Heterotrophic nitrogen removal in *Bacillus* sp. K5: involvement of a novel hydroxylamine oxidase
Yunlong Yang, Ershu Lin and Shaobin Huang

**ABSTRACT**

An aerobic denitrifying bacterium isolated from a bio-trickling filter treating NOx, *Bacillus* sp. K5, is able to convert ammonium to nitrite, in which hydroxylamine oxidase (HAO) plays a critical role. In the present study, the performance for simultaneous nitrification and denitrification was investigated with batch experiments and an HAO was purified by an anion-exchange and gel-filtration chromatography from strain K5. The purified HAO’s molecular mass was determined by SDS-PAGE and its activity by measuring the change in the concentration of ferricyanide, the electron acceptor. Results showed that as much as 87.8 mg L⁻¹ ammonium-N was removed without nitrite accumulation within 24 hours in the sodium citrate medium at C/N of 15. The HAO isolated from the strain K5 was approximately 71 KDa. With hydroxylamine (NH₂OH) as a substrate and potassium ferricyanide as an electron acceptor, the enzyme was capable of oxidizing NH₂OH to nitrite in vitro when the pH varied from 7 to 9 and temperature ranged from 25 °C to 40 °C. This is the first time that an HAO has been purified from the *Bacillus* genus, and the findings revealed that it is distinctive in its molecular mass and enzyme properties.

**Key words** | *Bacillus* sp., heterotrophic nitrifier, hydroxylamine oxidase, purification

**INTRODUCTION**

Biological methods for nitrogen removal have attracted more and more attention, in which nitrification and denitrification conducted by functional microorganisms contribute to the global nitrogen cycle. Conventionally, the nitrifying bacteria perform nitrification in aerobic environments, whereas the denitrifying bacteria perform denitrification under anaerobic conditions. Since the first aerobic denitrifier *Thiospheara pantotropha* (Robertson & Kuenen 1984) was found, however, simultaneous nitrification and denitrification (SND) have been proved to exist in many other bacteria such as *Alcaligenes faecalis* (Braber et al. 1992), *Providencia rettgeri* (Taylor et al. 2009), *Acinetobacter calcoaceticus* (Zhao et al. 2010), *Agrobacterium* sp. (Chen & Ni 2011), *Pseudomonas stutzeri* (Zhang et al. 2011), *Halomonas campisalis* (Guo et al. 2013) and *Chelatococcus daeguensis* (Yang et al. 2014). Although the nitrification mechanism in autotrophic nitrifiers has been investigated in detail (Arciero & Hooper 1993), the corresponding mechanisms involved in SND are not very clear, which might result from the characteristics of hydroxylamine oxidase (HAO).

As a key reaction of nitrification in autotrophic and heterotrophic microorganisms, hydroxylamine (NH₂OH) oxidation is catalyzed by HAO. NH₂OH is converted to nitrite (NO₂⁻) or nitrous oxide N₂O in at least one heterotrophic bacterium. Autotrophic bacteria can also produce both NO₂⁻ and N₂O but it has not yet been demonstrated that this is the result of NH₂OH oxidation (Otte et al. 1999). In order to clarify the characteristics of enzymes, some HAOs have been purified from both autotrophic and heterotrophic bacteria (Arciero & Hooper 1993; Zahn et al. 1994; Wehrfritz et al. 1996; Jetten et al. 1997; Zhang et al. 2014), and significant differences in molecular mass and structure have been found (Moir et al. 1996; Cedervall et al. 2009). To our knowledge, however, HAO in the *Bacillus* genus has not been reported.

Some isolates of the *Bacillus* genus such as *Bacillus subtilis* A1 (Yang et al. 2011), *Bacillus methylotrophicus* L7 (Yao et al. 2013), *Bacillus cereus* X7 (Yao et al. 2014) and *Bacillus licheniformis* (Takenaka et al. 2007) have been reported to be capable of nitrification. However, the further mechanisms of nitrification in these bacteria need to be addressed. Although *Bacillus* sp. K5 was an aerobic denitrifier isolated from a bio-trickling filter used for NOx treatment (unpublished data), the heterotrophic nitrogen...
removal capability in Bacillus sp. K5 was unknown, and it was unclear whether the HAO generated by Bacillus sp. K5 differed from those generated by the microorganisms reported previously. Therefore, Bacillus sp. K5 was characterized for its SND performance in the present work. Subsequently, the purification of HAO from this strain was performed using anion-exchange and gel-filtration chromatography, and some enzyme characteristics were analyzed. Our results provide a potential microbial resource for nitrogen removal in wastewater treatment, and also give insight into the mechanism of heterotrophic nitrification-aerobic denitrification in Bacillus sp. K5.

**METHODS**

**Microorganism and media**

Strain K5 was isolated from a bio-trickling filter used for NOx treatment in a power plant (Guangzhou, China), having been identified using 16s rDNA in a previous unpublished investigation. For short-term use, strain K5 was stored at −20 °C in 50% glycerol. For long-term use, strain K5 was freeze-dried into powder and stored at −80 °C.

The heterotrophic nitrification medium (HNM) comprised (g L⁻¹): sodium citrate (unless otherwise specified), 4; NH₃Cl, 0.8; KH₂PO₄, 0.5; Na₂HPO₄·7H₂O, 1; FeSO₄·7H₂O, 0.1; MgSO₄, 0.2; trace element solution, 2 ML per litre of HNM. The trace element solution was composed of (g L⁻¹): FeSO₄·7H₂O, 3; H₃BO₃, 0.01; Na₂MoO₄·2H₂O, 0.01; MnSO₄·H₂O, 0.02; CuSO₄·5H₂O, 0.01; ZnSO₄, 0.01 and ethylene diamine tetraacetic acid (EDTA), 0.5. All media had their pH maintained at 7.0–7.5 and were autoclaved at 115 °C for 20 min.

**Heterotrophic nitrogen removal experiments**

A single colony in a plate was inoculated into 30 mL LB medium in a 100 mL flask cultured at 30 °C for 12 hours in a shaker at 180 rpm. Then, a 5% (volume ratio) of cellular culture was transferred to 100 mL HNM in a 250 mL flask incubated at 30 °C for 24 hours in a shaker at 180 rpm. For the SND experiments in which the carbon to nitrogen (C/N) ratio and the pH were varied, sodium citrate was the carbon source. For the experiments in which the carbon source was varied, methanol, sodium acetate, sodium succinate and sodium citrate were used. In these experiments the pH was 8. In all the experiments ammonium chloride was used as the nitrogen source and the temperature was 30 °C. Each experiment was repeated three times.

Samples were taken periodically to determine the optical density at 600 nm (OD₆₀₀) using a spectrophotometer (UV-1100, MAPADA, China) and the concentrations of other ingredients including ammonium nitrogen (NH₄-N), nitrite nitrogen (NO₂-N) and nitrate nitrogen (NO₃-N) using Standard Methods (APHA 1998).

**Bacterial cultures for enzyme purification**

A loop of stock culture was inoculated into a 50 mL LB medium in a 250 mL shaking bottle. After cultivation for one day at 30 °C with a shaking speed of 180 rpm, the resultant culture (5%, v/v) used as the seed was transferred into a 500 mL Erlenmeyer flask containing HNM, and then was cultivated for 2 days at 30 °C with a shaking speed of 180 rpm. The expanding cultures were stored −20 °C for the preparation of the crude enzyme.

**Crude enzyme extraction**

The expanding cultures of 5 L were centrifuged for 15 min at 8,000 rpm and 4 °C. The cells were cleaned three times with sterile water and the supernatants were decanted. Subsequently, the cells were suspended in 100 mL Tris-HCl buffer of 20 mmol L⁻¹ (pH 8.0) in which 0.5 mol L⁻¹ sucrose, 1.3 mmol L⁻¹ EDTA and 50 mg lysozyme were included, and were incubated at 50 °C for 40 min. The suspensions were centrifuged at 10,000 rpm for 15 min at 4 °C. After the supernatants had been removed, the resultant precipitants were resuspended in 75 mL Tris-HCl buffer of 20 mmol L⁻¹ (pH 8.0), and were incubated for 5 min at 50 °C. The precipitates were centrifuged at 8,000 rpm for 30 min at 4 °C, and supernatants were obtained and stored at −20 °C for the purification of HAO.

**Enzyme purification**

The procedures for enzyme purification consisted of anion-exchange and gel-filtration chromatography. First of all, the crude enzyme solution was loaded onto an anion-exchange column (Sepharose CL-6B, GE Healthcare), equilibrated with 5 mmol L⁻¹ Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of 0–80 mmol L⁻¹ NaCl in the same buffer at a flow rate of 2.5 ml min⁻¹. Each fraction was collected and the corresponding HAO activity was measured, after which active fractions were concentrated by ultrafiltration. Afterwards, concentrates were applied to a gel-filtration column (Sephacryl S-100, GE Healthcare), equilibrated and eluted with 5 mmol L⁻¹ Tris-HCl buffer.
Effect of pH and temperature on the HAO activity

The purified HAO activity was investigated in a pH range of 5 to 10 and temperature range of 25 °C to 45 °C. To evaluate the effect of pH on the HAO activity, the buffer pHs were adjusted to 5, 6, 7, 8, 9 and 10 using 1 mol L$^{-1}$ HCl or 1 mol/L NaOH. For the determination of the optimal temperature, the reaction mixtures were incubated for 30 min at 25 °C, 30 °C, 37 °C, 40 °C and 45 °C. Each test was repeated three times.

RESULTS

SND by Bacillus sp. K5

Table 1 shows the time course of SND by strain K5. NH$_4^+$-N rapidly reduced from the initial 87.8 mg L$^{-1}$ to 0.75 mg L$^{-1}$ at 12 hours, and then could not be detected at 24 hours with the removal percentage (RP) being 100%. As expected, NO$_2^-$-N was produced, but it was reduced thoroughly after accumulating to the peak value of 0.8 mg L$^{-1}$, suggesting that strain K5 performs SND well without the accumulation of nitrite or the production of nitrate.

Influence of different factors on NH$_4^+$-N removal by Bacillus sp. K5

Several carbon sources were utilized to evaluate the nitrogen removal ability by K5 (Figure 1). The nitrification rate was greatest using sodium succinate and zero using methanol. Figure 2 shows the effect of C/N ratio on nitrogen removal. Up to C/N 15, the greater the C/N the greater nitri

HAO purification

The HAO of Bacillus sp. K5 was purified stepwise. Figure 4(a) exhibits the result for the crude enzyme fractionated by anion-exchange chromatography, in which three peaks appeared and the HAO activity was detected in peak II.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>OD$_{600}$</th>
<th>NH$_4^+$-N (mg L$^{-1}$)</th>
<th>NO$_2^-$-N (mg L$^{-1}$)</th>
<th>NO$_3^-$-N (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.025 ± 0.005</td>
<td>87.8 ± 0.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>0.31 ± 0.01</td>
<td>62.5 ± 1.2</td>
<td>0.2 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>1.05 ± 0.011</td>
<td>27.9 ± 0.9</td>
<td>0.8 ± 0.019</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>1.15 ± 0.01</td>
<td>0.75 ± 0.09</td>
<td>0.12 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>1 ± 0.006</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

OD$_{600}$: absorbance of cell cultures at 600 nm; ND: not detected.
The corresponding active fractions were subsequently subjected to gel-filtration chromatography to obtain two peaks (Figure 4(b)), but only the peak I showed the HAO activity.

**Analysis of HAO activity**

Table 2 displays the results for the HAO activity that was measured after each purification step. The yield of purified HAO was rather low, no more than 1.6% of the crude enzyme. In addition, the kinetic parameters of the purified enzyme were analyzed according to the following equation:

$$V = \frac{V_{\text{max}} [S]}{K_m + [S]}$$

where $V_{\text{max}}$ and $[S]$ represented the maximum velocity and substrate concentration, respectively, and $K_m$ is the half saturation constant. From this equation, a $V_{\text{max}}$ of 19.43 μmol min$^{-1}$ per mg protein and a $K_m$ of 0.19 mol L$^{-1}$ were observed. Meanwhile, the nitrite analysis was also conducted to verify the formation of nitrite, and results showed that nitrite was generated in the reaction mixtures containing active enzyme solution whereas there was almost no nitrite in the reaction mixtures in which the enzyme solution was inactivated before reaction (data not shown).

**Effect of pH and temperature on HAO activity**

Table 3 shows that pH exerted a significant effect on the HAO activity. The specific activity was greatest at pH 8 and no activity was detected below pH 7 or above pH 9. The temperature was also an important factor affecting the HAO activity, as seen in Table 4. The specific activity was...
greatest at 30 °C and there was no detected activity at temperatures above 40 °C.

**SDS-PAGE analysis**

The SDS-PAGE profile is shown in Figure 5, where the proteins with HAO activity obtained from anion-exchange and gel-filtration purification are in lanes 2 and 1, respectively. After anion-exchange chromatography, there were about 10 protein bands, from which the target protein was highlighted. After gel-filtration chromatography, only one band could be seen on SDS-PAGE and the corresponding molecular mass was estimated to be approximately 71 KDa. The results presented herein indicated that an HAO was successfully purified from *Bacillus* sp. K5.

**DISCUSSION**

Initially, as an aerobic denitrifier, *Bacillus* sp. K5 was isolated from a bio-trickling filter treating NOx, and the previous study showed that this strain had a good capability for denitrification under aerobic conditions (unpublished data), implying that this strain could be a potential heterotrophic nitrifier. It has been found that *Bacillus* strains perform simultaneous aerobic nitrification/denitrification. In *Bacillus methylotrophicus* strain L7, the maximum NH4+-N removal rate of 51.58 mg/L/d was obtained, and the optimal conditions for heterotrophic nitrification were sodium succinate as carbon source, C/N 6, pH 7–8 and 37 °C (Zhang et al. 2015). *Bacillus subtilis* A1 removed 58.4 ± 4.3% of NH4+-N within 60 hours of growth in acetate medium at a C/N of 6 (Yang et al. 2014). Although these results were different from the results observed in the present work (that the optimum conditions for heterotrophic nitrogen removal was C/N of 15 in the sodium citrate medium), *Bacillus* sp. K5 could remove as much as 87.8 mg/L NH4+-N within 24 hours (Table 1), and most importantly there was no nitrite and nitrate accumulation (not detected), which might be attributed to high activity of nitrite reductase (unpublished data) involved in K5 to result in nitrite being rapidly denitrified not nitrified. Therefore, strain K5 could also become a potential candidate for the wastewater treatment.

As a key enzyme in nitrogen removal, the mechanisms of HAO in autotrophic bacteria have been studied in detail (Fernandez et al. 2012), whereas they might be more complicated in heterotrophic bacteria because of terminal products containing both nitrite and N2O. The findings in this work

| Table 2 | Purification procedures and activities for HAO from *Bacillus* sp. K5 |
|---|---|---|---|---|---|---|
| Components | Total proteins (mg) | Total activities (μmol min⁻¹) | Specific activities (μmol min⁻¹ mg⁻¹) | Purification (fold) |
| Crude enzyme | 39.2 ± 0.2 | 0.161 ± 0.040 | 0.004 ± 0.001 | 1 |
| Anion-exchange eluate | 3.4 ± 0.2 | 0.048 ± 0.008 | 0.015 ± 0.005 | 3.75 |
| Gel-filtration eluate | 0.6 ± 0.0 | 0.022 ± 0.001 | 0.045 ± 0.003 | 11.25 |

| Table 3 | HAO activity at different pHs and 30 °C |
|---|---|---|---|---|---|---|---|
| Items | pH | 5 | 6 | 7 | 7.5 | 8 | 9 | 10 |
| Specific activity (μmol min⁻¹) | ND | ND | 0.008 ± 0.000 | 0.015 ± 0.007 | 0.022 ± 0.005 | 0.003 ± 0.002 | ND |
| Relative activity % | ND | ND | 35.40% ± 0.00 | 68.68% ± 0.30 | 100.00% ± 0.23 | 13.86% ± 0.09 | ND |

ND means not detected; ± represents three replicates.

| Table 4 | HAO activity at different temperatures and pH 8 |
|---|---|---|---|---|---|---|---|
| Items | Temperature (°C) | 25 | 30 | 37 | 40 | 45 |
| Specific activity (μmol min⁻¹) | ND | 0.014 ± 0.000 | 0.024 ± 0.003 | 0.015 ± 0.001 | 0.006 ± 0.001 | ND |
| Relative activity % | ND | 57.32% ± 0.02 | 100% ± 0.15 | 61.64% ± 0.03 | 26.52% ± 0.06 | ND |

ND means not detected; ± represents three replicates.
show that the HAO involved in *Bacillus* sp. K5 could oxidize NH$_2$OH to nitrite during the ammonium removal process, but whether HAO catalyzes NH$_2$OH to N$_2$O remains unknown for the reason that N$_2$O was not monitored.

Through anion-exchange and gel-filtration chromatography, a high-purity HAO was isolated from strain K5 in the present study. Results showed that the purified HAO has a molecular mass in the middle of the range of molecular masses (Table 5) but different from HAOs found in other bacteria. Furthermore it can be seen that K5 HAO has a different optimum temperature, pH range and maximum rate from other HAOs. This indicates that K5 HAO is a new enzyme distinct from these HAOs.

In addition, it is worth noting that the yield of protein was very low. The purified HAO after gel-filtration chromatography was only about 1.6% of the crude enzyme, which was much lower than that in previous reports (Shimamura *et al.* 2008; Zhang *et al.* 2014). The following reasons are probably responsible for the low HAO production. To begin with, the purification procedures adopted in the present study including the chromatography and concentration methods could cause production declines. Furthermore, the gene encoding NH$_2$OH oxidase might be inhibited during the cultivation of strain K5, leading to less HAO synthesis. Finally, there were some adverse products accumulated during strain growth and ammonium removal, which may contribute to HAO degradation. In spite of an important role HAO plays in ammonium removal, the primary structure and the corresponding catalysis mechanisms of HAO from *Bacillus* sp. K5 are still unclear. Therefore, further research should be done to address these problems in the future.

### CONCLUSIONS

This study showed that *Bacillus* sp. K5 has a good performance for ammonium removal without nitrite accumulation. A novel HAO was successfully purified from strain K5 by anion-exchange and gel-filtration chromatography. Although the HAO in this strain is different from others in molecular mass and enzyme properties, it can oxidize NH$_2$OH to nitrite, further confirming K5 could remove nitrogen through SND. However, whether this HAO could convert NH$_2$OH directly to N$_2$O needs to be addressed.

### ACKNOWLEDGEMENTS

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**Table 5 | HAOs in different microorganisms**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Thiosphaera pantotropha</th>
<th>Anammox KSU-1</th>
<th>Pseudomonas species</th>
<th>Nitrosomonas europaea</th>
<th>Pseudomonas PB16</th>
<th>Acinetobacter sp. Y16</th>
<th>Bacillus sp. K5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max rate (μmol min$^{-1}$ mg$^{-1}$)</td>
<td>0.129/0.99</td>
<td>9.6</td>
<td>3.6</td>
<td>UD</td>
<td>0.45</td>
<td>UD</td>
<td>19.43</td>
</tr>
<tr>
<td>pH range</td>
<td>UD</td>
<td>5.0–9.0</td>
<td>8.7*</td>
<td>UD</td>
<td>9*</td>
<td>6.0–8.5</td>
<td>7.0–9.0</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>UD</td>
<td>65 °C</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td>15 °C</td>
<td>30 °C</td>
</tr>
<tr>
<td>Size (KDa)</td>
<td>20</td>
<td>118</td>
<td>19</td>
<td>63</td>
<td>132</td>
<td>61</td>
<td>71</td>
</tr>
</tbody>
</table>

UD: not determined.
*Optimum pH.
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REFERENCES


