Original Article

Purification and Characterization of a Low-temperature Hydroxylamine Oxidase from Heterotrophic Nitrifier *Acinetobacter* sp. Y16*

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Abstract

Objective To purify a low-temperature hydroxylamine oxidase (HAO) from a heterotrophic nitrifying bacterium *Acinetobacter* sp. Y16 and investigate the enzyme property.

Methods A HAO was purified by an anion-exchange and gel-filtration chromatography from strain Y16. The purity and molecular mass were determined by RP-HPLC and SDS-PAGE. The HAO activity was detected by monitoring the reduction of potassium ferricyanide using hydroxylamine as substrate and ferricyanide as electron acceptor. The partial amino acid sequence was determined by mass spectrometry.

Results The low-temperature HAO with a molecular mass of 61 kDa was purified from strain Y16 by an anion-exchange and gel-filtration chromatography. The enzyme exhibited an ability to oxidize hydroxylamine in wide temperature range (4-40 °C) *in vitro* using hydroxylamine as substrate and ferricyanide as electron acceptor. It was stable in the temperature range of 4 to 15 °C and pH range of 6.0 to 8.5 with less than 30% change in its activity. The optimal temperature and pH were 15 °C and 7.5, respectively. Three peptides were determined by mass spectrometry which were shown to be not identical to other reported HAOs.

Conclusion This is the first study to purify a low-temperature HAO from a heterotrophic nitrifier *Acinetobacter* sp. It differs from other reported HAOs in molecular mass and enzyme properties. The findings of the present study have suggested that the strain Y16 passes through a hydroxylamine-oxidizing process catalyzed by a low-temperature HAO for ammonium removal.

Key words: Hydroxylamine oxidase; Purification; Heterotrophic nitrifier; *Acinetobacter* sp. Y16

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INTRODUCTION

Nitrification and denitrification by autotrophic and heterotrophic nitrifying bacteria are essential processes in the global nitrogen cycle[1-3]. Autotrophic nitrifying bacteria perform aerobic nitrification, whereas heterotrophic nitrifying bacteria can simultaneously perform aerobic nitrification and denitrification[4-6]. The nitrification mechanism of autotrophic nitrifying

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bacteria has been studied in detail[7]. The characteristics of heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* have been studied, and the nitrogen removal pathway has been proposed[6,8-9]. Recent reports have indicated that additional nitrogen removal pathways likely exist in heterotrophic microorganisms[10-15]. However, new nitrogen removal pathway is not very clear in new isolated genera.

Hydroxylamine oxidation catalyzed by hydroxylamine oxidase (HAO) is considered as a key reaction of nitrification in autotrophic and heterotrophic bacteria. HAO in autotrophic bacteria oxidizes hydroxylamine to nitrite, whereas the enzyme in heterotrophic bacteria oxidizes hydroxylamine to nitrite or to nitrous oxide[16]. Some HAOs are purified from autotrophic and heterotrophic nitrifying bacteria[8,17-21]. These HAOs differ from each other in their molecular mass and structure[22-25]. However, there is little knowledge about HAO in *Acinetobacter* sp.

Acinetobacter sp. Y16 is a newly isolated heterotrophic nitrifying bacterium from the Songhua River in winter, which can degrade low amounts of ammonia nitrogen (5 mg/L) at 4-15 °C. Several isolates of *Acinetobacter* genus named *Acinetobacter calcoaceticus* were reported to be capable of degrading NH_4^+-N[11,26]. However, they were just used in high ammonium degradation (initial ammonium concentration was 100-120 mg/L) at 30 °C. HAO is an important enzyme during nitrogen removal for nitrifying bacterium. However, it is unclear whether the HAO produced by a low-temperature bacterium differs from that of a normal-temperature bacterium. The present work describes the purification of a low-temperature hydroxylamine oxidase from *Acinetobacter* sp. by an anion-exchange and gel-filtration chromatography, and characterizes the effect of temperature, pH and different electron acceptors on the HAO activity. This study is helpful to unravel the mechanism of ammonium removal for *Acinetobacter* sp. Y16 at low temperature.

MATERIALS AND METHODS

Cultivation Organism

A stock culture of *Acinetobacter* sp. strain Y16 was stored at -70 °C. Continuous cultures were performed as described below. A loop of stock culture was inoculated onto an agar plate containing basic medium (NH_4Cl 0.5 g/L, CH_3COONa 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.05 g/L, K_2HPO_4 0.2 g/L, NaCl 0.12 g/L, $MnSO_4 \cdot 4H_2O$ 0.01 g/L, $FeSO_4$ 0.01 g/L, pH 7.4). The plate was cultivated at 4 °C for 5 d. A single colony was selected and inoculated in a test tube containing basic medium. The cultures were grown with shaking at 200 r/min for 5 d at 4 °C, used as the seed for expanding cultivation in a 500-mL Erlenmeyer flask. The expanding cultures were shaken at 200 r/min for 7 d at 4 °C, stored at 4 °C for the preparation of the crude extract.

Preparation of Crude Enzyme

Two liters of cultures were harvested by centrifugation at 5 000 × g for 10 min at 4 °C. The cells were suspended in 200 mL of 10 mmol/L Tris-HCl buffer (pH 7.5) containing 150 mmol/L NaCl and centrifuged at 10 000 × g for 30 min at 4 °C. The cells were resuspended in 10 mL of 10 mmol/L Tris-HCl buffer (pH 7.5) containing 0.5 mol/L sucrose, 0.5 mmol/L EDTA and 50 mg of lysozyme and incubated at 15 °C for 40 min. The suspensions were centrifuged at 10 000 × g at 4 °C for 30 min. The precipitants were resuspended in 5 mL of 20 mmol/L Tris-acetate buffer (pH 7.5) containing 150 mmol/L KCl, 200 mmol/L sucrose, and 10 mmol/L magnesium acetate and incubated at 15 °C for 5 min. After centrifugation at 10 000 × g at 4 °C for 30 min, the solutions were condensed by ultrafiltration and filtered by 0.45 μm filter membrane for purification of HAO.

Enzyme Purification

Enzyme purification was carried out with AKTA Purifier Instrument. The crude enzyme solution was loaded onto an anion-exchange column (HiTrap™ DEAE FF 1 mL, GE Healthcare), then equilibrated with 0.05 mol/L Tris-HCl buffer (pH 7.5) and eluted with a linear gradient of 0 to 2 mol/L NaCl in the same buffer. Each fraction was collected and analyzed for HAO activity. The active fractions were condensed by ultrafiltration and applied to a gel-filtration column (Superdex™75 10/300GL, GE Healthcare) equilibrated and eluted with the buffer (0.05 mol/L phosphate and 0.15 mol/L NaCl, pH 7.5). Each fraction on the peak top was collected and analyzed for HAO activity again. The active fractions were concentrated with TCA method for SDS-PAGE[27].

Enzyme Assays

The HAO activity was determined by monitoring the absorbance change at 400 nm due to the reduction of potassium ferricyanide using hydroxylamine as substrate and ferricyanide as electron

acceptor. The reaction mixture (mL^{-1}) contained 50 μmol Tris-HCl pH 7.5, 1 μmol $K_3Fe(CN)_6$, 4 μmol EDTA, 2 μmol NH_2OH and 40 μL enzyme solution, incubated at 15 ℃ for 30 min while enzyme was added, followed by absorbance measuring at 400 nm.

Effect of pH, Temperature, and Electron Acceptor on HAO Activity

The purified HAO activity was tested at the temperature range 4-50 °C with pH range 5.0-9.0. For determining optimal pH, a different 50 μmol Tris-HCl buffer was added into the enzyme reaction mixture. The buffer was adjusted to pH 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, and 9.0 with 1 mol/L HCl or 1 mol/L NaOH, respectively. To determine the optimal temperature, the reaction mixtures were incubated at 4 °C, 10 °C, 15 °C, 20 °C, 30 °C, 40 °C, and 50 °C for 30 min, respectively. To test alternative electron acceptor, potassium ferricyanide was replaced by horse heart cytochrome c in the reaction mixtures. Each test was repeated three times.

SDS-PAGE and Molecular Mass Determination

After each purification, the purity of the HAO was checked by SDS-PAGE. SDS-PAGE was carried out using 12% (w/v) polyacrylamide gel on Mini protein gel equipment (Bio-Rad, U.S.A). The protein samples were denatured by incubation in the protein loading buffer with SDS and β-mercaptoethanol (Sigma) at 100 ℃ for 5 min. 10 μL of the denatured sample was added into the gel hole. The gel was run at 80 V for about 3 h. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 for 3 h at room temperature, followed by methanol and acetic acid solution (4:1 v/v) destaining. A low molecular mass marker (from 14.3 kDa-97.2 kDa) was used to determine the molecular mass of the protein. The molecular mass of the purified HAO was measured by comparing the migration distance of the single protein band to marker protein (Auoto Chemi System, USA). Protein concentration was determined using the method of Bradford[27]. Bovine serum albumin was used as standard. Each analysis was repeated three times.

Purity Assay of the Purified Protein

The purity of the fraction containing single protein band was analyzed by high performance liquid chromatography. The fraction was loaded onto a C18 reversed phase column (4.6×150 mm, 5 μm) and eluted with a gradient mixed mobile phase A and B. The mobile phase A was 0.1% (v/v) trifluoroacetic acid (TFA)-water solution and B was 0.1% (v/v) TFA-acetonitrile solution. The detection wavelength was 280 nm and column temperature was 25 °C.

Amino Acid Sequence Analysis

The single protein band in SDS-PAGE was excised from the gel and washed twice with sterile water. The protein was digested with trypsin. The polypeptides generated by trypsin digestion were subjected to ABI 4800 MALDI-TOF-TOF-MS. The mass data were analyzed with GPS 3.6 (Applied Biosystems) and Mascot 2.1 (Matrix Science). The peptide sequence was confirmed by MS/MS and compared to those of other HAOs in NCBI. This work was completed by Sangon Biotech (Shanghai) Co., Ltd.

RESULTS

Purification of HAO

The crude enzyme was fractionated on an anion-exchange chromatography into two effusion peaks I, II and two elution peaks III, IV (Figure 1A). HAO activity was detected in peak IV. Peak IV was

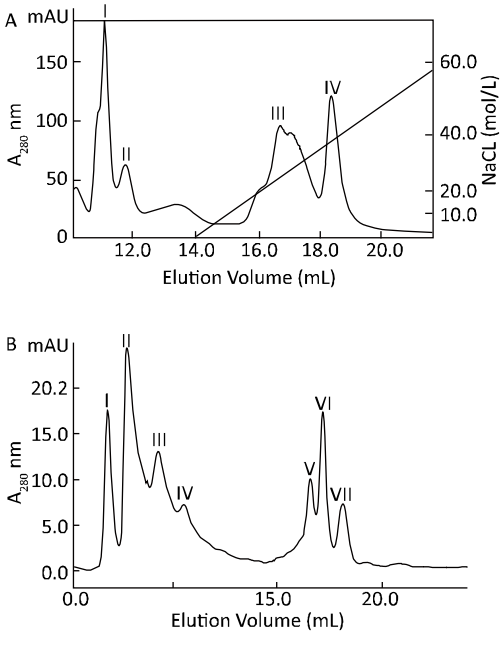

Figure 1. A. Anion exchange chromatography of the crude enzyme on a HiTrap™ DEAE FF column; Peak I and II: effusion eluate; Peak III and IV: elution eluate. B. Gel filtration chromatography of the elution peak IV on a superdex™75 10/300 GL column.

separated on a gel-filtration chromatography into seven absorbed peaks (Figure 1B). Only the peak VI exhibited the HAO activity.

Activity Analysis of HAO

The HAO activity was detected after each purification step. The results are shown in Table 1. The HAO activity increased with the proceeding of the purification process. The HAO activity of the crude enzyme was 0.03 μmol/min/mg. It reached approximately 3.3 and 10.3 times as high as the crude enzyme after the anion-exchange and gel-filtration purification. The yield of purified HAO was lower, no more than 3% of the crude enzyme.

Effect of pH, Temperature, and Electron Acceptor on HAO Activity

The purified HAO activity fluctuated with the variation of reaction temperature (Table 2). The enzyme activity was the highest at 15 °C (0.31 μmol/min/mg). It dropped slowly from 0.31 μmol/min/mg to 0.23 μmol/min/mg with the decrease of temperature from 15 °C to 4 °C. However, when the reaction temperature exceeded 15 °C, the enzyme activity decreased drastically. The HAO activity was only 0.03 μmol/min/mg at 50 °C, which was 9.6% of HAO activity at 15 °C. The purified HAO displayed higher activity at lower temperature (4-15 °C). The effect of pH on the HAO activity was examined by assaying the enzyme activity in different pH buffers (Table 3). The HAO activity was shown to differ in different pH conditions, which was higher between pH 6.0 and pH 8.5. The optimal activity was pH 7.5. Below pH 6.0 and above pH 8.5, the enzyme activity decreased drastically. Using cytochrome c as the electron acceptor to replace potassium ferricyanide in reaction mixtures, no HAO activity was observed.

Table 1. Purification Procedure of Hydroxylamine Oxidase from *Acinetobacter* sp. Y16

Purification Step	Total Protein (mg)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Purification Fold
Crude enzyme	6.5±0.35	0.20±0.08	0.03±0.02	1
Anion exchange eluate	0.8±0.08	0.08±0.04	0.10±0.05	3.3
Gel filtration elutae	0.2±0.06	0.06±0.03	0.31±0.06	10.3

***Note*.** The results were obtained in 3 replicates.

Table 2. The Activity of HAO in Different Reaction Temperatures

Items	Temperature (°C)						
	4	10	15	20	30	40	50
Specific activity (μmol/min/mg)	0.23±0.04	0.27±0.06	0.31±0.05	0.20±0.03	0.14±0.02	0.09±0.02	0.03±0.02
Relative activity %	74.1±0.34	87.1±0.29	100±0.00	64.5±0.58	45.1±0.39	29.0±0.42	9.6±0.24

***Note*.** The results were obtained in 3 replicates.

Table 3. The Activity of HAO in Different pH Conditions

Items	pH						
	5.0	6.0	7.0	7.5	8.0	8.5	9.0
Specific activity (μmol/min/mg)	0.04±0.02	0.26±0.06	0.27±0.08	0.31±0.05	0.26±0.07	0.25±0.03	0.12±0.03
Relative activity %	12.9±0.36	83.6±0.53	87.1±0.47	100±0.00	83.6±0.31	80.6±0.44	38.7±0.28

***Note*.** The results were obtained in 3 replicates.

SDS-PAGE Analysis of HAO

Figure 2 shows the SDS-PAGE profiles of the protein with HAO activity obtained from the crude enzyme, anion-exchange purification (peak IV) and gel filtration (peak VI). The crude enzyme exhibited more protein bands than those of ion-exchange and gel-filtration purification. After ion-exchange chromatography, the protein bands decreased from 22 to 11. The low molecular weight protein disappeared (<30 KDa). After gel-filtration chromatography, only one band was present in peak VI on SDS-PAGE with a molecular mass of approximately 61 kDa. The purity of the protein was 97.6% by RP-HPLC. The results testified that a HAO was successfully purified from the strain Y16, and the purity was in accordance with the purpose of this study.

Amino Acid Sequence Analysis of Purified HAO

The purified HAO was digested by trypsin. The N-terminal sequence of the HAO could not be determined, but three polypeptide sequences were determined by mass spectrometry analysis (Figure 3): LPSGLYQFR (peptide 1), VTVPHEDFIAK (peptide 2), and SEFGPLPDQSMHEK (peptide 3). Their sequences were compared with other HAO sequences from protein databases in NCBI, including *Nitrosomonas* sp. 1S79A3 (GI:218761596), *Paracoccus denitrificans* PD1222 (GI:119385214), *Nitrosococcus halophilus* Nc-4 (GI:292491205), *Anaeromyxobacter* sp. FW109-5 (GI:153003821), and *Campylobacter curvus* 525.92 (GI:153793136), which were not identical to the reported HAOs.

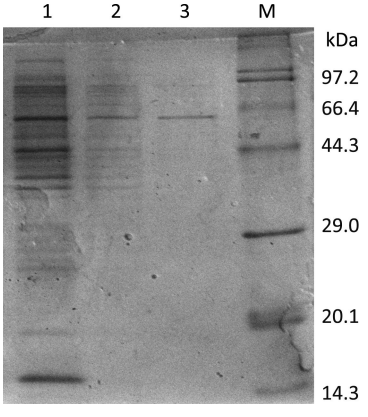

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of HAO from the crude, peak IV and peak VI with HAO activity. Lane M: low molecular mass markers from TaKaRa Biotechnology (China); Lane 1: the crude HAO from the crude enzyme; Lane 2: purified HAO (elution peak IV) from the anion-exchange chromatography; Lane 3: purified HAO (peak VI) from the gel-filtration chromatography.

DISCUSSION

Acinetobacter sp. strain Y16 was isolated from the Songhua River in winter, which was a heterotrophic nitrifying bacterium. The previous study showed that the strain grew rapidly at low temperatures (4-15 °C), whereas it grew slowly at normal temperature (20-37 °C) using NH_4^+-N, NO_2^- and NO_3^- as nitrogen source and CH_3COONa as carbon source. Moreover, the strain Y16 could remove a small amount of nitrogen at low temperatures. Less intermediates (nitrite and nitrate) and more terminal products (N_2) were detected using NH_4^+-N as nitrogen source during the bacteria growth (data not shown). NH_2OH is related to nitrogen cycle and serves as starting material for nitrite and ammonia formation or as critical intermediate in their interconversion[28]. HAO was considered to be a key enzyme during the nitrogen removal process, which can oxidize NH_2OH to nitrite or nitrous oxide. The finding of the present study indicated that the strain Y16 could oxidize NH_2OH to nitrite catalyzed by a low-temperature HAO during ammonium removal at low temperatures. However, it is unknown whether HAO catalyzes NH_2OH to nitrous oxide without monitoring nitrous oxide production.

In the present study, a highly pure HAO was isolated from an *Acinetobacter* sp. strain Y16 by an anion-exchange and gel-filtration chromatography. The purified HAO is distinct from those of autotrophic and heterotrophic bacteria in molecular mass. HAO isolated from *Acinetobacter* sp. Y16 is an approximately 61 kDa monomer. However, the HAOs published are a 63 kDa homotrimer from *N. europaea*[22], a 20 kDa periplasmic monomer from *Thiosphaera pantotropha*[8], a homodimer of 68 kDa subunits from *Pseudomonas* PB16[24] and a 118 kDa homodimer composed of a 53 kDa subunit from anammox bacterium strain KSU-1[25]. Partial amino acid sequence analysis of polypeptides of the purified HAO showed that no homology to the reported HAOs was published in database. The size and amino acid sequence of an enzyme decide its structure and function. The different size and amino

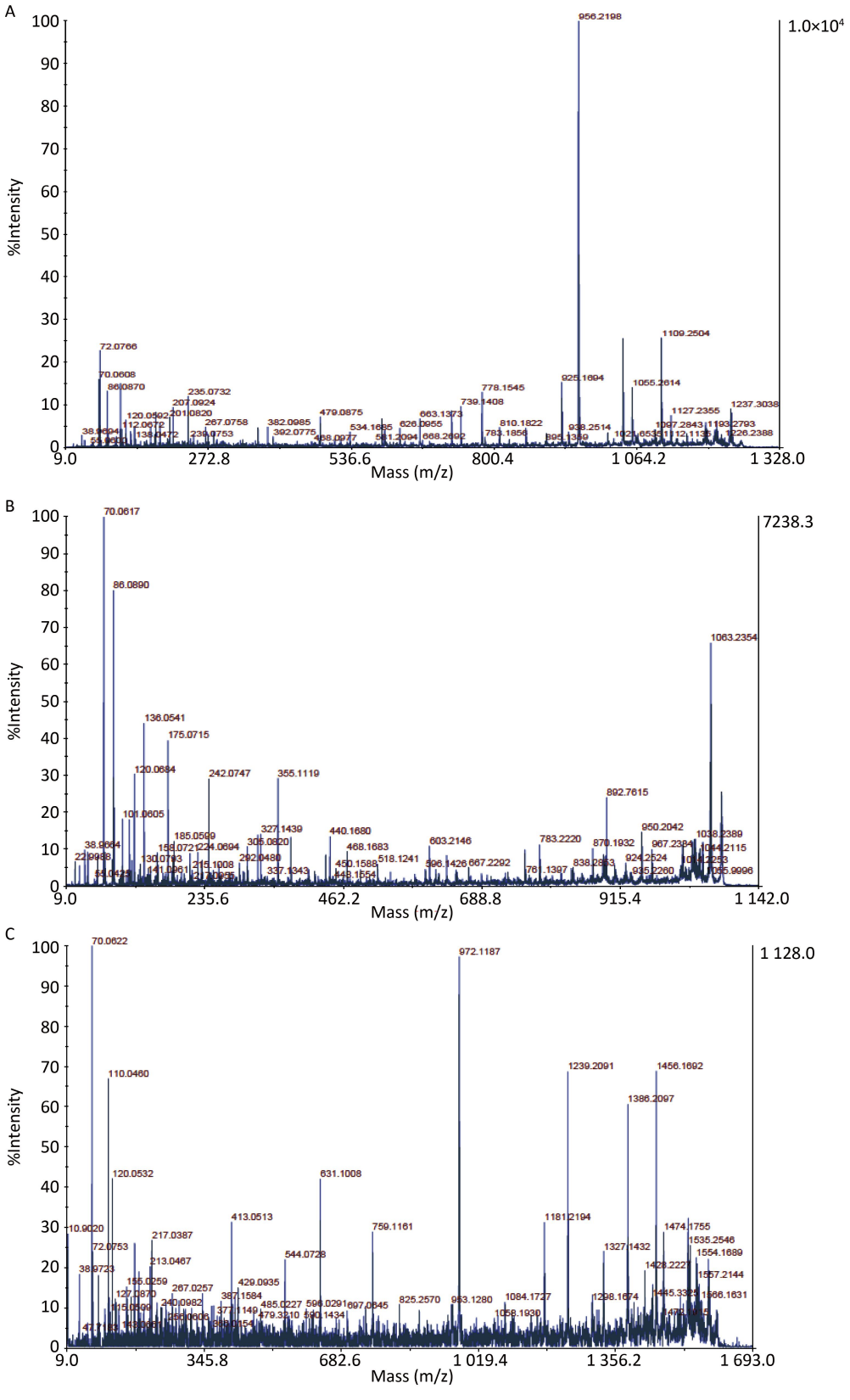

Figure 3. MS/MS spectrum of three peptides. A. Peptide 1; B. peptide 2; C. peptide 3.

acid sequence of the HAO from the strain Y16 imply that it may be distinct from other published HAOs.

The purified HAO has special properties. Cytochrome is the electron acceptor for most HAOs [8,18,21]. However, in this study, the purified HAO showed a high activity with $K_3Fe(CN)_6$ as electron acceptor and no activity with cytochrome C as electron acceptor was found. This character is similar to HAO from *Alcaligenes faecali*[19]. HAO activity has been tested in *Acinetobacter calcoaceticus* HNR with cytochrome C as an electron acceptor[11]. This study indicated that the HAO purified from *Acinetobacter* sp. Y16 was not the same as that from *Acinetobacter calcoaceticus* HNR. In the present research, HAO activity in the crude enzyme from the strain Y16 was 0.2 μmol/min, which was much higher than that of *Acinetobacter calcoaceticus* HNR (0.05 μmol/min). The purified HAO has the ability to oxidize hydroxylamine at low temperature (4-15 °C) *in vitro*. The optimal activity temperature (15 °C) is lower than that of other HAOs. Munetaka et al. reported that the maximum activity of HAO from anammox bacterium was at 65 °C[25]. Moreover, the temperature stability of the enzyme is also different from Munetaka's report which identified that the HAO kept below 60 °C maintaining more than 90% of the initial activity[25], whereas the HAO from the present work only retained 9.6% of the initial activity incubating at 50 °C. In addition, the purified HAO from strain Y16 is not consistent with other HAOs in pH stability. The HAO from the strain Y16 kept stable activity in pH 6.0 and 8.5, whereas the HAO from the anammox bacterium was stable between pH 5.0 and 9.0. The yield of the purified HAO appeared to be 3% of the crude protein. The results indicated that a little HAO was extracted from the strain Y16. As we speculated, two reasons might cause the lower HAO production. On the one hand, the purification process reduced the yield of HAO. On the other hand, most of HAOs were consumed by ammonia removal reaction during the strain growth. The HAO played a crucial role during ammonia nitrogen removal process at low temperature. However, the primary structure of the purified HAO from *Acinetobacter* sp. Y16 is still unknown. It is unclear why the HAO has activity at low temperatures. This is a problem needed to be addressed in the future.

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