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Heterotrophic nitrification and aerobic denitrification at low temperature by a newly isolated bacterium, *Acinetobacter* sp. HA2

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HIGHLIGHTS

- Psychrotrophic heterotrophic nitrifying–aerobic denitrifying strain isolated.
- Much higher tolerance to low temperature than common mesophiles.
- Efficient removal of NH_4^+–N, NO_2^-–N and NO_3^-–N at 10 °C under aerobic conditions.
- N_2 produced but no N_2O detected during nitrification–denitrification.
- Simultaneous removal of COD and TN in an open aeration reactor.

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ABSTRACT

A psychrotrophic heterotrophic nitrifying–aerobic denitrifying bacterium was newly isolated and identified as *Acinetobacter* sp. with phenotypic and phylogenetic analysis. The strain possessed excellent tolerance to low temperature with 20 °C as its optimum and 4 °C as viable. Moreover, ammonium, nitrite and nitrate could be removed efficiently under low-temperature, solely aerobic conditions with little accumulation of intermediates. The average removal rate at 10 °C reached as high as 3.03, 2.51 and 1.88 mg N L^{-1} h^{-1} for ammonium, nitrite and nitrate respectively. N_2 was produced through heterotrophic nitrification and aerobic denitrification via nitrite but N_2O was never detected in the whole process. Nitrogen balance analysis indicated that N_2 and intracellular nitrogen were two major fates of the initial ammonium, accounting for 32.4 and 49.2%, respectively. Further aerated batch test demonstrated efficient removal of COD and TN from synthetic wastewater, which implied promising practical application of the present strain.

1. Introduction

Biological process is widely used in wastewater treatment plants for its ease of implementation, high-efficiency, and cost benefit. Nitrogen removal, as an integral component of wastewater treatment, normally involves nitrification process carried out by autotrophic nitrifiers under aerobic conditions and denitrification process by heterotrophic denitrifiers under anoxic conditions. However, biological treatment faces great challenges in the winter months because low temperature have severe inhibition effect on activated sludge microorganisms, especially on autotrophic nitrifiers. Previous studies demonstrated that nitrification was strongly inhibited at temperatures of 10 °C or less (Ilies and Mavinic, 2001; Sundaresan and Philip, 2008). Moreover, the saturated dissolved oxygen concentration increases with decreasing temperature, which put extra pressure on the conduct of anoxic denitrification at low temperature.

In order to enhance wastewater treatment efficiency under cold temperature, countermeasures such as lower organic loading, higher sludge recycle ratio and extended hydraulic resident time are always adopted in engineering practices. However, limited improvements had been achieved with acceptable construction investment and operation cost. Immobilization technology could increase resistance of microorganisms to low temperature (Yang et al., 1997). Nevertheless, the practical application of immobilized microorganism technology would meet new problems, including higher cost, limited service life of carriers, and additional requirements on configuration, operation and maintenance of the bioreactor.

In recent years, heterotrophic nitrifying–aerobic denitrifying microorganisms capable of nitrification and denitrification simultaneously under aerobic conditions have drawn increasing atten-

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tion (Chen and Ni, 2011; Khardenavis et al., 2007; Zhu et al., 2012). Good adaptation of these functional bacteria to conventional activated sludge systems led to efficient removal of total nitrogen in a single aeration phase (Joo et al., 2006; Kulkarni, 2013) without the need of either carrier or additional constructions. Such kind of bacteria holds considerable promise for effective nitrogen removal at low temperature due to (1) faster growth rate and better cold-adaptation compared with autotrophic nitrifiers; and (2) denitrification capability under relatively high dissolved oxygen concentration (Yao et al., 2013). However, very limited researches have been done in this area. *Pseudomonas stutzeri* YZN-001 (Zhang et al., 2011) was reported to be able to remove ammonium at low temperature, but its nitrite and nitrate removal capability was only investigated at 30 °C. More microbial resources with excellent tolerance to low temperature must be exploited and characterized.

In previous research, a psychrotrophic consortium capable of heterotrophic nitrification and aerobic denitrification was successfully enriched (Yao et al., 2013). In the present study, a psychrotrophic bacterium was newly isolated from the obtained consortium and its temperature characteristics were specially evaluated. Nitrogen removal performance and metabolic mechanisms of the strain were further investigated under aerobic conditions at 10 °C (typical wastewater temperature during wintertime of northern China). Potential applications were also exemplified with efficient treatment of synthetic wastewater in an open aeration reactor.

2. Methods

2.1. Medium

A modified Bromothymol blue (BTB) medium (Takaya et al., 2003) used for bacteria isolation contained (per liter of distilled water): 1.0 g L-asparagine, 2.73 g sodium acetate, 1.0 g KNO_3, 1.0 g KH_2PO_4, 0.2 g $CaCl_2{\cdot}2H_2O$, 1.0 g $MgSO_4{\cdot}7H_2O$, 0.006 g $FeSO_4{\cdot}7H_2O$, 16–18 g agar and 1 ml BTB solution (1% in ethanol); pH 7.0–7.3. A basal medium used for bacteria cultivation contained (per liter of distilled water): 2.73 g sodium acetate, 0.306 g NH_4Cl, 0.088 g KH_2PO_4, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.006 g $FeSO_4{\cdot}7H_2O$ and 1 ml of a trace element solution. The components of the trace element solution were as follows (per liter of distilled water): 0.35 g EDTA, 0.2 g $ZnSO_4{\cdot}7H_2O$, 0.1 g $CuSO_4{\cdot}5H_2O$, 0.2 g $MnSO_4{\cdot}7H_2O$, 0.09 g $Co(NO_3)_2{\cdot}6H_2O$, 0.1 g H_3BO_3 and 0.1 g Na_2MoO_4.

Each medium was autoclaved for 20 min at 121 °C before use.

2.2. Isolation and identification

Isolation of functional bacteria was newly completed from the psychrotrophic heterotrophic nitrifying–aerobic denitrifying consortium obtained previously (Yao et al., 2013). The sludge sample (10 ml) was transferred to a 250 ml flask containing sterilized 0.9% NaCl solution (90 ml) and glass beads and then shaken at 200 rpm to obtain homogeneous bacterial suspension. Gradient dilutions were then performed and resultant suspensions of different concentration were spread on BTB medium plates. The plates were incubated at temperature as low as 4 °C for a couple of days until visible blue colonies (positive result) had formed. Separate colonies were isolated and purified by repeated streaking on the BTB medium plates. Afterwards, pure isolates obtained went through a rapid screening on BTB medium plates and then were cultivated in basal medium for detailed nitrogen removal capability evaluation. Selected isolate with excellent performance was suspended in 25% glycerol solution and stored at −80 °C.

The 16S rRNA gene of the isolate was PCR amplified using bacterial universal primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1492 (5′-TTGGYTACCTTGTTACGACT-3′). PCR products were sequenced on both strands in Invitrogen Inc. (Beijing, China). The sequence alignment was performed using the Basic Local Alignment Search Tool (BLAST). A neighbor-joining tree was constructed in MEGA 5.05 program using neighbor-joining (NJ) method with 1000 bootstrap replicates and the maximum composite likelihood model.

2.3. Temperature characteristics

To evaluate the temperature characteristics of the isolate, its growth conditions at different temperatures were investigated in shake flask experiments. 150 ml of the basal medium was placed in triplicate 250 ml shake flasks, inoculated with 5 ml pre-culture of the isolate and then cultivated at 10, 15, 20, 25 and 30 °C respectively. During incubation, 3 ml culture was removed periodically for the determination of cell optical density (OD_{600}).

Experimental growth data were then fitted using the logistic model (OriginPro 8.0) as described in the following equation:

$$\mathrm{Log}N = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} \tag{1}$$

where x is time (h); N is the population of the organism at time x (CFU/ml), calculated from 1 OD = 1×10^8 CFU/ml; A_1, A_2, x_0 and p are parameters.

To obtain the inflection point of the curve, the second derivative of the function with respect to x was calculated:

$$\frac{dy}{dx} = (A_2 - A_1) \cdot p \cdot \frac{1}{x_0} \cdot \left(\frac{x}{x_0}\right)^{p-1} \left[1 + \left(\frac{x}{x_0}\right)^p\right]^{-2} \tag{2}$$

$$\frac{d^2y}{dx^2} = (A_2 - A_1) \cdot p \cdot \frac{1}{x_0} \left\{ \frac{p-1}{x_0} \cdot \left(\frac{x}{x_0}\right)^{p-2} \cdot \left[1 + \left(\frac{x}{x_0}\right)^p\right]^{-2} - 2\left(\frac{x}{x_0}\right)^{2p-2} \cdot \frac{p}{x_0} \cdot \left[1 + \left(\frac{x}{x_0}\right)^p\right]^{-3} \right\} \tag{3}$$

At the inflection point, where $x = x_i$, the second derivative is equal to zero:

$$\frac{d^2y}{dx^2} = 0 \rightarrow x_i = x_0 \left(\frac{p-1}{p+1}\right)^{\frac{1}{p}} \tag{4}$$

Then, μ_m (the maximum specific growth rate) can be obtained by calculating the first derivative at the inflection point.

2.4. Nitrogen removal performance at low temperature

Performance evaluation was also carried out in shake flask experiments with the same liquid volume and inoculation amount as described in Section 2.3. Basal medium was adopted to investigate the ammonium removal performance at temperature as low as 10 °C. Furthermore, nitrite and nitrate were respectively used instead of ammonium as the sole nitrogen source in the basal medium to assess the denitrification performance of the isolate. During incubation, 5 ml culture was removed periodically and centrifuged (6000 rpm, 10 min) to obtain supernatant for the measurement of ammonium, hydroxylamine, nitrite and nitrate.

2.5. Gas detection in sealed serum bottles

100 ml of basal medium was placed in triplicate 300 ml glass serum bottles and inoculated with 5 ml bacterial suspension of the isolate. These bottles were then fully aerated with pure oxygen

gas, tightly sealed with a rubber septum and cultivated at 10 °C and 120 rpm. The system without the bacterial inoculum was used as a control. Gas samples (250 μl) were collected periodically using a gas-tight syringe to detect N_2, N_2O and O_2 by gas chromatography. Cultures were sampled at the starting and ending points and analyzed for the concentration of ammonium, hydroxylamine, nitrite and nitrate.

2.6. Treatment of synthetic wastewater in aerated batch test

Wastewater treatment was performed in four identical 1 L glass beakers at 10 °C. Synthetic wastewater was prepared by dissolving the following in 1 L of tap-water: 0.153 g NH_4Cl (40 mg/L NH_4^+–N), 0.044 g KH_2PO_4, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.006 g $FeSO_4{\cdot}7H_2O$ and 0.2 g $NaHCO_3$. Moreover, 0.27, 0.40, 0.54 and 1.08 g sodium acetate was supplied with corresponding COD concentration of 200, 300, 400 and 800 mg/L respectively. The isolated strain was inoculated into synthetic wastewater at dosage of 5% (V/V) and then aeration batch test commenced. Air was supplied by an adjustable gas pump and divided into four branches by a gas distributor. DO concentrations measured periodically during the test were ranged from 1.0 and 3.6 mg/L. Samples were taken at regular intervals for the measurement of COD, ammonium, nitrite and nitrate.

2.7. Analytic methods

The cell optical density was measured at 600 nm (OD_{600}) using a spectrophotometer (UV-1800, Shimadzu, Japan). NH_4^+–N, NO_2^-–N and NO_3^-–N were measured by Nessler's reagent spectrophotometry, N-(1-naphthalene)-diaminoethane spectrophotometry and ultraviolet spectrophotometric method respectively (APHA, 1998). Hydroxylamine was analyzed according to Frear and Burrell (1955). Total nitrogen (TN) in the medium was calculated by adding ammonium, hydroxylamine, nitrite and nitrate. Intracellular nitrogen content was calculated from the weight (mg/L) of dry biomass and the nitrogen content (%) in it. The dry biomass was obtained by drying the thallus at 105 °C overnight after centrifugation (6000 rpm, 10 min). Nitrogen content was detected by an element analyzer (Vario EL, Elementar, Germany). N_2O and N_2 were determined according to Zeng et al. (2003). Gas samples were injected into gas chromatography–mass spectrometer (GC–MS, 6890N-5973, Agilent, USA) equipped with a HP-Plot 5A molecular sieve column (30 m × 0.32 mm × 25 μm). SIM mode was chosen to detect the characteristic peaks of N_2 and N_2O. The carrier gas was He with a flow rate of 3 ml/min. For N_2 and N_2O, the column temperatures were 35 and 200 °C, respectively. COD was determined by closed reflux titrimetric method 5220C (APHA, 1998).

3. Results and discussion

3.1. Isolation and identification

The isolation medium contained BTB which was able to indicate pH increase during denitrification process (Takaya et al., 2003). Thirty-six distinct positive colonies were isolated from BTB medium plates incubated at 4 °C. According to the size of the blue color ring generated by the purified isolates on the BTB medium plates, 16 strains were screened. The strains were further tested for nitrogen removal capability under aerobic conditions at 10 °C. A strain which demonstrated more than 80% ammonium removal efficiency within 24 h with little accumulation of oxidized nitrogen was selected and named as HA2.

HA 2 is Gram-negative, non-motile, rod-shaped bacterium with size of (0.4–0.7) μm ∗ (0.8–1.3) μm, and exists individually, in pairs or short chains. Its colony on the solid medium is off-white, round, salient, smooth surface, and with regular edge. Nearly full-length 16S rRNA gene (1417 bp) of strain HA2 was sequenced and deposited in GenBank under accession number of KC422446. A phylogenetic tree was constructed based on 16S rRNA gene sequence of the isolate and some other phylogenetically related strains (Fig. 1). The results indicated that HA2 was most closely related to *Acinetobacter* sp. B2070 (similarity 99.7%). Therefore, HA 2 is proposed to be an *Acinetobacter* species.

3.2. Temperature characteristics of strain HA2

Fig. 2A depicted the growth curves of the isolate at temperature 10, 15, 20, 25 and 30 °C, respectively. It was shown that the strain grew well under all temperature conditions tested. The OD_{600} value increased rapidly right after inoculation and the stationary phase was reached in 12 h (20 °C)–24 h (10 °C). For further quantitative analysis of bacterial growth, the number of the bacterium over time was fitted with the logistic model shown in Eq. (1). The fitted curves were presented in Fig. 2B and the values of relevant parameters were summarized in Table 1; statistical values of adjusted coefficient of multiple determination R^2_{adj} and calculated values of μ_m were also listed in the table. In general, experimental data were well fitted with the logistic model, obtaining R^2_{adj} ranging from 0.996 to 0.998. At the temperature 20 °C, μ_m reached the peak value which was comparable with that reported in pervious literature, e.g. 0.2 h^{-1} for *Alcaligenes faecalis* No. 4 (Joo et al., 2005). When temperature exceeded 20 °C, the specific growth rate declined slightly; at 30 °C, the strain was still able to grow rapidly. When temperature fell below 20 °C, an obvious decline trend in growth rate was observed. Yet, the strain exhibited a relatively high growth rate at temperature as low as 10 °C, higher than the rate of 0.03–0.05 h^{-1} by autotrophic nitrifier *Nitrosomonas europaea* even at moderate temperature (Gupta and Gupta, 2001) Table 2.

It is notable that the cardinal temperatures for the present strain were quite different from that for mesophilic microorganisms: (1) the optimum growth temperature for strain HA2 is 20 °C, while it is 30–37 °C for mesophilic bacteria; (2) strain HA2 grew well with no lag phase at 10 °C and additional spread-plate experiment indicated it was able to flourish at 4 °C. In contrast, mesophiles normally show little growth or begin to grow after a long lag period at 10 °C and cannot grow at all at 4 °C (Shen, 2006). As a whole, strain HA2 was identified as a psychrotrophic bacterium.

Up to now, nearly all reported heterotrophic nitrifying–aerobic denitrifying bacteria were mesophiles. Table 2 summarized the temperature dependency of the strains in previous studies (Joo et al., 2005; Song et al., 2011; Zhang et al., 2011, 2012). It was demonstrated that the reported isolates obtained the best growth/performance at 30–37 °C and were severely or totally inhibited at temperature as low as 10 °C. As a result, the present bacterium which was much more cold-tolerant can offer a new microbial resource for nitrogen removal at low temperature.

3.3. Ammonium removal performance at low temperature

Ammonium removal capability of the strain HA2 was investigated under aerobic conditions at 10 °C, as depicted in Fig. 3. Ammonium concentration decreased dramatically during the whole test period and eventually reached zero around 25 h. The batch test was well described by zero-order reaction, indicating a lack of a lag period in ammonium removal at temperature as low as 10 °C. By contrast, at 20 °C a long period where there was no ammonium removal was reported for the strain *P. stutzeri* YZN-001 (Zhang et al., 2011). Moreover, the average specific ammonium

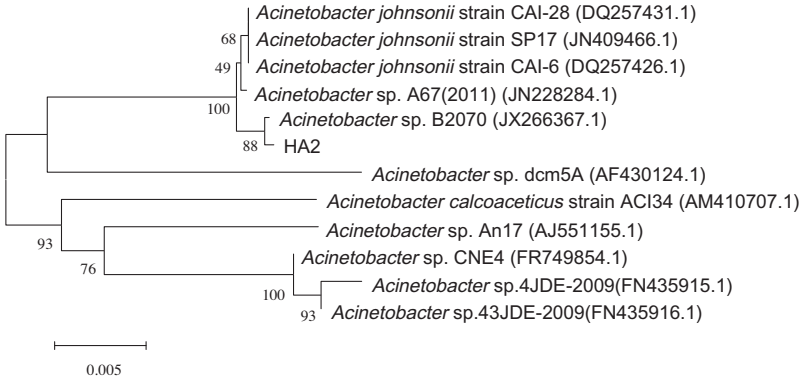

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence of strain HA2 and other reference sequences.

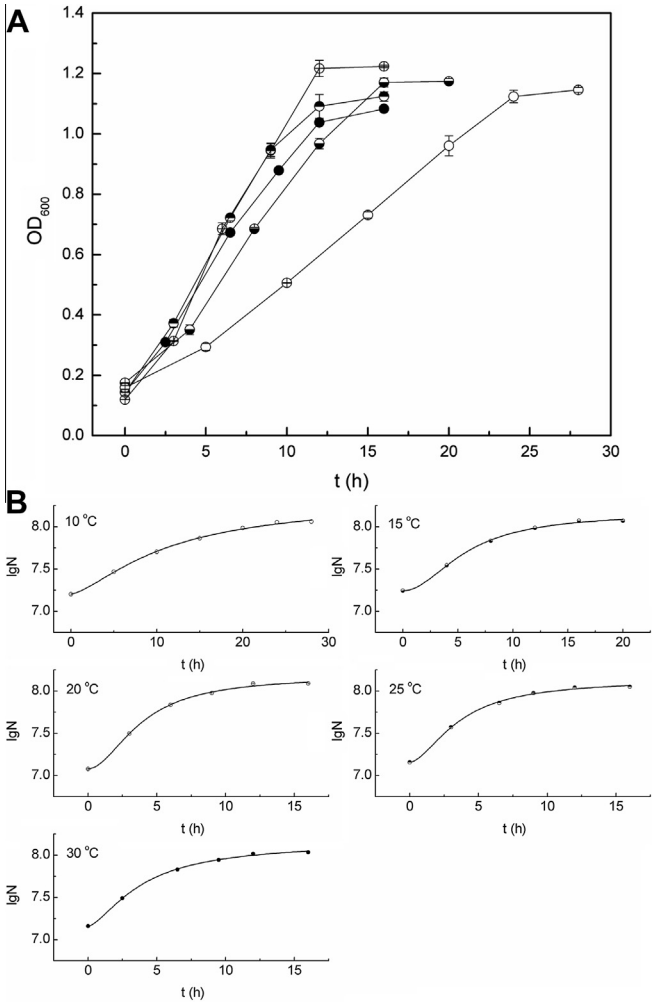

Fig. 2. Growth curves of strain HA2 at various culture temperatures ranging from 10 to 30 °C. (A) line chart of the experimental data; (B) fitted curves using the logistic model. Symbols: closed circles, 30 °C; circles filled with upper half, 25 °C; circles with across in the middle, 20 °C; circles filled with bottom half, 15 °C; open circles, 10 °C.

removal rate of the strain was calculated to be 3.03 mg N L^{-1} h^{-1}. This value was over 100% higher than the removal rate of 1.48 mg L^{-1} NH_4^+–N h^{-1} obtained by *P. stutzeri* YZN-001 at the same temperature 10 °C (Zhang et al., 2011), and even equivalent or higher compared with those obtained by other heterotrophic nitrifying–aerobic denitrifying bacteria under moderate temperature 28–30 °C (Chen et al., 2012; Su et al., 2006; Yang et al., 2011).

Table 1
Parameter values, statistical analysis of fittings and calculated μ_m of the logistic model for the growth curves.

T (°C)	A_1	A_2	x_0	p	R^2	μ_m
30	7.204	8.301	11.123	1.465	0.998	0.153
25	7.244	8.156	5.749	1.985	0.997	0.166
20	7.078	8.175	3.892	1.881	0.996	0.179
15	7.157	8.134	3.617	1.717	0.997	0.103
10	7.159	8.151	3.944	1.527	0.998	0.060

The intermediates hydroxylamine and nitrite were only present in trace amounts during the removal of ammonium, while the concentration of nitrate increased to a maximum of 2.27 mg/L at 10 h and then declined. As a result, total nitrogen was effectively and completely removed under aerobic conditions, which is of great significance to practical applications in wastewater treatment. Total nitrogen removal under aerobic conditions by pure isolate was generally linked to heterotrophic nitrification and simultaneously aerobic denitrification in previous studies (Daum et al., 1998; Robertson et al., 1988). Hence, aerobic denitrification capability of the present isolate was investigated in the following.

3.4. Nitrite and nitrate removal performance at low temperature

Aerobic denitrification capability were evaluated using nitrite and nitrate as sole nitrogen source respectively as shown in Fig. 4A and B. A lag period was observed with both compounds at the very beginning, which probably resulted from the conversion from nitrite or nitrate to NH_4^+–N for bacterial assimilation. After that, the strain began to grow rapidly accompanied with significant decrease of nitrite/nitrate. The removal efficiency of nitrite and nitrate reached the peak value of 83.6% and 80% when the growth of the strain reached the stationary phase at 28 h and 34 h respectively. Nitrite slightly accumulated with the decline of nitrate and then reduced to a final concentration of 1.99 mg/L. Moreover, higher growth rate and cell yield were obtained with nitrite as N-source compared with nitrate. Similar results were also reported in previous studies (Zhao et al., 2010). One possible explanation might be the lower oxidation state of N in nitrite made it easier for assimilatory reduction to support bacterial growth. Moreover, the relatively high nitrite concentration of 80 mg/L did not have inhibitory effect on strain HA2, while growth of the strain *Pseudomonas* sp. yy7 (Wan et al., 2011) was poor with an initial nitrite concentration of 50 mg/L. The calculated average nitrite and nitrate removal rate at 10 °C was as high as 2.51 and 1.88 mg N L^{-1} h^{-1}, respectively. It was interesting that the present strain performed at low temperature even better than most bacterial strains at moderate temperature, such as *Rhodococcus* sp.

Table 2
Temperature dependency of heterotrophic nitrifying–aerobic denitrifying bacteria in several studies.

Microorganism	Optimum temperature (°C)	Growth under low temperature	References
A. faecalis No.4	37	Delayed at 20 °C	Joo et al. (2005)
P. stutzeri YZN-001	30–37	Delayed at 10 °C	Zhang et al. (2011)
Bacillus sp. YX-6	30	Disabled at 10 °C	Song et al. (2011)
Bacillus methylotrophicus L7	37	Not mentioned	Zhang et al. (2012)
Acinetobacter sp. HA2	20	Grow well at 10 °C	This study

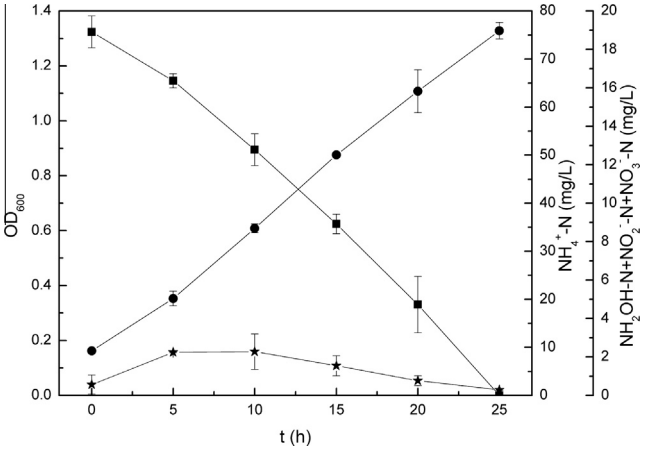

Fig. 3. Ammonium removal performance of strain HA2 at 10 °C. Symbols: circles, OD_{600}; squares, NH_4^+–N; pentagrams, NH_2OH–N + NO_2^-–N + NO_3^-–N.

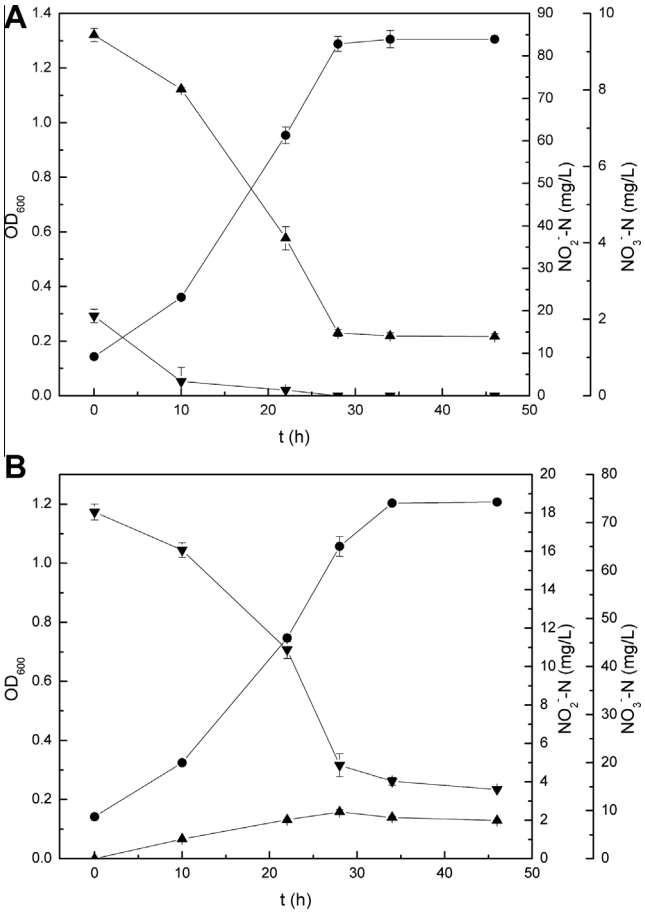

Fig. 4. Nitrite (A) and nitrate (B) removal performance of strain HA2 at 10 °C. Symbols: circles, OD_{600}; upward triangles, nitrite; downward triangles, nitrate.

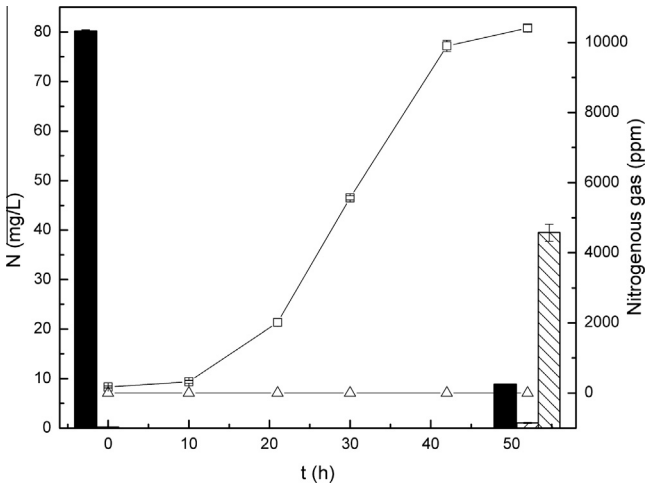

Fig. 5. Gas production during the heterotrophic nitrification and aerobic denitrification process. Symbols: open squares, N_2; open triangles, N_2O; closed columns, ammonium; columns with leftward streak, NH_2OH–N +NO_2^-–N+NO_3^-–N; columns with rightward streak, intracellular nitrogen.

CPZ24 (Chen et al., 2012) with nitrate removal rate of 0.93 mg L^{-1} h^{-1} at 30 °C and *Pseudomonas* sp. yy7 (Wan et al., 2011) with nitrite removal rate of 18.20 mg l^{-1} d^{-1} at 25 °C.

Richardson et al. (1998) indicated that the heterotrophic nitrification process and aerobic denitrification process were coupled via nitrite or hydroxylamine. Among very limited research on *Acinetobacter* species, the pathway of *Acinetobacter calcoaceticus* HNR was presumed to be via hydroxylamine based on the fact that hydroxylamine can be utilized but neither nitrite nor nitrate (Zhao et al., 2010). In comparison, for *Acinetobacter* sp. HA2, conversion of nitrate was observed during the process of ammonium removal and both nitrite and nitrate could be denitrified efficiently under aerobic conditions. Furthermore, the NAP and nirS gene responsible for aerobic reduction of nitrate and nitrite respectively were successfully amplified. As a consequence, ammonium removal pathway for *Acinetobacter* sp. HA2 was proposed to be via nitrite, that is NH_4^+–NH_2OH–NO_2^-–N_2O–N_2, as reported in several studies (Chen and Ni, 2012; Richardson et al., 1998; Robertson et al., 1988).

3.5. Gas production during heterotrophic nitrification and aerobic denitrification

To further confirm the nitrogen removal mechanism of *Acinetobacter* sp. HA 2, gas detection during heterotrophic nitrification–aerobic denitrification process was conducted. In the whole period, oxygen was continuously detected by qualitative analysis with GC–MS, indicating aerobic conditions were maintained throughout the experiment. Production of nitrogenous gas was monitored and depicted in Fig. 5. The concentration of N_2 increased over time and eventually stabilized at about 10,400 ppm (13 mg/L), while N_2O was never detected. The production of N_2 further confirmed the occurrence of dissimilatory nitrogen removal under aerobic conditions by strain HA2. Additionally, N_2O was known as a greenhouses

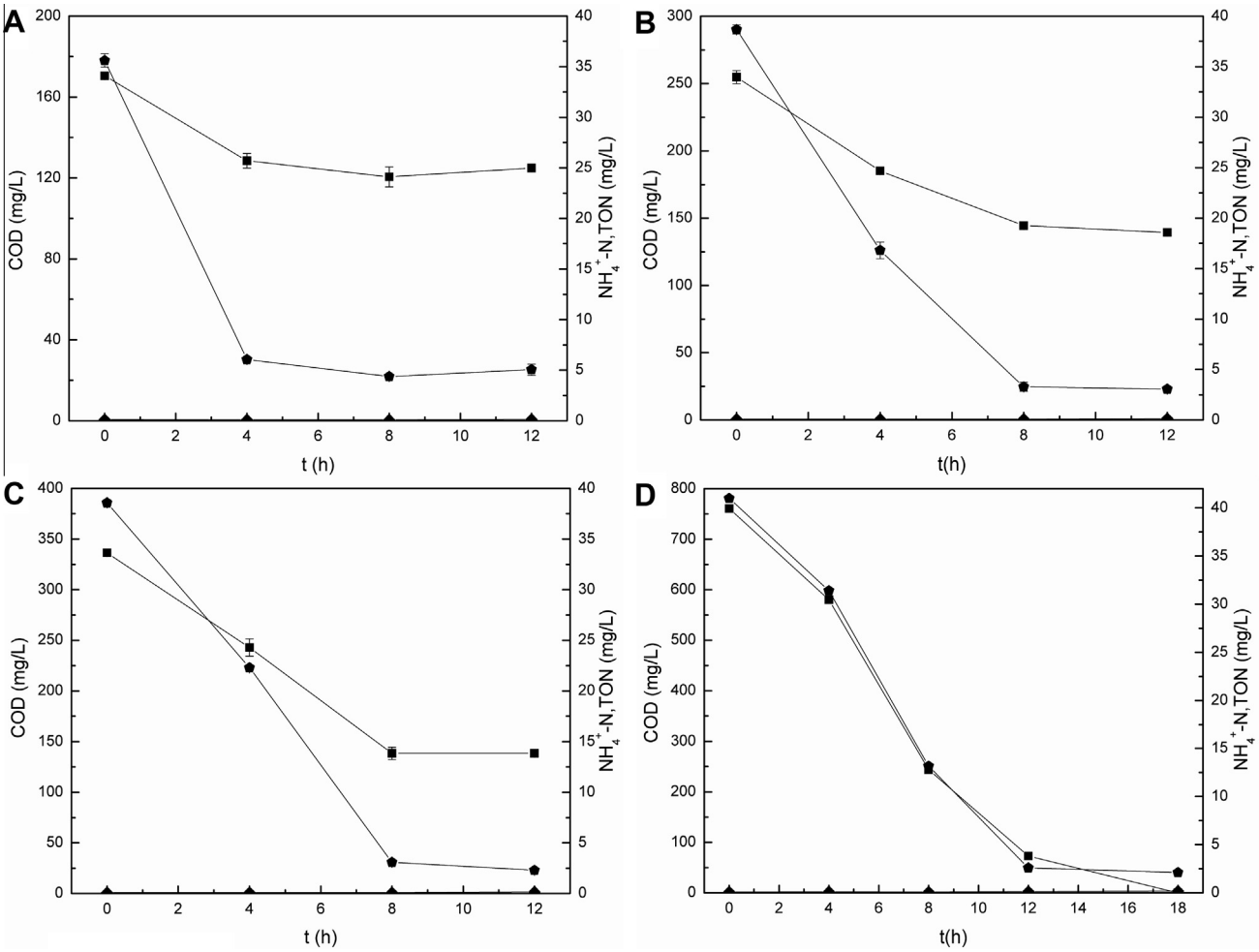

Fig. 6. Treatment of synthetic wastewater with initial COD concentration of 200 mg/L (A), 300 mg/L (B), 400 mg/L (C) and 800 mg/L (D) in aerated batch test at 10 °C. Symbols: pentagons, COD; squares, N_4^+–N; diamonds, NO_2^-–N+NO_3^-–N.

gas and responsible for the depletion of ozone-layer (Perez-Ramirez et al., 2003). Many known denitrifiers produced mainly N_2O (Matsuzaka et al., 2003), equivalent amounts of N_2 and N_2O (Robertson et al., 1995) or small percentage of N_2O (Su et al., 2006) under aerobic conditions. Therefore, the present strain is of great superiority in practical application due to negligible emission of N_2O.

Analysis of water samples at the starting and ending points (Fig. 5) indicated that ammonium was significantly reduced with a removal efficiency of 88.96% and minor accumulation of intermediates (hydroxylamine, nitrite and nitrate). Intracellular nitrogen via assimilation and gaseous product N_2 via nitrification–denitrification accounted for 49.2% and 32.4% of the initial ammonium respectively. In previous studies, it was 24.8% and 24% for *Bacillus* strains (Kim et al., 2005), 52.1% and 40.2% for *A. calcoaceticus* HNR (Zhao et al., 2010), and 54.6% and 28.9% for *A. faecalis* NR (Zhao et al., 2012). Nitrogen balance analysis indicated that 6.1% of N-source could not be accounted for, which could be due to measurement errors originated from the adoption of different analytical methods (Zhao et al., 2012).

3.6. Treatment efficiency of synthetic wastewater

Batch test was carried out to evaluate performance of strain HA2 in an open aeration reactor for potential engineering applications. The removal of COD and nitrogen from wastewater with different carbon-source concentration at 10 °C was depicted in Fig. 6. The results showed that both COD and ammonium could be removed efficiently with negligible accumulation of oxidized nitrogen, indicating simultaneous removal of organics and TN could be successfully achieved. The TN removal efficiency was closely related to the organic carbon source. It could reach 26.72%, 45.29%, 58.88% and even 100% with initial COD concentration of 200, 300, 400 and 800 mg/L, respectively. This phenomenon was also reported in previous studies (Joo et al., 2005; Patureau et al., 2000) with general explanation that carbon source was essential for the cell growth and nitrogen removal process of heterotrophic microorganisms. It was also worth mentioning that TN removal rate did not show significant difference (around 2.1–2.3 mg L^{-1} - h^{-1}) with varying concentration of organic carbon unless the carbon was completely consumed. This demonstrated that the growth of strain HA2 was not limited by low carbon-source concentration, which made it applicable in low-strength wastewater treatment.

4. Conclusions

In this study, *Acinetobacter* sp. HA2 capable of heterotrophic nitrification and aerobic denitrification at low temperature was successfully isolated. The strain possessed excellent cold resistance and was able to remove ammonium, nitrite and nitrate at 10 °C with high average removal rate of 3.03, 2.51 and 1.88 mg N L^{-1} h^{-1}

respectively. N_2 was produced as the end product under aerobic conditions but N_2O was never detected. This is the first systematic research on psychrotrophic heterotrophic nitrifying–aerobic denitrifying bacterium and is of primary importance to nitrogen removal in winter seasons.

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