2019; Ouyang et al., 2020).

**Nitrogen Balance Analysis in Process of Heterotrophic Nitrification and Aerobic Denitrification**

The discrepancy results of the nitrogen balance among, NH$_4^+\text{-N}$, NO$_3^-\text{-N}$, and NO$_2^-\text{-N}$ were shown in Table 1. When NH$_4^+\text{-N}$ was used as the sole nitrogen source, about 29.34% ± 0.18 of initial TN was removed as the nitrogenous gas after 24 h incubation. Only 0.72 ± 0.06 mg/L of NH$_4^+\text{was}$ remained in the nitrification system. There were low concentrations of NH$_2$OH, NO$_3^-\text{-N}$, and organic-N accumulated. The nitrite was not detected even though the NH$_2$OH was produced as 5.47 mg/L, which was different from that there was about 0.05–2.22 mg/L nitrite generation during the NH$_2$OH oxidation process. This may be that a small amount of accumulation nitrite was rapidly converted into nitrogenous gas. Therefore, the nitrification pathway could be inferred as NH$_4^+\text{-N} \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_3^-\text{-N} \rightarrow \text{NO}_2^-\text{-N} \rightarrow \text{nitrogenous gas}$. When the NO$_3^-\text{-N}$ and NO$_2^-\text{-N}$ were separately used as the sole nitrogen, the nitrogen loss efficiency reached 26.71 ± 0.36% and 23.72 ± 0.88%, respectively. The nitrogen loss efficiency was considerably higher than that of 14.88% by *Paracoccus denitrificans* Z195 (Zhang et al., 2020) and 19.63% by *Stenotrophomonas maltophilia* DQ01 (Jia et al., 2019) when they were processed with nitrate alone. The NO$_2^-\text{-N}$ accumulation was detected during NO$_2^-\text{-N}$ transformation process. Thus, the denitrification pathway could be deduced as NO$_3^-\text{-N} \rightarrow \text{NO}_2^-\text{-N} \rightarrow \text{nitrogenous gas}$.

**Enzyme Analysis**

To further analyze the nitrogen removal mechanism of strain EM-A1, AMO, HAO, NR, and NiR activities were examined. After cell lysis of strain EM-A1, the specific activities of AMO, HAO, NR, and NiR were detected as 0.37, 0.88, 0.45, and 0.70 U/mg protein, respectively (Table 1). Medhi et al. (2017) found that the extracellular-specific activities of AMO, NR, and NiR were 0.07, 0.023, and 0.122 U/mg protein, while they were 0.01, 0.006, and 0.01 U/mg protein intracellularly under aerobic condition in a strain of *Paracoccus denitrificans* ISTOD1. The specific activities of AMO, NR, and NiR in strain EM-A1 were greater than the sum of the intracellular and extracellular related specific activities in *Paracoccus denitrificans* ISTOD1. Additionally, the specific HAO activity (0.88 U/mg protein) in strain EM-A1 was higher than those detected in *A. calcoaceticus* HNR (0.051 U/mg protein) (Zhao et al., 2010a) and *Pseudomonas* PB16 (0.47 U/mg protein) (Jetten et al., 1997). The target heterotrophic nitrification and aerobic denitrification enzymes were successfully expressed and detected, which further confirmed that strain EM-A1 could be capable of full heterotrophic nitrification and aerobic denitrification. Combined with the results of nitrogen balance analysis, the possible pathway for nitrogen removal was identical to that of NH$_4^+\text{-N} \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_3^-\text{-N} \rightarrow \text{NO}_2^-\text{-N} \rightarrow \text{nitrogenous gas}$. which was performed by *B. thuringiensis* WXN-23 (Xu et al., 2021).

**CONCLUSION**

*B. thuringiensis* EM-A1 was isolated from the biogas digester by using NH$_2$OH as the sole N source. The optimal conditions for NH$_2$OH removal were sucrose, C/N 40, temperature 30°C, pH 6.95, inoculant concentration of 0.58 × 10$^8$ CFU, and rotation speed of 0 rpm. *B. thuringiensis* EM-A1 efficiently removed low concentrations (20 mg/L) of NH$_4^+\text{-N}$, NO$_3^-\text{-N}$, and NO$_2^-\text{-N}$ within 48 h. Combined with the results of intermediates accumulation, nitrogen balance analysis, specific activities of AMO, HAO, NR, and NiR, the possible pathway for nitrogen removal by strain EM-A1 was NH$_4^+\text{-N} \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_3^-\text{-N} \rightarrow \text{NO}_2^-\text{-N} \rightarrow \text{nitrogenous gas}$. Therefore, strain EM-A1 could be a promising candidate for inorganic nitrogen removal from wastewater.

### Table 1 | Nitrogen balance of ammonium, nitrite, and nitrate removal by strain EM-A1.

| Substance  | Initial N (mg/L) | Final N (mg/L) | Intracellular-N (mg/L) | N lose (%) |
|------------|------------------|----------------|------------------------|------------|
| NH$_4^+\text{-N}$ | 54.88 ± 0.47     | 0.72 ± 0.06    | 5.47 ± 0.594           | 129 ± 0.61 |
| NO$_2^-\text{-N}$ | 57.95 ± 0.58     | 4.38 ± 0.45    | 0                      | 5.45 ± 0.23 |
| NO$_3^-\text{-N}$ | 58.18 ± 0.57     | 4.35 ± 0.06    | 0                      | 5.78 ± 1.11 |

Values represent mean ± SD of triplicates (n = 3). Nitrogen loss efficiency of strain EM-A1 was NH$_4^+\text{-N} \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_3^-\text{-N} \rightarrow \text{NO}_2^-\text{-N} \rightarrow \text{nitrogenous gas}$.
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GenBank (accession number: MW551532).

AUTHOR CONTRIBUTIONS

TW: Investigation, Experimental design, Experimental operation, Data calculation, Paper writing. MC: Investigation, Experimental design, Data calculation, Experimental operation, Figure construction. XL: Investigation, Experimental operation, Data calculation. FC: Experimental operation. TH: Investigation, Experimental idea, Conceptualization, Funding acquisition, Writing-review, Paper revisions. ZL: Investigation.

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