frontiers in Environmental Science

ORIGINAL RESEARCH
published: 19 January 2022
doi: 10.3389/fenvs.2021.818316

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

*Tingjiang Wang, Mengping Chen, Xiwen Liang, Fali Chen, Tengxia He* and Zhu Li*

*Key Laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), Collaborative Innovation Center for Mountain Ecology Agro-Bioengineering (CICMEAB), College of Life Sciences/Institute of Agro-Bioengineering, Guizhou University, Guiyang, China*

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 150 rpm, pH 9.58, and inoculant concentration of $0.58\times10^8$ 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 $NO_2^-$-N and $NO_3^-$-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 $NH_4^+$, $NO_3^-$, and $NO_2^-$ 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**

OPEN ACCESS

***Edited by:***
*Likun Wang,*
*Chinese Academy of Sciences (CAS), China*

***Reviewed by:***
*Lei Yang,*
*Xi'an University of Architecture and Technology, China*
*Ang Li,*
*Harbin Institute of Technology, China*

****Correspondence:***
*Tengxia He*
*txhe@gzu.edu.cn*

***Specialty section:***
*This article was submitted to Water and Wastewater Management, a section of the journal Frontiers in Environmental Science*

***Received:*** *19 November 2021*
***Accepted:*** *21 December 2021*
***Published:*** *19 January 2022*

***Citation:***
*Wang T, Chen M, Liang X, Chen F, He T and Li Z (2022) The Alkali-Tolerant Bacterium of* Bacillus thuringiensis *EM-A1 Can Effectively Perform Heterotrophic Nitrification and Aerobic Denitrification. Front. Environ. Sci. 9:818316. doi: 10.3389/fenvs.2021.818316*

## 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 ($NH_2OH$) is an inevitable intermediate in the process of heterotrophic nitrification (Chen and Ni, 2011; Chen and Ni, 2012; Huang et al., 2013). Excessive $NH_2OH$ 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 $NH_2OH$ strongly inhibited the activities of nitrite-oxidizing bacteria, *Nitrobacter*, and *Nitrobacter agilis*, respectively (Soler-Jofra et al., 2021). Unfortunately, the $NH_2OH$ could accumulate to 4.3 mg/L from ammonium oxidation in partial nitritation granular airlift, which could result in an increase of ammonium accumulation in a continuous partial nitrification airlift reactor (Poot et al., 2016; Soler-Jofra et al., 2021). Therefore, exploiting the bacteria that can effectively remove $NH_2OH$ is important to improve the wastewater treatment efficiency. However, the studies on nitrifying or denitrifying bacteria that can effectively remove $NH_2OH$ are rarely reported (Zhao et al., 2010b; Chen et al., 2014; Liu et al., 2019; Wang and He, 2020), because it is difficult to provide electrons to denitrifying bacteria through $NH_2OH$ 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 $NH_2OH$ 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 $NH_2OH$ 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 monooxygenase, hydroxylamine oxidoreductase, nitrate reductase, and nitrite oxidoreductase involved in the removal of $NH_4^+$-N, $NH_2OH$, $NO_3^-$-N, and $NO_2^-$-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 $NH_2OH$, 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 $NH_2OH.HCl$, 3.6763 sodium citrate, 0.04 $MgSO_4$, 1.5 $KH_2PO_4$, 0.009 $Fe_2(SO_4)_3$, 0.014 $CaCl_2$, and 3.5 $K_2HPO_4$. The pH was adjusted to 7.2.

The screening medium for $NH_2OH$ (g/L) consisted of 0.0992 $NH_2OH.HCl$, 3.6763 sodium citrate, 3.5 $K_2HPO_4$, 1.5 $KH_2PO_4$, 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 $NaNO_2$, 0.2 $CaCl_2{\cdot}7H_2O$, 1 $MgSO_4{\cdot}7H_2O$, 1 $KH_2PO_4$, 0.5 $FeCl_2{\cdot}6H_2O$, 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).

The basic medium (BM) (g/L) used to evaluate heterotrophic nitrification and aerobic denitrification consisted of 3.6763 sodium citrate, 1.5 $KH_2PO_4$, 0.144 $KNO_3$, 0.0944 $(NH_4)_2SO_4$ or 0.0986 $NaNO_2$, 0.04 $MgSO_4$, 0.009 $Fe_2(SO_4)_3$, and 0.014 $CaCl_2$. The pH was adjusted to 7.2. All media were autoclaved at 121°C for 30 min.

## 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′-AGAGTTTGACCTGGCTAG-3′) and 1492R (5′-GGTTACCTTTTTTGTTACGACTT-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 $NH_2OH$. 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 $NH_2OH$ 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 $NH_2OH$ 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, $NH_2OH$, 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 $NH_2OH$ 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 × 100%, where L1 and L2 represent the initial and final concentrations of $NH_2OH$ (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 × *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, $NH_2OH$, $NO_2^-$-N, or $NO_3^-$-N and centrifuged at 6,000 × *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 20 ml reaction solution contained potassium ferricyanide, Tris–HCl buffer (pH 7.5), $NH_2OH$, 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 μmol 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). $NH_2OH$ 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 guganensis* strain 4-n-1 (Motamedi and Jafari, 2020). In addition, the low TN removal efficiency by strain

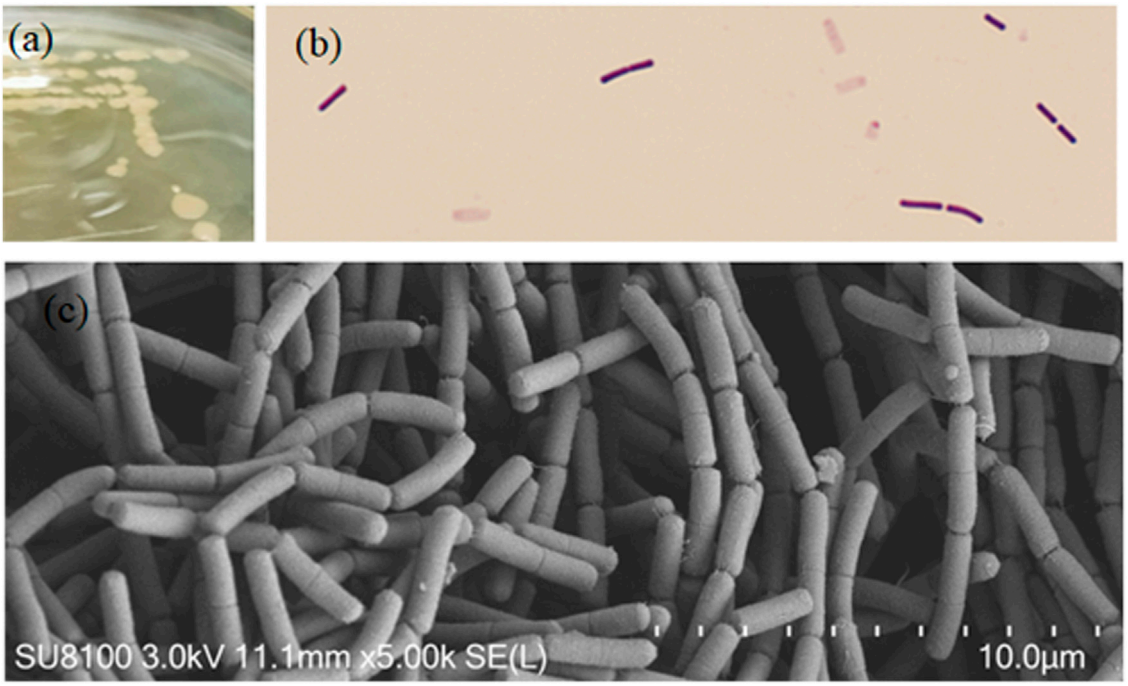


**FIGURE 1 |** The morphologies of the strain EM-A1. Colony on LB plates **(A)**, gram staining reaction by optical microscope **(B)**, and cells under the scanning electron microscope **(C)**.

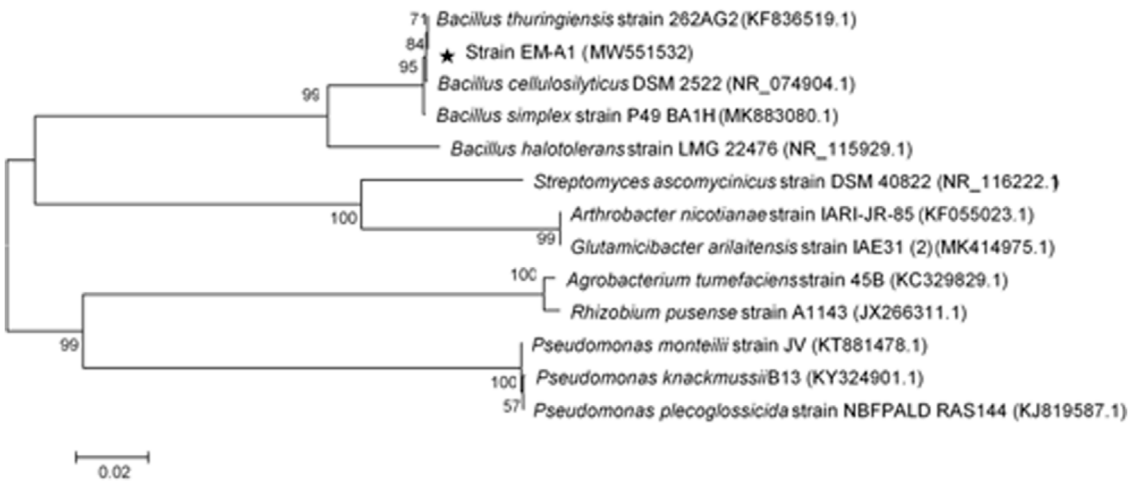


**FIGURE 2 |** Neighbor-joining phylogenetic tree of the strain EM-A1 and related bacteria based on 16S rRNA sequence comparisons.

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 ± 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 ± 0.73 mg/L. Meanwhile, $NO_2^-$-N accumulation was hardly detected during $NO_3^-$-N removal, which contradicted the theory that

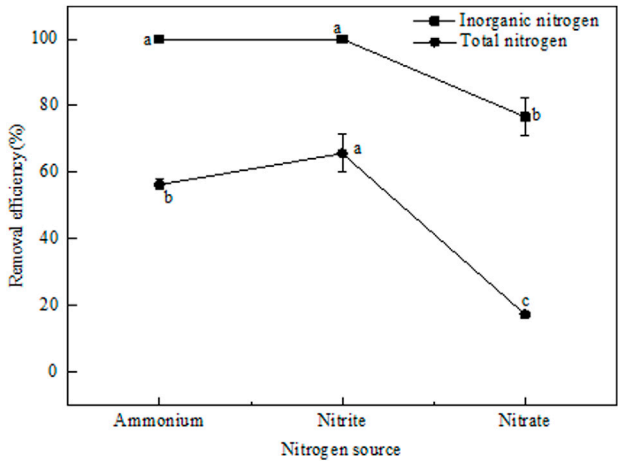


**FIGURE 3 |** Nitrogen removal efficiency of strain EM-AI under aerobic conditions using ammonium and nitrite as sole nitrogen source. Values are presented as the means ± SD of the results of three replicates (error bars). Different letters indicate significant differences between treatments at $p < 0.05$.

## Effects of Different Factors on $NH_2OH$ and TN Removal Capacity

### Effect of Inoculum Concentration on $NH_2OH$ 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 $NH_2OH$ 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 $NH_2OH$ 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 $NH_2OH$ and TN concentration decreased to 2.6 ± 0.68 mg/L and 18.02 ± 0.53 mg/L. The removal efficiencies of $NH_2OH$ 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 ± 0.04 mg/L, which was different from the heterotrophic nitrifying bacteria *Pseudomonas putida* Y-9, which transformed the whole $NH_2OH$ to $NO_2^-$ (Huang et al., 2019). A

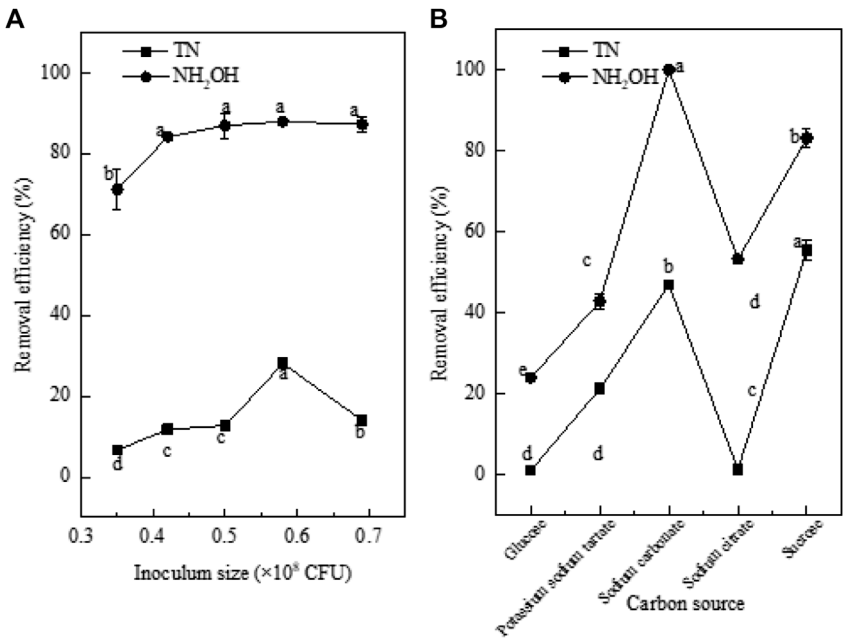


**FIGURE 4 |** Effect of factors on hydroxylamine removal ability of strain EM-A1. Inoculum size **(A)**, carbon source **(B)**. Values are presented as the means ± SD of the results of three replicates (error bars). Different letters indicate significant differences between treatments at $p < 0.05$.

$NO_2^-$-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.

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 $NH_2OH$ and

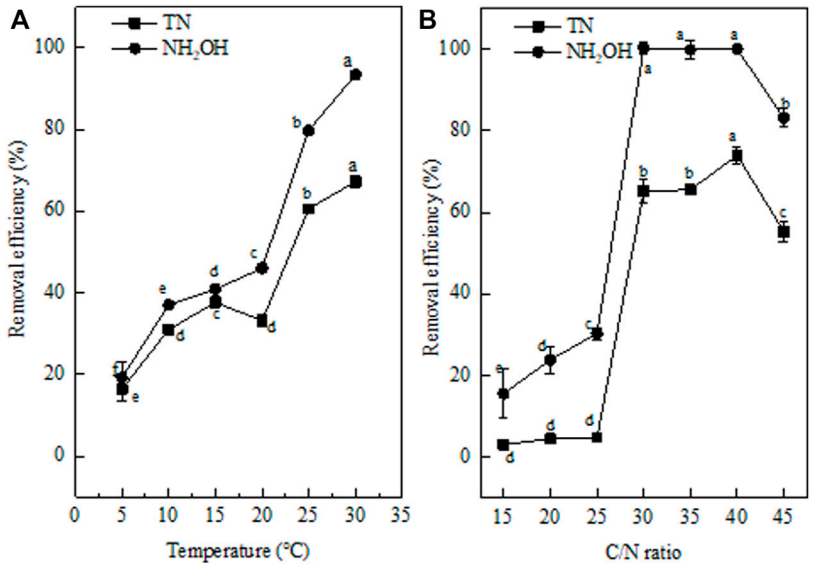


**FIGURE 5 |** Effect of factors on hydroxylamine removal ability of strain EM-A1. Temperature **(A)**, C/N ratio **(B)**. Values are presented as the means ± SD of the results of three replicates (error bars). Different letters indicate significant differences between treatments at $p < 0.05$.

TN, the inoculum concentration of strain EM-A1 needs to be maintained at $0.58 \times 10^8$ CFU.

## Effect of C Source on $NH_2OH$ 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 $NH_2OH$ 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 $NH_2OH$ 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 $NH_2OH$ (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 $NH_2OH$ 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 ± 0.0.1 mg/L), and $NO_3^-$-N (1.64 ± 0.02 mg/L) observed during $NH_2OH$ 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.

## $NH_2OH$ 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 $NH_2OH$ and TN at different temperatures was investigated. The N removal of strain EM-1 occurred over a wide temperature range (from 5°C to 30°C; **Figure 5A**). Increasing the temperature significantly enhanced the $NH_2OH$ and TN removal efficiencies. The $NH_2OH$ removal efficiency showed a gentle upward trend (from 19.25 to 46.04%) 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 $NH_2OH$ and TN removal was continuously enhanced, and the maximum $NH_2OH$ (93.44%) and TN (67.18%) removal efficiencies were observed at 30°C. Meanwhile, the low concentrations of $NH_2OH$ (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 $NH_2OH$ conversion by strain EM-A1. The $NH_2OH$ 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 $NH_2OH$ removal efficiency at 35°C was

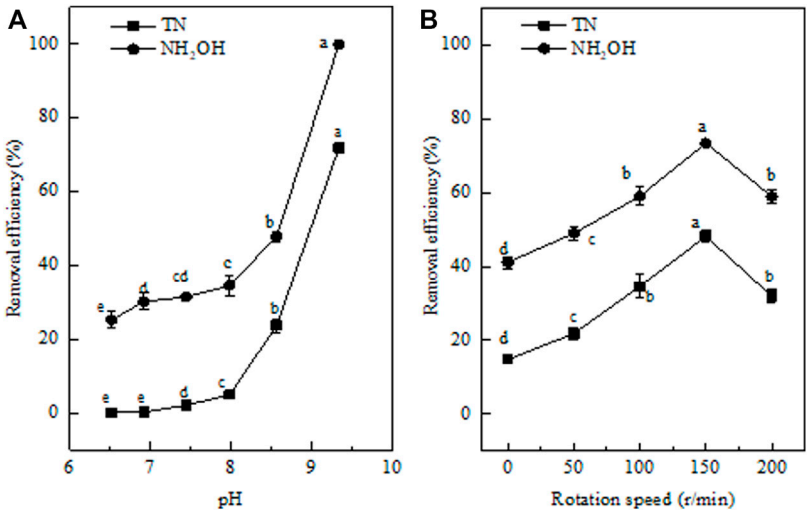


**FIGURE 6 |** Effect of factors on hydroxylamine removal ability of strain EM-A1. pH **(A)**, rotation speed **(B)**. Values are presented as the means ± SD of the results of three replicates (error bars). Different letters indicate significant differences between treatments at $p < 0.05$.

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).

## $NH_2OH$ 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 $NH_2OH$ (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 $NH_2OH$ 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 $NH_2OH$ 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 $NH_2OH$ oxidation process, which indicated that $NH_2OH$ 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 $NH_2OH$ 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 $NH_2OH$ and TN increased with increasing pH. When the pH was 6.52, strain EM-A1 had the lowest removal efficiencies of $NH_2OH$ and TN, which were 25.31 and 0.1%, respectively. When the pH rose from 6.52 to 8.56, the removal efficiencies of $NH_2OH$ and TN significantly improved to 47.84 and 23.72%, respectively. These results showed that higher pH was more beneficial for $NH_2OH$ and TN removal by strain EM-A1. When the pH increased from 8.56 to 9.33, the $NH_2OH$ and TN removal efficiencies increased rapidly, and maximum $NH_2OH$ 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 $NH_2OH$ 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

**TABLE 1 |** Nitrogen balances of ammonium, nitrite and nitrate removal by strain EM-A1.

| Substances | Initial N (mg/L) | Final N (mg/L) | | | | | Intracellular-N (mg/L) | N lose (%) |
|---|---|---|---|---|---|---|---|---|
| | | $NH_4^+$-N | $NH_2OH$-N | $NO_2^-$N | $NO_3^-$-N | Organic-N | | |
| $NH_4^+$-N | 54.88 ± 0.47 | 0.72 ± 0.06 | 5.47 ± 0.59 | 0 | 1.29 ± 0.61 | 23.54 ± 0.15 | 13.59 ± 0.6 | 29.34 ± 0.18 |
| $NO_2^-$N | 57.95 ± 0.58 | 4.38 ± 0.4 | 0 | 5.45 ± 0.23 | 4.67 ± 0.28 | 19.72 ± 1.02 | 16.24 ± 0.18 | 23.72 ± 0.88 |
| $NO_3^-$-N | 58.18 ± 0.57 | 4.35 ± 0.96 | 0 | 5.78 ± 1.11 | 11.24 ± 0.38 | 10.10 ± 0.81 | 11.28 ± 0.61 | 26.71 ± 0.36 |

*Values represent mean ± S.D., of triplicates (n = 3). Final organic-N = final soluble TN – (final $NH_4^+$-N) – (final $NO_3^-$-N) – (final $NO_2^-$-N) – (final $NH_2OH$). Intracellular-N = (final TN – final soluble TN). % N removal = [(initial TN) – (final $NH_4^+$-N) – (final $NO_3^-$-N) – (final $NO_2^-$-N) – (final organic-N) – (final intracellular-N) – (final $NH_2OH$)]/initial TN × 100%.*

EM-A1 was decreased with the increasing rotation speeds (**Figure 6B**).

For instance, when the rotation speed was stationary, the lowest $NH_2OH$ and TN removal efficiencies were achieved of 41.16 and 14.83%, respectively. The inorganic nitrogen of $NO_2^-$-N (0.80 ± 0.04 mg/L), $NO_3^-$-N (0.39 ± 0.36 mg/L), and $NH_4^+$-N (6.73 ± 0.0.15 mg/L) were accumulated. The relative high level of $NH_4^+$-N production in $NH_2OH$ 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 $NH_2OH$ removal efficiency increased from 48.99 to 59.07%. The highest $NH_2OH$ 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 $NH_2OH$ 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, $NH_4^+$-N, $NO_3^-$N, and $NO_2^-$-N were shown in **Table 1**. When $NH_4^+$-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 $NH_4^+$ was remained in the nitrification system. There were low concentrations of $NH_2OH$, $NO_3^-$N, and organic-N accumulated. The nitrite was not detected even though the $NH_2OH$ 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 $NH_2OH$ 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 $NH_4^+$-N →$NH_2OH$ → $NO_3^-$-N→ $NO_2^-$-N → nitrogenous gas. When the $NO_3^-$-N and $NO_2^-$-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 conspicuously 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 $NO_2^-$-N accumulation was detected during $NO_2^-$-N transformation process. Thus, the denitrification pathway could be deduced as $NO_3^-$-N→ $NO_2^-$-N → 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 $NH_4^+$-N → $NH_2ON$ → $NO_2^-$-N → $NO_3^-$-N→ $NO_2^-$-N →$N_2O$ → $N_2$, 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 $NH_2OH$ as the sole N source. The optimal conditions for $NH_2OH$ removal were sucrose, C/N 40, temperature 30°C, pH 9.58, inoculant concentration of 0.58 × $10^8$ CFU, and rotation speed of 150 rpm. *B. thuringiensis* EM-A1 efficiently removed low concentrations (20 mg/L) of $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-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 $NH_4^+$-N → $NH_2ON$ → $NO_2^-$-N → $NO_3^-$-N→ $NO_2^-$-N → nitrogen gas. Therefore, strain EM-A1 could be a promising candidate for inorganic nitrogen removal from wastewater.

## 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.

## FUNDING

This work was supported by the Guizhou Province Science and Technology Planning Project (Qianke He Foundation-ZK (2021) General 233), the National Natural Science Foundation of China (No. 42007223), the Natural Science Research Young Talents Project of Provincial Education Department (No. Guizhou Education and KY word (2021) 086), Gui Da Zhuan Ji He Zi (2019) 04 and Gui Da cultivation (2019)50, the Science and Technology plan Project of Guizhou Province (Support of Guizhou Science and Technology Cooperation (2021)193). Undergraduate "SRT Plan" project, (Guida SRT (2021) No. 271) to TW.

## REFERENCES

Chen, M., Ding, C., He, T., Zhang, M., and Wu, Q. (2022). Efficient Hydroxylamine Removal through Heterotrophic Nitrification by Novel Bacterium Glutamicibacter Arilaitensis EM-H8. *Chemosphere* 288 (Pt 1), 132475. doi:10.1016/j.chemosphere.2021.132475

Chen, M., Wang, W., Feng, Y., Zhu, X., Zhou, H., Tan, Z., et al. (2014). Impact Resistance of Different Factors on Ammonia Removal by Heterotrophic Nitrification-Aerobic Denitrification Bacterium Aeromonas Sp. HN-02. *Bioresour. Technol.* 167, 456–461. doi:10.1016/j.biortech.2014.06.001

Chen, Q., and Ni, J. (2012). Ammonium Removal by Agrobacterium Sp. LAD9 Capable of Heterotrophic Nitrification-Aerobic Denitrification. *J. Biosci. Bioeng.* 113 (5), 619–623. doi:10.1016/j.jbiosc.2011.12.012

Chen, Q., and Ni, J. (2011). Heterotrophic Nitrification-Aerobic Denitrification by Novel Isolated Bacteria. *J. Ind. Microbiol. Biotechnol.* 38 (9), 1305–1310. doi:10.1007/s10295-010-0911-6

Chen, W., Ding, X. L., He, Y. L., Mo, Y., Xu, L. J., and Gao, S. F. (2011). Isolation and Identification of Nitrite Degrading Bacteria and Their Degradation Characteristics. *Environ. Sci. Technol.* 34 (S2), 37–41.

Chen, Z. H., Xu, S. S., Li, X., and Liu, F. X. (2015). The Influence of pH on the Efficiency of Anammox Reaction. *Environ. Prot. Chem. industry* 35 (2), 121–126.

Feng, S., Xie, S., Zhang, X., Yang, Z., Ding, W., Liao, X., et al. (2012). Ammonium Removal Pathways and Microbial Community in GAC-Sand Dual media Filter in Drinking Water Treatment. *J. Environ. Sci.* 24 (9), 1587–1593. doi:10.1016/s1001-0742(11)60965-0

Frear, D. S., and Burrell, R. C. (1955). Spectrophotometric Method for Determining Hydroxylamine Reductase Activity in Higher Plants. *Anal. Chem.* 27 (10), 1664–1665. doi:10.1021/ac60106a054

Gao, J., Zhu, T., Liu, C., Zhang, J., Gao, J., Zhang, J., et al. (2020). Ammonium Removal Characteristics of Heterotrophic Nitrifying Bacterium Pseudomonas Stutzeri GEP-01 with Potential for Treatment of Ammonium-Rich Wastewater. *Bioproc. Biosyst Eng* 43 (6), 959–969. doi:10.1007/s00449-020-02292-x

Guo, L., Chen, Q., Fang, F., Hu, Z., Wu, J., Miao, A., et al. (2013). Application Potential of a Newly Isolated Indigenous Aerobic Denitrifier for Nitrate and Ammonium Removal of Eutrophic lake Water. *Bioresour. Technol.* 142, 45–51. doi:10.1016/j.biortech.2013.05.021

Hammer, D. A., and Knight, R. L. (1994). Designing Constructed Wetlands for Nitrogen Removal. *Water Sci. Technol.* 29 (4), 15–27. doi:10.2166/wst.1994.0148

Hans, B. (1994). Use of Constructed Wetlands in Water Pollution Control: Historical Development, Present Status, and Future Perspectives. *Water Sci. Technol.* 30 (8), 209–223. doi:10.2166/wst.1994.0413

He, T., Ye, Q., Sun, Q., Cai, X., Ni, J., Li, Z., et al. (2018). Removal of Nitrate in Simulated Water at Low Temperature by a Novel Psychrotrophic and Aerobic Bacterium, Pseudomonas Taiwanensis Strain J. *Biomed. Res. Int.* 2018, 4984087. doi:10.1155/2018/4984087

He, T., Xie, D., Ni, J., Li, Z., and Li, Z. (2020). Nitrous Oxide Produced Directly from Ammonium, Nitrate and Nitrite during Nitrification and Denitrification. *J. Hazard. Mater.* 388, 122114. doi:10.1016/j.jhazmat.2020.122114

He, T. X., Wu, Q. F., Ding, C. Y., Chen, M. P., and Zhang, M. M. (2021). Hydroxylamine and Nitrite are Removed Effectively by Streptomyces Mediolani Strain EM-B2. *Ecotoxicol. Environ. Saf.* 224, 112693.

Heil, J., Vereecken, H., and Brüggemann, N. (2016). A Review of Chemical Reactions of Nitrification Intermediates and Their Role in Nitrogen Cycling and Nitrogen Trace Gas Formation in Soil. *Eur. J. Soil Sci.* 67 (1), 23–39. doi:10.1111/ejss.12306

Hong, P., Wu, X., Shu, Y., Wang, C., Tian, C., Wu, H., et al. (2020). Bioaugmentation Treatment of Nitrogen-Rich Wastewater with a Denitrifier with Biofilm-Formation and Nitrogen-Removal Capacities in a Sequencing Batch Biofilm Reactor. *Bioresour. Technol.* 303, 122905. doi:10.1016/j.biortech.2020.122905

Hu, X. F., Su, J. F., Ali, A. J., Wang, Z., and Wu, Z. Z. (2021). Heterotrophic Nitrification and Biomineralization Potential of Pseudomonas Sp. HXF1 for the Simultaneous Removal of Ammonia Nitrogen and Fluoride from Groundwater. *Bioresour. Technol.* 323, 124608. doi:10.1016/j.biortech.2020.124608

Huang, X., Li, W., Zhang, D., and Qin, W. (2013). Ammonium Removal by a Novel Oligotrophic Acinetobacter Sp. Y16 Capable of Heterotrophic Nitrification-Aerobic Denitrification at Low Temperature. *Bioresour. Technol.* 146, 44–50. doi:10.1016/j.biortech.2013.07.046

Huang, X., Xu, Y., He, T., Jia, H., Feng, M., Xiang, S., et al. (2019). Ammonium Transformed into Nitrous Oxide via Nitric Oxide by Pseudomonas Putida Y-9 under Aerobic Conditions without Hydroxylamine as Intermediate. *Bioresour. Technol.* 277, 87–93. doi:10.1016/j.biortech.2019.01.040

Huang, Y. (2009). *Study on Metabolism Pathway of Nitrogen Removel by a New Strain Shanghai Jiao*. Shanghai: Tong University.

Jain, R. M., Mody, K. H., Keshri, J., and Jha, B. (2014). Biological Neutralization and Biosorption of Dyes of Alkaline Textile Industry Wastewater. *Mar. Pollut. Bull.* 84 (1-2), 83–89. doi:10.1016/j.marpolbul.2014.05.033

Jetten, M. S. M., de Bruijn, P., and Kuenen, J. G. (1997). Hydroxylamine Metabolism in Pseudomonas PB16 Involvement of a Novel. *Antoni. Leeuw* 71, 69–74. doi:10.1023/a:1000145617904

Jia, Y., Zhou, M., Chen, Y., Luo, J., and Hu, Y. (2019). Carbon Selection for Nitrogen Degradation Pathway by Stenotrophomonas Maltophilia: Based on the Balances of Nitrogen, Carbon and Electron. *Bioresour. Technol.* 294, 122114. doi:10.1016/j.biortech.2019.122114

Jiang, A. X. (2000). The Mechanism and Countermeasures of Nitrogen Pollution in Water Environment. *China's Popul. Resour. Environ.* 10 (S2), 77–78.

Jiang, T. S., Yang, Q., Shang, H. T., Gan, Y. P., Bai, Y., and Wang, H. C. (2007). The Influence of Temperature and pH Value on Activated Sludge Method for Nitrogen and Phosphorus Removal. *Chin. J. Environ. Eng.* 1 (9), 10–14.

Lang, X., Li, Q., Ji, M., Yan, G., and Guo, S. (2020). Isolation and Niche Characteristics in Simultaneous Nitrification and Denitrification Application of an Aerobic Denitrifier, Acinetobacter Sp. YS2. *Bioresour. Technol.* 302, 122799. doi:10.1016/j.biortech.2020.122799

Lei, X., Jia, Y., Chen, Y., and Hu, Y. (2019). Simultaneous Nitrification and Denitrification without Nitrite Accumulation by a Novel Isolated Ochrobactrum Anthropic LJ81. *Bioresour. Technol.* 272, 442–450. doi:10.1016/j.biortech.2018.10.060

Li, D., Liu, R., Cui, X., He, M., Zheng, S., Du, W., et al. (2021). Co-culture of Bacteria and Microalgae for Treatment of High Concentration Biogas Slurry. *J. Water Process Eng.* 41, 102014. doi:10.1016/j.jwpe.2021.102014

Liu, G., and Wang, J. (2013). Long-term Low Do Enriches and Shifts Nitrifier Community in Activated Sludge. *Environ. Sci. Technol.* 47 (10), 5109–5117. doi:10.1021/es304647y

Liu, Y., Ai, G.-M., Wu, M.-R., Li, S.-S., Miao, L.-L., and Liu, Z.-P. (2019). Photobacterium Sp. NNA4, an Efficient Hydroxylamine-Transforming Heterotrophic Nitrifier/aerobic Denitrifier. *J. Biosci. Bioeng.* 128 (1), 64–71. doi:10.1016/j.jbiosc.2018.12.014

Liu, Y., Hu, T., Song, Y., Chen, H., and Lv, Y. (2015). Heterotrophic Nitrogen Removal by Acinetobacter Sp. Y1 Isolated from Coke Plant Wastewater. *J. Biosci. Bioeng.* 120 (5), 549–554. doi:10.1016/j.jbiosc.2015.03.015

Medhi, K., Singhal, A., Chauhan, D. K., and Thakur, I. S. (2017). Investigating the Nitrification and Denitrification Kinetics under Aerobic and Anaerobic Conditions by *Paracoccus Denitrificans* ISTOD1. *Bioresour. Technol.* 242, 334–343. doi:10.1016/j.biortech.2017.03.084

Miao, L. (2011). *Isolation of Heterotrophic Nitrifying Bacteria and Characters of Removal of Ammonia*. Changsha: Central South University.

Motamedi, H., and Jafari, M. (2020). Screening Heterotrophic Ammonia Removal and Aerobic Denitrifying Bacteria from Wastewater of Ammonia Production Units of a Petrochemical Industry. *Curr. Microbiol.* 77 (9), 2207–2214. doi:10.1007/s00284-020-02065-5

Nie, H. H., Zhao, Q. F., Li, C. H., Wang, Y. N., and Zhang, D. M. (2013). Isolation, Identification and Denitrification Characteristics of a Strain of Nitrogenous Nitrogen Removal Bacteria. *Microbiol. China* 40 (11), 2146–2155.

Ouyang, L., Wang, K., Liu, X., Wong, M. H., Hu, Z., Chen, H., et al. (2020). A Study on the Nitrogen Removal Efficacy of Bacterium Acinetobacter Tandoii MZ-5 from a Contaminated River of Shenzhen, Guangdong Province, China. *Bioresour. Technol.* 315, 123888. doi:10.1016/j.biortech.2020.123888

Poot, V., Hoekstra, M., Geleijnse, M. A. A., van Loosdrecht, M. C. M., and Pérez, J. (2016). Effects of the Residual Ammonium Concentration on NOB Repression during Partial Nitritation with Granular Sludge. *Water Res.* 106, 518–530. doi:10.1016/j.watres.2016.10.028

Ren, Y.-X., Yang, L., and Liang, X. (2014). The Characteristics of a Novel Heterotrophic Nitrifying and Aerobic Denitrifying Bacterium, Acinetobacter Junii YB. *Bioresour. Technol.* 171, 1–9. doi:10.1016/j.biortech.2014.08.058

Shammas, N. K. (1986). Interactions of Temperature, pH, and Biomass on the Nitrification Process. *Journal (Water Pollution Control Federation)* 58 (1), 52–59. doi:10.2307/25042841

Soler-Jofra, A., Pérez, J., and van Loosdrecht, M. C. M. (2021). Hydroxylamine and the Nitrogen Cycle: A Review. *Water Res.* 190, 116723. doi:10.1016/j.watres.2020.116723

Su, J. f., Ma, M., Huang, T. l., Ma, F., Shao, S. c., Lu, J. s., et al. (2016). Characteristics of Autotrophic and Heterotrophic Denitrification by the Strain Pseudomonas Sp. H117. *Geomicrobiology J.* 34 (1), 45–52. doi:10.1080/01490451.2015.1137661

Taylor, E. T., and Bishop, P. L. (1989). Distribution and Role of Bacterial Nitrifying Populations in Nitrogen Removal in Aquatic Treatment Systems. *Pergamon* 23 (8), 947–955. doi:10.1016/0043-1354(89)90167-x

Wan, W., He, D., and Xue, Z. (2017). Removal of Nitrogen and Phosphorus by Heterotrophic Nitrification-Aerobic Denitrification of a Denitrifying Phosphorus-Accumulating Bacterium Enterobacter cloacae HW-15. *Ecol. Eng.* 99, 199–208. doi:10.1016/j.ecoleng.2016.11.030

Wang, H. Y., Ma, F., Su, J. F., Zuo, W., Zhang, X. X., and Zhang, J. (2007). The Effect of Different Carbon Sources and Carbon-Nitrogen Ratios on the Denitrification Performance of an Aerobic Denitrifying Bacteria. *Acta Scientiae* (06), 968–972.

Wang, Q., and He, J. (2020). Complete Nitrogen Removal via Simultaneous Nitrification and Denitrification by a Novel Phosphate Accumulating Thauera Sp. Strain SND5. *Water Res.* 185, 116300. doi:10.1016/j.watres.2020.116300

Wang, X. W., Kong, Q. X., Jin, M., Song, N., Zheng, J. L., Gu, C. Q., et al. (2003). Effect of pH and Aeration on Nitrification of Nitrifying Bacteria. *J. Prev. Med. Chin. PLA* 21, 319–321.

Wei, R., Hui, C., Zhang, Y., Jiang, H., Zhao, Y., and Du, L. (2021). Nitrogen Removal Characteristics and Predicted Conversion Pathways of a Heterotrophic Nitrification-Aerobic Denitrification Bacterium, Pseudomonas aeruginosa P-1. *Environ. Sci. Pollut. Res.* 28 (6), 7503–7514. doi:10.1007/s11356-020-11066-7

Wu, Y. (2018). *Study on Nitrogen Status of Urban Surface Water and Nitrogen Utilization Mechanism of Aquatic Plants*. Tianjin: Tianjin University.

Xia, L., Li, X., Fan, W., and Wang, J. (2020). Heterotrophic Nitrification and Aerobic Denitrification by a Novel Acinetobacter Sp. ND7 Isolated from Municipal Activated Sludge. *Bioresour. Technol.* 301, 122749. doi:10.1016/j.biortech.2020.122749

Xing, C. Y., Fan, Y. C., Chen, X., Guo, J. S., Shen, Y., Yan, P., et al. (2020). The Effect of Hydroxylamine on Anammox Sludge Community. *Environ. Sci.* 41 (7), 3365–3372. doi:10.13227/j.hjkx.201911244

Yan, N., Jin, X. B., and Zhang, J. Q. (2002). Comparison of Methanol and Glucose as Carbon Sources in the Denitrification Process. *J. Shanghai Normal Univ. (Natural Sci. Edition)* 31 (03), 41–44. doi:10.13227/j.hjkx.201911244

Ye, J., Zhao, B., An, Q., and Huang, Y.-S. (2016). Nitrogen Removal by Providencia Rettgeri Strain YL with Heterotrophic Nitrification and Aerobic Denitrification. *Environ. Technol.* 37 (17), 2206–2213. doi:10.1080/09593330.2016.1146338

Zhang, D., Li, W., Huang, X., Qin, W., and Liu, M. (2013). Removal of Ammonium in Surface Water at Low Temperature by a Newly Isolated Microbacterium Sp. Strain SFA13. *Bioresour. Technol.* 137, 147–152. doi:10.1016/j.biortech.2013.03.094

Zhang, H., Li, S., Ma, B., Huang, T., Qiu, H., Zhao, Z., et al. (2020). Nitrate Removal Characteristics and 13C Metabolic Pathways of Aerobic Denitrifying Bacterium Paracoccus Denitrificans Z195. *Bioresour. Technol.* 307, 123230. doi:10.1016/j.biortech.2020.123230

Zhang, M., Pan, L., Su, C., Liu, L., and Dou, L. (2021). Simultaneous Aerobic Removal of Phosphorus and Nitrogen by a Novel Salt-Tolerant Phosphate-Accumulating Organism and the Application Potential in Treatment of Domestic Sewage and Aquaculture Sewage. *Sci. Total Environ.* 758, 143580. doi:10.1016/j.scitotenv.2020.143580

Zhang, Q.-L., Liu, Y., Ai, G.-M., Miao, L.-L., Zheng, H.-Y., and Liu, Z.-P. (2012). The Characteristics of a Novel Heterotrophic Nitrification-Aerobic Denitrification Bacterium, Bacillus Methylotrophicus Strain L7. *Bioresour. Technol.* 108, 35–44. doi:10.1016/j.biortech.2011.12.139

Zhang, Q. L., Wang, H., Zhang, L. Q., Li, J., and Tang, X. Q. (2008). The Impact of Nitrate Nitrogen Pollution in Drinking Water on Human Health. *Groundwater* 30 (01), 57–59.

Zhao, B., Cheng, D. Y., Tan, P., An, Q., and Guo, J. S. (2018). Characterization of an Aerobic Denitrifier Pseudomonas Stutzeri Strain XL-2 to Achieve Efficient Nitrate Removal. *Bioresour. Technol.* 250, 564–573. doi:10.1016/j.biortech.2017.11.038

Zhao, B., He, Y. L., Hughes, J., and Zhang, X. F. (2010a). Heterotrophic Nitrogen Removal by a Newly Isolated Acinetobacter Calcoaceticus HNR. *Bioresour. Technol.* 101 (14), 5194–5200. doi:10.1016/j.biortech.2010.02.043

Zhao, B., He, Y. L., and Zhang, X. F. (2010b). Nitrogen Removal Capability through Simultaneous Heterotrophic Nitrification and Aerobic Denitrification byBacillussp. LY. *Environ. Technol.* 31 (4), 409–416. doi:10.1080/09593330903508922

Zou, S., Yao, S., and Ni, J. (2014). High-efficient Nitrogen Removal by Coupling Enriched Autotrophic-Nitrification and Aerobic-Denitrification Consortiums at Cold Temperature. *Bioresour. Technol.* 161, 288–296. doi:10.1016/j.biortech.2014.03.066

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.