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Ammonium reduction kinetics in drinking water by newly isolated *Acinetobacter* sp. HITLi 7 at low temperatures

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ABSTRACT

The *Acinetobacter* sp. HITLi 7 was isolated from the Songhua River and shown to be capable of heterotrophic nitrification ability at 2°C. To predict the ammonium reduction performance in drinking water at low temperature, the kinetics of strain HITLi 7 were investigated using Monod kinetic models. The results of calculations showed that the substrate half saturation constant K_s was 9.9 mg/L of total ammonium, and the maximum specific rate μ_{max} was 7.9×10^{-4} h^{-1} at 8°C, C/N 2, pH 6.0 while shaking at 100 r/min. The effects of temperature (2, 5 and 8°C), C/N (2, 4, and 10), and pH (6.0, 7.0, and 7.5) on kinetic parameters were also evaluated. K_s and μ_{max} increased consistently with an increase in temperature and decreased as C/N ratio increased. The specific affinity a^0 ($a^0 = \mu_{max}/K_s$) for ammonium was the highest for a C/N of 10. This value was 2.1-fold higher than the affinity observed for a C/N of 2. The results demonstrated that the affinity of HITLi 7 for ammonium was higher when a sufficient carbon source was present. The maximum ammonium reduction rate was 0.18 mg NH_4^+-N/L/h at a C/N of 10. These results suggest that HITLi 7 may be used for ammonium removal in drinking water at low temperatures.

Keywords: Heterotrophic nitrification bacterium; Kinetics; Low temperature; Drinking water

1. Introduction

Recently, the ammonium concentration of source water in China has increased because of the wide use of pesticides and fertilizers. Ammonium concentration is higher in winter because biological activities decrease at low temperatures. Songhua River is the main water source for the citizens of Harbin, China. During winter, the water temperature remains between 1 and 6°C for four months. In 2013, the ammonium concentration was 1.1 mg/L in the winter, about 4.2 times higher than in summer. Because ammonium may be hazardous to human health, efforts are underway to reduce ammonium in drinking water at low temperatures [1,2]. In 2006, the Ministry of Health of the P.R. China issued the new Standards for Drinking Water Quality (GB5749-2006), which require an ammonium concentration lower than 0.5 mg/L to guarantee safe drinking water. At normal temperatures, conventional ammonium reduction processes rely on breakpoint chlorination, which may stimulate the formation of undesirable chlorinated

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by-products [3,4]. Ammonium reduction by autotrophic nitrifiers, which are sensitive to environmental factors, is restrained when the temperature is below 10℃ [5–7]. Therefore, a new method for effectively removing ammonium at low temperatures is required.

Research has shown that heterotrophic nitrification micro-organisms play an important role in the global nitrogen cycle. Previous studies have focused on isolation of heterotrophic nitrification micro-organisms, the pathway of nitrogen reduction, activity of key enzymes, and application in wastewater treatment [8–13]. Ammonium reduction by heterotrophic nitrification micro-organisms is advantageous because the micro-organisms grow quickly [14,15] and adapt easily to their environment [16]. Zhang et al. [17] demonstrated that *Pseudomonas stutzeri* YZN-001 oxidizes 106.3 ± 7.8 mg/L NH_4^+-N after 14 d at 4℃. In that study, the ammonium reduction rate was 0.3 mg-NH_4^+-N/L/h. However, the isolated heterotrophic nitrifying micro-organisms were used mainly to treat wastewater with high carbon and nitrogen concentrations. Zhang et al. [18] isolated *Microbacterium* SFA13 that was able to perform heterotrophic nitrification at 10℃. In that study, the *Microbacterium* was attached to activated carbon for removing ammonium from drinking water. However, the temperature of the Songhua River may be as low as 1–6℃ in winter and *Microbacterium* SFA1 may not be used to remove ammonium at such a low temperature.

In previous work, the strain HITLi 7, which has the ability to grow at 2℃, was isolated from the Songhua River during the winter. The optimal growth temperature was found to be between 8 and 20℃. The 16S rRNA gene was sequenced and submitted to GenBank with the accession number KC843488. The 16S rRNA gene sequences had 98% similarity to *Acinetobacter lwoffii (A. lwoffii)*. However, the DNA–DNA hybridization between the strain HITLi 7 and *A. lwoffii* was only 36.2%. The bacterial identification results showed that HITLi 7 was a new species in the genus *Acinetobacter*. However, the ammonium reduction characteristics of the strain were not studied. Moreover, the ammonium reduction efficiency of heterotrophic nitrification micro-organisms is reportedly affected by environmental factors, such as temperature, C/N ratio, pH value, and dissolved oxygen concentration (DO) [19,20]. The effect of environmental factors on ammonium reduction efficiency by strain HITLi 7 remains unknown. Kinetics of biodegradation reflects the biodegradation characteristics [21–23]. In this study, the kinetics of HITLi 7 at low temperatures were calculated using Monod kinetic models. In addition, the effects of environmental factors on the kinetic parameters were evaluated. The purpose of this study was to optimize the conditions for ammonium reduction by the strain HITLi 7. Our results may be useful for the biological treatment of drinking water ammonium reduction at low temperatures.

2. Materials and methods

2.1. Micro-organism

The 16S rRNA gene sequences of strain HITLi 7 were submitted to GenBank with accession number KC843488 (http://www.ncbi.nlm.nih.gov/nuccore/KC843488). The sequences were 98% similar to *A. lwoffii*. Thus, the strain was identified as a new species in the *Acinetobacter* genus. However, only 36.2% DNA–DNA hybridization was observed between strain HITLi 7 and *A. lwoffii*. The bacterial identification results suggested that strain HITLi 7 was a new species in the *Acinetobacter* genus. Strain *Acinetobacter* nitrificans HITLi 7 was deposited under number Korean Collection for Type Cultures (KCTC) 32411 in the general collection of micro-organisms in the KCTC and under number China General Microbiological Culture Collection Center (CGMCC) NO.1.12528 in the CGMCC.

2.2. Strain cultivation

Cultures of *Acinetobacter* sp. strain HITLi 7 were maintained in sterile synthetic basic medium containing 0.38 g/L NH_4Cl, 2.0 g/L CH_3COONa, 0.05 g/L $MgSO_4{\cdot}7H_2O$, 0.2 g/L K_2HPO_4, 0.12 g/L NaCl, 0.01 g/L $MnSO_4{\cdot}4H_2O$, and 0.01 g/L $FeSO_4$ at pH 7.0 at 2℃.

2.3. Ammonium reduction performance

Strain HITLi 7 was cultivated in synthetic basic medium for 7–10 d at 2℃ while shaking at 100 r/min (DO ≈ 3.5 mg/L). When the concentration exceeded 10^7 cells/mL, one liter of the liquid culture was collected and centrifuged at 9,000 r/min for 5 min. The pellet was collected and washed with sterile deionized water three times. The collected bacteria were then inoculated into two sterile 500 mL batches of ammonium solution (about 5 mg/L ammonium) with sodium acetate as a carbon source at 8℃, C/N 2, and pH 6.0 while shaking at 100 r/min. Sterile ammonium solution without inoculate served used as a control. The residual ammonium concentration was measured after shaking for 0, 2, 4, 6, 8.5, 24, 29, 47.5, 57.5, 81.25, 104.5, and 124.5 h. The hydroxylamine, nitrate, and nitrite concentration were measured after shaking for 0, 8.5, 24, 47.5, 104.5, and 124.5 h.

2.4. The effect of environmental factors on ammonium reduction by the strain HITLi 7

Strain HITLi 7 was cultivated as described in the previous section. To investigate the effects of temperature, C/N ratio, and pH on ammonium reduction by strain HITLi 7, temperatures of 2, 5 and 8 ℃ were tested; C/N ratios of 2, 4, and 10 were tested; and pH of 6.0, 7.0, and 7.5 were tested while shaking at 100 r/min (DO ≈ 3.5 mg/L). After 0, 2, 4, 6, 8, 10, 24, 30, 48, 58, 72, 80, 105, and 125 h, 5 mL of the solution was sampled to measure the residual ammonium concentration.

2.5. Analytical methods

The ammonium concentration was measured colorimetrically according to water quality–determination of ammonium–Nessler's reagent colorimetric method (GB7479-87). NH_2OH was measured using indirect spectrophotometry [24]. Nitrite and nitrate were measured using N-(1-naphthalene)-diaminoethane photometry and phenol disulphonic acid, respectively, according to the State Environmental Protection Administration of China [25]. The optical density of the strain HITLi 7 was measured by spectrophotometry at a wavelength of 600 nm. The microbial biomass was measured after drying at 105℃ for 2 h. The formula for the ammonium reduction rate is $((S_0-S_t)/S_0)\times 100\%$, where S_0 is the initial concentration of NH_4^+-N and S_t is the concentration of NH_4^+-N at time t.

3. Results

3.1. Ammonium reduction

Acinetobacter sp. HITLi 7 was cultivated in ammonium solution with an NH_4^+ concentration of about 5 mg/L at 8℃, C/N 2, and pH 6.0 while shaking at 100 r/min g (DO ≈ 3.5mg/L). The ammonium concentration dynamics were tested over 125 h. The results are shown in Fig. 1. Ammonium, the sole N-source in the solution, decreased dramatically during the first 30 h. The ammonium reduction rate reached a maximum of 0.04 mg NH_4^+-N/L/h with an initial microbial biomass of 76.0 ± 0.4 mg/L. The biomass of *Acinetobacter* sp. HITLi 7 remained nearly constant during degradation (data not shown), indicating no visible growth of the strain. After 48 h, the ammonium concentration remained nearly constant. The concentration of hydroxylamine was low during ammonium degradation by *Acinetobacter* sp. HITLi 7. After 24 h, the hydroxylamine concentration reached a maximum of 0.02 mg/L and then decreased. However, as the hydroxylamine concentration decreased, the nitrate concentration increased, suggesting that hydroxylamine was converted to nitrate during this process. During ammonium degradation, very little nitrite was detected.

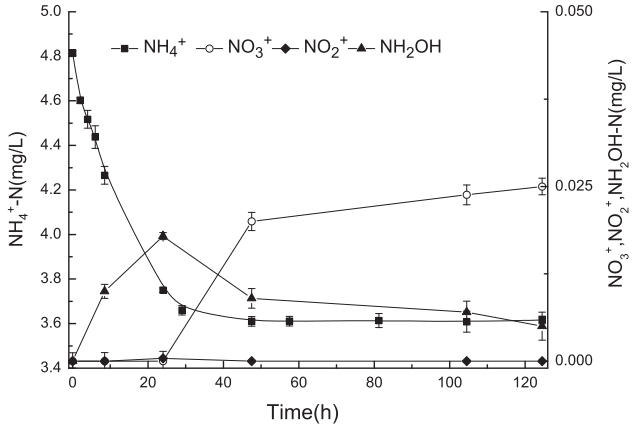

Fig. 1. Changes in nitrogen compounds by HITLi 7 under optimum conditions (8℃, C/N 2, pH 6.0 while shaking at 100 r/min). Error bars: mean ± S.D. of two replicates.

3.2. Effects of environmental factors on ammonium reduction

3.2.1. Effect of temperature on ammonium reduction

Ammonium reduction of *Acinetobacter* sp. HITLi 7 was investigated at different temperatures when cultivated in ammonium solution with an NH_4^+ concentration of about 5 mg/L at C/N 2 and pH 6.0 while shaking at 100 r/min. Temperatures of 2, 5 and 8℃ were investigated to determine the optimal growth temperature of strain HITLi 7 in source water during the winter. The ammonium concentration dynamics were tested over 125 h. The ammonium concentration decreased drastically during the first 48 h at 2–8℃ (Fig. 2(a)). For an initial microbial biomass (X_{H0}) of 120.0 ± 2.9 mg/L, 34 and 39% of NH_4^+-N was removed at 2 and 5℃, respectively. For an X_{H0} of 76.0 ± 0.4 mg/L, 24% of NH_4^+-N was removed at 8℃ (Fig. 2(a)). The maximum ammonium reduction rates, which were calculated based on the maximum slopes of ammonium reduction, were 0.09, 0.15, and 0.11 mg NH_4^+-N/L/h, at 2, 5 and 8℃, respectively.

3.2.2. Effect of C/N ratio on ammonium reduction

The influence of C/N ratio on ammonium reduction by *Acinetobacter* sp. HITLi 7 was investigated in

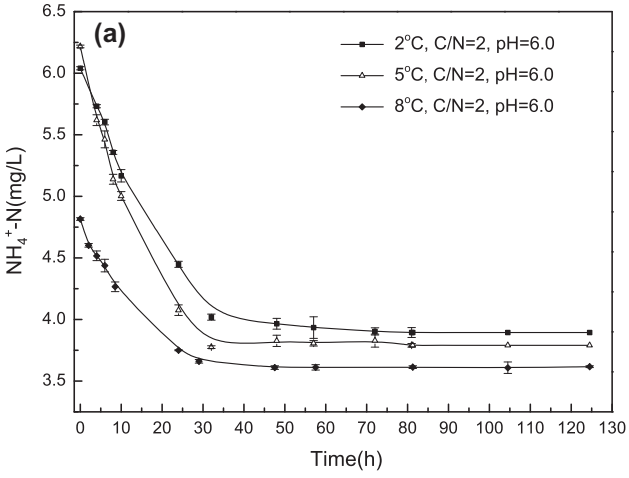

Fig. 2(a). The effects of temperature on ammonium removal of HITLi 7. Error bars: mean ± S.D. of two replicates.

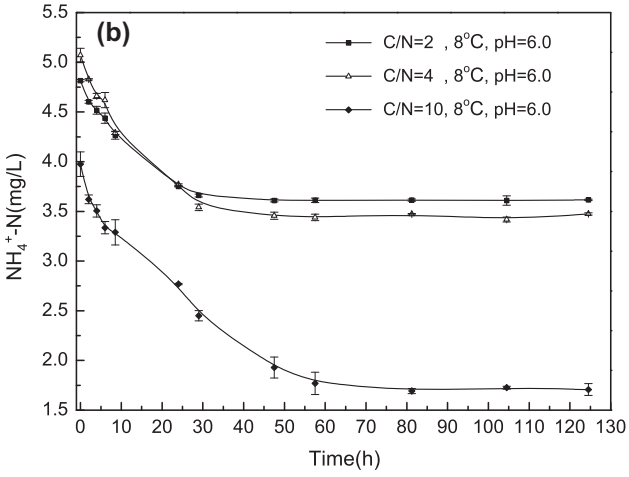

Fig. 2(b). The effects of C/N ratio on ammonium removal of HITLi 7. Error bars: mean ± S.D. of two replicates.

ammonium solution with an NH_4^+ concentration of about 5 mg/L at 8℃ and pH 6.0 while shaking at 100 r/min. The concentration of acetate served as the carbon source and was adjusted to produce C/N ratios of 2, 4, and 10, respectively. The results are shown in Fig. 2(b). Ammonium degraded rapidly for a C/N of 2 and 4. However, a long ammonium degradation period was observed for C/N = 10 (Fig. 2(b)). For an initial microbial biomass (X_{H0}) of 76.0 ± 0.41 mg/L, 25, 31, and 57% of NH_4^+-N were removed by strain HITLi 7 at a C/N of 2, 4, and 10, respectively (Fig. 2(b)). The maximum ammonium reduction rate at a C/N of 2, 4, and 10 was 0.11, 0.12, and 0.18 mg NH_4^+-N/L/h, respectively.

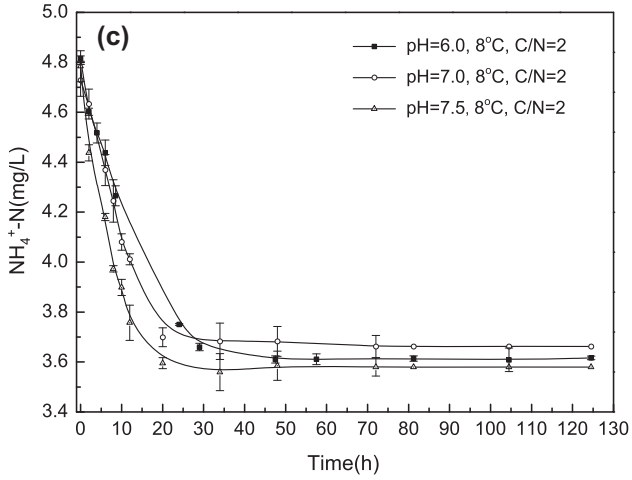

Fig. 2(c). The effects of pH on ammonium removal of HITLi 7. Error bars: mean ± S.D. of two replicates.

3.2.3. Effect of pH value on ammonium reduction

The effect of pH 6.0, 7.0, and 7.5 on ammonium reduction by *Acinetobacter* sp. HITLi 7 was investigated at 8℃ and C/N 2 while shaking at 100 r/min. As shown in Fig. 2(c), the consumption of ammonium stopped after 34 h. For an initial microbial biomass (X_{H0}) of 232.0 ± 5.7 mg/L, 22 and 26% of NH_4^+-N were removed at pH 7.0 and 7.5, respectively. For an initial microbial biomass (X_{H0}) of 76.0 ± 0.4 mg/L, the ammonium reduction rate was 24% in 47.5 h at pH 6.0. The final ammonium reduction rates at pH 6.0, 7.0, and 7.5 were nearly equivalent. However, the degradation rate in a slightly acidic environment pH 6.0 decreased because the initial microbial biomass was one-third of initial microbial biomass at pH 7.0 and 7.5.

3.3. Ammonium reduction kinetics

Ammonium reduction by *Acinetobacter* sp. HITLi 7 was similar to the research of Martens-Habbena et al. [26]. The ammonium reduction behavior could be described by the Monod equation, which describes three stages: an approximately linear decrease, a nonlinear decrease, and a plateau (Fig. 2). Thus, the kinetics of strain HITLi 7 were analyzed using the integrated Monod equation [27]. The residual ammonium concentration (S_s), the initial ammonium concentration (S_0), the corresponding sampling time (t), and the initial biomass concentration (X_{H0}) were obtained using the data shown in Fig. 2. The kinetic parameters (μ_{max}, Y_H, K_s) were calculated using nonlinear curve fitting with the Origin 8.5.1 software (OriginLab, USA). The curve is shown in Fig. 3. K_s was calculated

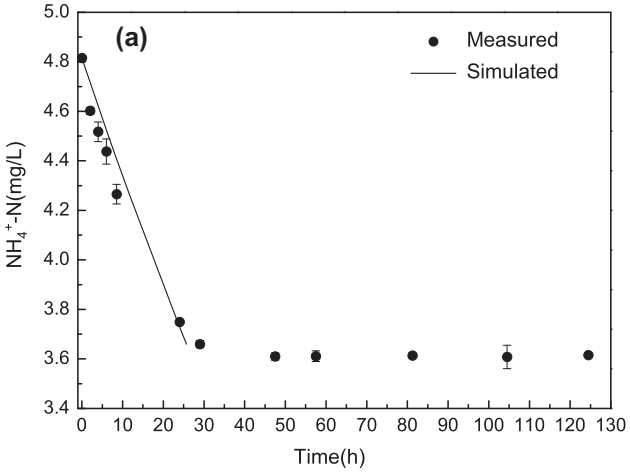

Fig. 3(a). Ammonium removal kinetics of HITLi 7. Ammonium uptake of HITLi 7 upon transfer to fresh medium containing 4.8 mg/L ammonium at 8 ℃, C/N ratio 2, pH = 6.0 while shaking at 100 r/min. Error bars: mean ± S.D. of two replicates.

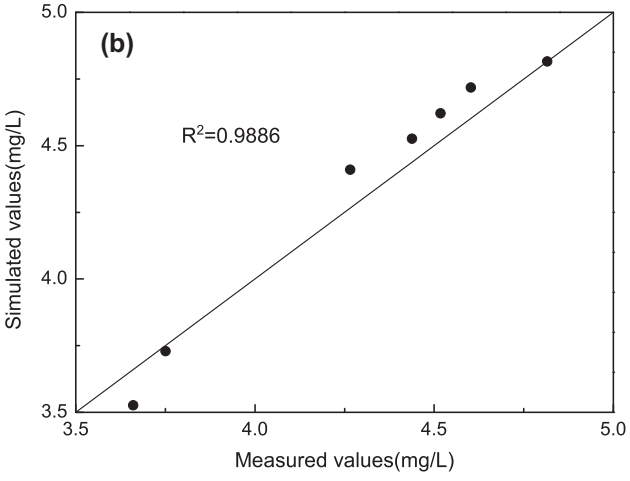

Fig. 3(b). Ammonium removal kinetics of HITLi 7. Simulated ammonium concentrations using the estimated parameters (μmax = 7.9 × 10^{-4} h^{-1}, K_s = 9.9 mg/L, YH = 0.4 mg N_X/mg Ns). Error bars: mean ± SD of two replicates.

to be 9.9 mg/L total ammonium, μ_{max} was calculated to be 7.9×10^{-4} h^{-1}, and Y_H was calculated to be 0.4 mg N_X/mg N_s. Correlation coefficient reached to 0.9886.

For temperatures of 2, 5 and 8℃, the μ_{max} was calculated to be 4.7, 6.1 and 7.9×10^{-4} h^{-1}, and K_s was calculated to be 4.8, 5.3, and 9.9 mg NH_4^+/L, respectively (Fig. 4). The specific affinity a^0 at 2, 5 and 8℃ was 0.10, 0.12, and 0.08 L/g wet cells/h (Fig. 5).

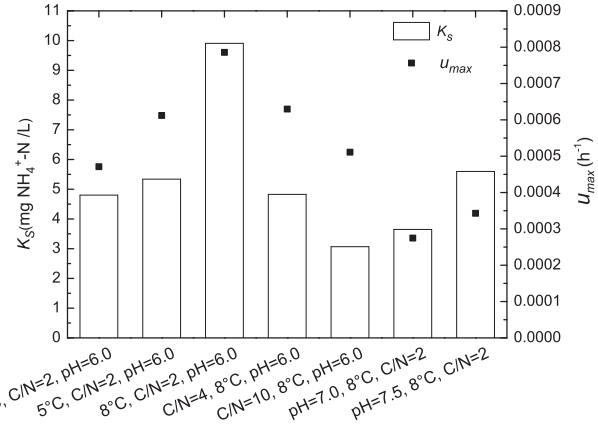

Fig. 4. Estimated kinetic parameters of HITLi 7 under different conditions.

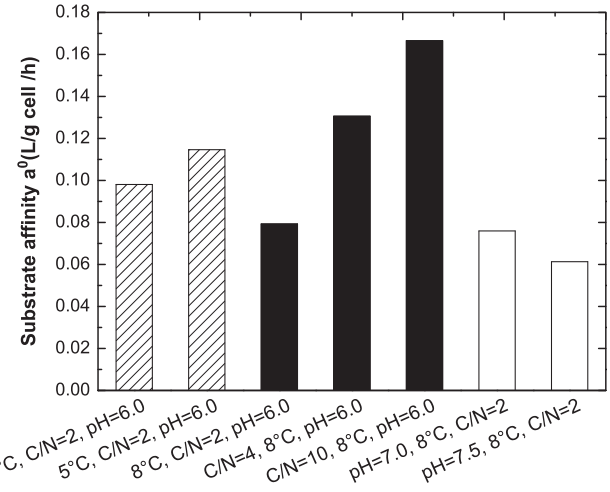

Fig. 5. Specific affinity a^0 of HITLi 7 under different conditions.

For C/N ratios of 2, 4, and 10, the calculated μ_{max} was 7.9, 6.3, and 5.1×10^{-4} h^{-1}, respectively, and K_s was 9.9, 4.8, and 3.1 mg NH_4^+-N/L, respectively. Although the final ammonium reduction rate was higher for higher C/N ratios, μ_{max} was slightly lower for higher C/N ratios. The specific affinity a^0 at C/N 2, 4, and 10 was 0.08, 0.13, and 0.17 L/g wet cells/h, respectively (Fig. 5).

The calculated μ_{max} was 7.9, 2.8, and 3.4×10^{-4} h^{-1}, and K_s was 9.9, 3.6, and 5.6 mg/L total ammonium at pH 6.0, 7.0, and 7.5, respectively.The value of μ_{max} reached a maximum at pH 6.0, decreased for pHs between 6.0 and 7.0, and increased for pHs between 7.0 and 7.5. The specific affinity a^0 at pH 6.0, 7.0, and 7.5 was 0.08, 0.08, and 0.06 L/g wet cells/h (Fig. 5).

4. Discussion

The nitrification process has been described as follows: $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$ [28–30]. NH_2OH was found to be an intermediate and was detected during the heterotrophic nitrification process [9,12,18,20]. In this work, conversion of NH_2OH was observed during NH_4^+-N degradation (Fig. 1). During the first 24 h, NH_2OH increased while NH_4^+-N decreased, indicating the oxidation of NH_4^+-N to NH_2OH. Subsequently, a high concentration of NO_3^- was detected while NH_2OH decreased, indicating the transformation of NH_2OH to NO_3^-. However, the total nitrogen content of products (NH_2OH, NO_2^-, and NO_3^-) was not equal to the total reduction of NH_4^+-N. *Acinetobacter* sp. HITLi 7 exhibited ammonium reduction behavior similar to *Alcaligenes faecalis* No. 4 and *Acinetobacter calcoaceticus* HNR [9,13]. In addition, *Acinetobacter* sp. HITLi 7 did not exhibit visible growth during NH_4^+-N degradation. Thus, we hypothesize that NO_3^- was denitrified simultaneously by the strain HITLi 7. This hypothesis should be tested in the future work.

Although the biomass at 8˚C was 63% of the biomass at 5˚C, the ammonium reduction rates were similar at two temperatures (Fig. 2(a)). A higher temperature may improve other biological characteristics of the strain, such as transportation of NH_4^+ into cells. Although the temperature of cultivation was below 10˚C, a delay in ammonium reduction was not observed. This phenomenon was different from the observations of Joo et al. and Zhang et al. [9,17]. These results indicate that *Acinetobacter* sp. HITLi 7 has the ability to remove ammonium at low temperatures.

In this study, the maximum ammonium reduction rates were much lower for *Acinetobacter* sp. HITLi 7 (0.18 mg NH_4^+-N/L/h) than for *A. faecalis* strain NR (26.9 mg NH_4^+-N/L/h) and *Providencia rettgeri* strain YL (18.7 mg-NH_4^+-N/L/h) [12,31] because of poor nutrition, low biomass, and low temperature. In the previous studies, percentage (%) and velocity (mg NH_4^+-N/L/h) were often used to describe ammonium reduction efficiency, However, these characteristics neglect the effects of biomass. Therefore, this study required an analysis of the kinetics of ammonium reduction by strain HITLi 7.

The kinetic parameters of ammonium reduction by *Acinetobacter* sp. HITLi 7 were calculated for different growth conditions. K_s of heterotrophic strain HITLi 7 was similar to the K_s of *Nitrosomonas eutropha* and *Nitrosococcus oceani* at about 30˚C [26]. In previous work, specific affinity a^0 ($a^0 = \mu_{max}/K_s$) was used as a rate constant that defines the accumulation of substrate in terms of mass over time [32]. This parameter is the best measure of the relative ability of bacteria to degrade the substrate and can be used to evaluate the size of a specific population [33]. A high a^0 indicates that the bacteria have a high affinity for the objective substrate. *Acinetobacter* strain HITLi 7 demonstrated a high affinity at 5˚C. K_s and μ_{max} increased consistently with an increase in temperature (Fig. 4), indicating that strain HITLi 7 performs heterotrophic nitrification more efficiently at higher temperatures. These results were expected, as the optimal growth temperature of strain HITLi 7 is 8˚C.

As shown in Fig. 4, μ_{max} did not increase with an increase in C/N ratio. The results demonstrated that a high C/N provided a sufficient carbon source for sustaining ammonium metabolism, but the ammonium degradation velocity remained constant. In this study, the specific affinity a^0 was the highest for a C/N of 10. This specific affinity value was 2.1-fold higher than the affinity observed for C/N of 2 (Fig. 5), and was equivalent to the a^0 of glucose metabolized by *Sphingomonas* sp. strain RB 2256 [32]. Thus, the optimum C/N ratio for ammonium reduction by HITLi 7 is 10.

According to data from the Ministry of Environmental Protection of the People's Republic of China, the average permanganate index of the Songhua River in winter from 2009 to 2013 was 5.8 ± 1.6 mg/L; the average TOC was about 7.3 ± 1.3 mg/L; the average ammonium concentration was 0.7 ± 0.1 mg/L; and the C/N ratio was between 2 and 10. An 800 mg/L biomass of *Acinetobacter* sp. HITLi 7 could remove 0.2 mg/L NH_4^+-N in 30 min even at low temperatures and under conditions of poor nutrition (2˚C, C/N 2), according to theoretical calculations. Currently, we are conducting a biological enhanced activated carbon (BEAC) process at 2˚C to verify the ammonium reduction efficiency of *Acinetobacter* sp. HITLi 7. The empty bed contact time of the BEAC process is set to 30–45 min. When the initial ammonium concentration is 0.70 ± 0.05 mg/L, 0.20 ± 0.03 mg/L NH_4^+-N may be removed by the BEAC process in 45 min at 2˚C (unpublished data). Therefore, *Acinetobacter* sp. HITLi 7 may play an effective role in ammonium reduction at low temperatures. The BEAC process inoculated with *Acinetobacter* sp. HITLi 7 may serve as an effective drinking water treatment process.

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