The characteristics of a novel heterotrophic nitrifying and aerobic denitrifying bacterium, *Acinetobacter junii* YB

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**Highlights**

- *Acinetobacter junii* YB was isolated from activated sludge and identified.
- The strain exhibited efficient removal of ammonium at varying culture conditions.
- Hydroxylamine, nitrite and nitrate could be utilized by strain YB.
- The strain showed capacities for aggregation and hydrophobicity.

**Abstract**

A novel heterotrophic nitrifying bacterium was isolated from activated sludge and was identified as *Acinetobacter junii* YB. The strain exhibited efficient heterotrophic nitrification–aerobic denitrification ability at a broad range of ammonium loads and had the capability to utilize hydroxylamine, nitrite and nitrate as a sole nitrogen source. Based on the nitrogen removal and enzyme assay, the nitrogen removal pathway was speculated to be achieved through heterotrophic nitrification coupled with aerobic denitrification. In addition, single-factor experiments showed that efficient heterotrophic nitrification and growth of strain YB occurred with succinate as the carbon source, pH 7.5, 37 °C, and high C/N ratio and dissolved oxygen. Furthermore, the new isolate showed capacities for aggregation and hydrophobicity. Regular variations of the flocculating ability and relative hydrophobicity were observed during the whole cultivation. The ability to perform heterotrophic nitrification–aerobic denitrification and cell aggregation demonstrated the great potential of the strain YB for future applications.

**Article Info**

Article history:
Received 27 July 2014
Received in revised form 9 August 2014
Accepted 11 August 2014
Available online 19 August 2014

Keywords:
Heterotrophic nitrification–aerobic denitrification
*Acinetobacter junii* YB
Nitrogen removal pathway
Cell aggregation

**1. Introduction**

Currently, the most common approach for nitrogen removal from wastewater is based on the processes of aerobic autotrophic nitrification and anaerobic heterotrophic denitrification (Joo et al., 2005). However, this conventional system tends to be time-consuming due to the low rate of nitrification and demands vast expanses of space to separate the aerobic and anoxic tanks (Khin and Annachhatre, 2004). Additionally, the autotrophs are vulnerable to high loads of ammonium and organic matter, which restrict their applications in treating high-strength ammonium wastewater (Joo et al., 2005). To overcome these limitations, several novel nitrogen removal processes have been developed in the past few years, including partial nitrification, aerobic denitrification, anaerobic ammonium oxidation, and its combined system (Ahn, 2006).

Since the bacterium capable of heterotrophic nitrification was firstly isolated from the natural environment (Verstra and Alexandre, 1972), an increasing number of heterotrophic nitrifying bacteria have been isolated and characterized (Joo et al., 2005; Zhang et al., 2011; Chen et al., 2012; Huang et al., 2013; Guo et al., 2013; Padhi et al., 2013; Yao et al., 2013). These special heterotrophic bacteria exhibit higher growth rates than autotrophs, and can use organic substrates as sources of carbon and energy to convert ammonium into nitrogenous gas under aerobic conditions (Zhao et al., 2010, 2012). Furthermore, the alkalinity generated during denitrification can partly compensate for the acidification caused by nitrification (Zhang et al., 2012). Therefore, simultaneous nitrification and denitrification (SND) could be achieved for low operation costs and highly accelerated rates of nitrogen removal in a single reactor.

Previous studies have shown that the different isolated strains have distinct characteristics of heterotrophic nitrification–aerobic denitrification (Chen and Ni, 2012; Joo et al., 2005; Kim et al.,...
Several researchers have reported that some heterotrophic nitrifiers had the ability to perform heterotrophic nitrification and produced N₂ simultaneously, while aerobic denitrification of nitrite and nitrate was not observed (Joo et al., 2005; Zhao et al., 2010, 2012). On the other hand, some other strains, reportedly, not only had the capability to perform heterotrophic nitrification, but also could utilize nitrite and nitrate as the source of nitrogen for growth and as an energy source for denitrification (Chen et al., 2012; Padhi et al., 2013). These are two main pathways for the process of heterotrophic nitrification–aerobic denitrification. One exhibits a full nitrification and denitrification pathway (Robertson et al., 1988; Huang et al., 2013), while the other demonstrates denitrification via hydroxylamine, rather than nitrite and nitrate (Zhao et al., 2010, 2012). To date, the nitrogen removal pathway under aerobic conditions for the process of heterotrophic nitrification–aerobic denitrification is still unclear. It is necessary to further investigate the nitrogen transformation pathways of other heterotrophic nitrifying strains. Moreover, the aggregation of microbial cells is an important step towards biofilm development and the flocculation of activated sludge, and regulates the performance of biomass-water separation, which is crucial to the overall treatment performance of the process. Nevertheless, the investigations examining the aggregation capability in heterotrophic nitrifying and aerobic denitrifying bacteria are rare. Only Klebsiella pneumoniae CF-S9 strain (Padhi et al., 2013) was examined to have the ability to form cell flocs in the presence of ammonia, nitrite and nitrate, but the changes of flocculating properties during the whole process were not discussed in detail.

Although a number of heterotrophic nitrifying bacteria have been reported, the heterotrophic nitrifying bacteria with high nitrification and denitrification ability are still urgently required to meet the demand for high-strength ammonium wastewater treatment. Furthermore, understanding the characteristics of these bacteria in detail is essential for maintaining highly efficient treatment. In this study, a novel heterotrophic nitrifying and aerobic denitrifying bacterium was isolated from a high-strength ammonium treatment system in laboratory. The aim of this work was to determine the heterotrophic nitrification characteristics of the isolate under different culture conditions. In addition, the denitrification and possible nitrogen removal pathway under aerobic conditions was investigated. Additionally, the visual aggregation degree, flocculating ability (FA) and relative hydrophobicity (RH) of the strain were determined during the whole cultivation to elucidate the flocculating properties and subsequent biomass enrichment of the isolate for future practical applications.

### 2. Methods

#### 2.1. Medium

The basal medium (BM) used for bacteria isolation and heterotrophic nitrifying study consisted of the following components (per liter): 0.47 g of (NH₄)₂SO₄, 5.49 g of sodium succinate, 50 mL of trace elements solution, and pH 7.5. The trace elements solution contained (per liter): 5 g of K₂HPO₄, 2.5 g of MgSO₄·7H₂O and NaCl, 0.05 g of MnSO₄·4H₂O and FeSO₄·7H₂O. The denitrification medium (DM-1&2) used for nitrate and nitrite reduction studies contained (per liter): 0.72 g KNO₃ (0.49 g NaNO₃), 5.62 g of sodium succinate, 7.9 g Na₂HPO₄·7H₂O, 1.5 g KH₂PO₄, 0.1 g MgSO₄·7H₂O, and 2 mL trace elements solution. The trace element solution contained (g/L): Na₂EDTA, 50.0; ZnSO₄·7H₂O, 2.2; CaCl₂, 5.5; MnCl₂·4H₂O, 5.06; FeSO₄, 5.0; CuSO₄·5H₂O, 1.57 and CoCl₂·6H₂O, 1.60. Solid media were prepared from growth media with the addition of 2% agar. All chemicals were of analytical grade.

#### 2.2. Isolation and identification of bacterial strains

Bacteria were isolated from a laboratory-scale sequencing batch reactor (SBR) with a working volume of 20 L. The SBR was seeded with sludge obtained from a piggery wastewater treatment system (Xi’an, China), and was fed with synthetic wastewater (100 mg/L NH₄–N, 1200 mg/L COD). The reactor was operated with hydraulic retention time 24 h, sludge retention time 20 days, temperature 30°C, dissolved oxygen (DO) 1.5–3.5 mg/L and pH 7.5. After cultivation for about 90 days, the removal percentages of COD, NH₄–N and TN were as high as 93.7%, 91.8% and 78.3%, respectively.

The sludge sample (10 mL) was transferred to sterile 0.9% NaCl solution (90 mL) in 250 mL Erlenmeyer flask and then shaken at 160 rpm to obtain homogeneous suspensions. Gradient dilutions were then performed, and the resultant bacterial suspensions were spread onto agar basal medium plates and incubated at 30°C until visible colonies were formed. Purified isolates were obtained by repeated streaking on fresh agar plates. The isolates were picked and individually tested for nitrification and denitrifying activity. The strain with highest nitrification and denitrifying ability was suspended in 25% glycerol solution and stored at −80°C.

The morphology of the bacterium after 24 h cultivation was examined with a scanning electron microscopy (SEM) (JSM-6510LV, Japan). Fixation, dehydrating and drying methods of biological specimen preparation for SEM were followed according to Hazrin-Chong and Manefield (2012). The 16S rRNA gene of the isolate was PCR amplified using bacterial universal primers F27 (5′-AGAGTTTGATCATGGCTACG-3′) and R1492 (5′-TACGGT–TACC TTTGATCAGACTT-3′) (Huang et al., 2013), and sequenced by Sangon Biotech (shanghai, China) Co., Ltd. The sequence was compared with that of other microorganisms by way of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). A phylogenetic tree was constructed in MEGA 5.0 program using the neighbor-joining method.

#### 2.3. Single-factor experiments to study the influencing factors on the nitrification performance

The heterotrophic nitrification characteristics of the isolated strain were investigated under different culture conditions, including carbon source, C/N ratio, pH, temperature, DO and ammonia concentration. In the carbon source experiments, glucose, sucrose, sodium acetate and sodium citrate were used as the sole carbon source instead of sodium succinate in the BM, respectively. The effect of C/N on ammonia removal was examined by adjusting the ratio to 0, 2, 5, 10 and 15. To determine the effects of pH, incubation temperature and DO on ammonia removal, the initial pH was adjusted to 4.5, 6, 7, 8, 9, 10 and 11 by the addition of 1 mol/L HCl or 1 mol/L NaOH; the temperature was set at 10, 20, 30 and 37°C and the shaking speed was adjusted to 80, 120, 160 and 200 rpm in the BM. For the ammonium load experiments, the initial ammonium concentrations were adjusted to approximately 50, 200, 500 and 1000 mg/L on the basis of N content. All of the above experiments were conducted in triplicate with the inoculation size of 1% (v/v) in 250 mL Erlenmeyer flasks with 100 mL sterile medium, and non-seeded samples experiments were also conducted as controls. Unless the single-factor was adjusted as experimental design, the medium with a constant ammonium concentration (100 mg/L) was incubated at 30°C, pH 7.5, C/N 10 and 160 rpm for 48 h. Samples were taken from the bottles periodically to determine the OD₆₀₀, ammonium nitrogen (NH₄–N), hydroxylamine (NH₂·OH), nitrite nitrogen (NO₂–N), nitrate nitrogen (NO₃–N), total nitrogen (TN) and pH.
2.4. Utilization of hydroxylamine, nitrite and nitrate

To observe the nitrogen removal pathway of the isolate, three nitrification intermediates, hydroxylamine, nitrite and nitrate were used as the sole N-source under the aerobic culture conditions. Hydroxylamine (50 mg/L) was added to BM instead of (NH₄)₂SO₄ as the nitrogen source, nitrite and nitrate were adjusted to 200 mg/L in DM-182. For each compound, the amount of succinate as the carbon source was changed to a C/N ratio of 10. Then, the 1 mL of cell suspension was inoculated into triplicate 250 mL Erlenmeyer flasks with 100 mL sterile medium and was aerobically incubated at 30 °C and 160 rpm. Samples were taken from the bottles periodically to determine the OD₆₀₀, NH₄⁻–N, NH₂OH, NO₂⁻–N, NO₃⁻–N, TN and pH.

2.5. Enzyme assay

Strain YB was harvested after cultivation for 24 h in BM by centri- fugation at 8000 g (4 °C, 15 min), and then resuspended in a potassium phosphate buffer (20 mM, pH 7.4). The bacterial suspensions were lysed using ultrasonication treatment, and cell-free extracts were obtained by centrifugation at 10,000 g, 4 °C for 5 min. Formation of nitrite from nitrate, and reduction of nitrite and NH₄–OH in the reaction mixture was taken as a measure for nitrate reductase (NR), nitrite reductase (NiR) and hydroxylamine oxidase (HAO) activity respectively (Zhao et al., 2010). Protein concentration in the cell-free extract was determined by Lowry method (Lowry et al., 1951). The specific activity (U/mg) was defined as the amount of enzyme that catalyzed the transformation of 1 μmol of the substrate per minute by the amount of protein in mg.

2.6. The flocculation and hydrophobicity tests of the isolate

The 1 mL cell suspension was placed into 100 mL BM and DM-182 in 250 mL flasks for 48 h. The visual aggregation assay was performed as previously described, and the scoring criterion was done for testing auto aggregation of the bacteria (Padhi et al., 2013). The scoring criteria were as follows: 0, no aggregates in suspension; (1) small uniform aggregates in a turbid suspension; (2) easily visible aggregates in a turbid suspension; (3) clearly visible aggregates after settling and leaving a clear supernatant; and (4) large flocs of aggregates after instant settling and leaving a clear supernatant. At the same time, samples were taken from the bottles periodically to determine the FA and RH of the isolate. The FA was determined as the reflocculation ability of the isolate after disruption, as described by Liu et al. (2007), and RH was measured as adherence to hexadecane (Padhi et al., 2013).

2.7. Analytical methods

The growth of the isolate was measured by spectrophotometry at 600 nm (OD₆₀₀). Ammonium, nitrite and nitrate were measured by Nessler’s reagent spectrophotometry, N-(1-naphthalene)diaminoothiole spectrophotometry and ultraviolet spectropho- metric method respectively (APHA, 1998). Hydroxylamine was analyzed according to Prear and Burrell (1955). TN was determined by UV spectrophotometry, and intracellular nitrogen content was calculated by subtracting the TN of inoculated medium following centrifugation (4 °C, 15 min, 4000g) from the TN of non-centrifuged medium.

2.8. Statistical analysis

Results are presented as means ± SD (standard deviation of means). Data were analyzed by one-way ANOVA with Tukey’s HSD test (P < 0.05) using SPSS Statistics 19 (IBM Corporation, Armonk, NY, USA).

3. Results and discussion

3.1. Isolation and identification

Twenty purified strains from the enrichment medium were tested for their capabilities to carry out heterotrophic nitrification and aerobic denitrification. One isolate, named as YB, demonstrated high efficiency for ammonium and nitrate removal in BM and DM-1, and no ammonia removal and growth of the isolate were observed in the absence of organic carbon source. Therefore, the heterotrophic capability of the strain was confirmed. The colony of strain YB in the agar plate was rounded, off-white, salient, smooth surface and with regular edge. Strain YB was Gram-negative and appeared as short rods with a size of 0.4 μm × (1.0–1.4) μm. The partial 16S rRNA sequence of strain YB was determined and deposited in the GenBank database (GenBank ID: # KJ623740). The BLAST results indicated that strain YB was closely related to members of genus Acinetobacter, and showed the highest similarity (99%) to Acinetobacter junii strain WS14. A neighbor-joining phylogenetic tree was constructed based on partial 16S rRNA of the isolate, some members of Acinetobacter and other strains with heterotrophic nitrogen removal capability (Fig. 1). The result further confirmed the identification of strain YB as A. junii. There is currently no report about the heterotrophic nitrification and aerobic denitrification of this species.

3.2. Factors affecting the heterotrophic nitrification performance

3.2.1. Carbon source, temperature, dissolved oxygen and pH

Carbon compounds usually serve as energy and electron sources for heterotrophic bacteria. As shown in Fig. 2a, the ammonium removal and cell growth were significantly affected by different carbon sources (P < 0.05). Among the five carbon sources tested, minimal growth of the strain and the worst nitrification performance were observed in the cases of glucose and sucrose. When succinate, acetate and citrate were used as sole carbon sources, strain YB exhibited efficient ammonium removal ability and growth, where NH₄–N was completely removed in 25, 25 and 36 h, respectively; meanwhile, the OD₆₀₀ reached the peaks at 1.2, 1.35 and 1.1, respectively. By comparison, higher ammonium removal rate and TN removal efficiency were observed in succinate than the other media. Accordingly, sodium succinate was employed in subsequent research.

Fig 2b shows that higher temperature significantly promoted the heterotrophic nitrifying ability of the isolate (P < 0.05). The variation of NH₄–N and OD₆₀₀ were marginal at 10 °C, but as the tem- perature increased from 20 °C to 37 °C, the NH₄–N was completely removed in 9, 25 and 25 h, respectively. The trend of microbial growth was consistent with ammonium removal, which was faster with the rising of temperature, but a longer lag phase was required for the growth at lower temperatures. The ammonium removal characteristics of strain YB at 20, 30 and 37 °C were similar to Alcaligenes faecalis No. 4 (Joo et al., 2005) and Pseudomonas stutzeri YZN-001 (Zhang et al., 2011). The increase of NH₄–N removal observed after 24 h at 37 °C was accompanied by a decrease of OD₆₀₀ from 1.2 to 0.8, which was caused by cellular lysis at the decay phase. The excellent heterotrophic nitrification at higher temperatures might be due to the increase of the enzyme activity.

The effect of DO on ammonium removal was controlled by adjusting the rotation speed of the shaker. As shown in Fig. 2c, the higher shaking speed significantly promoted the oxidation of ammonium and cellular growth (P < 0.05). This finding.
contradicted the theory that the heterotrophic nitrifying activity would be inhibited when the DO was lower or higher than the critical DO point (Zhang et al., 2012; Huang et al., 2013), but was consistent with Agrobacterium sp. LAD9 (Chen and Ni, 2012). The NH₄⁺–N was nearly completely removed after 24 h cultivation, except for 80 rpm, where only 54.02% of ammonium was removed after 24 h. NH₄⁺–N was effectively removed both at 160 rpm and 200 rpm, but the improvement of ammonium removal was minimal when the shaking speed was increased from 160 rpm to 200 rpm. The peaks of the logarithmic growth phase cultivated at 160 rpm and 200 rpm appeared at about 24 h simultaneously, but the decline phase at 200 rpm was much earlier than that at 160 rpm.

Fig. 1. The phylogenetic tree derived from neighbor-joining analysis of partial 16S rRNA gene sequence.

Fig. 2. Effect of carbon source (a), temperature (b), shaking speed (c) and pH (d) on ammonium removal and the growth of strain YB. Solid symbols, NH₄⁺–N; Open symbols, OD₆₀₀. Values are means ± SD (error bars) for three replicates.
alkaline condition was harmful to the growth of strain YB. There was almost no OD\textsubscript{600} increase at pH of 4 and 11. The efficient removal of ammonium at the slightly alkaline environment may be caused by more free ammonia contained in the medium, which is used preferentially by Ammonia monooxygenase (Mevel and Prieur, 2000). These pH values were coincidental with the actual wastewater, which is helpful for practical applications.

3.2.2. C/N ratio

The influence of five different C/N ratios on ammonium removal in the BM was investigated in shaking cultures (Fig. 3). Significant differences were observed among C/N ratios of 2–15 (\(P < 0.05\)). The NH\(_4^+\)–N was completely removed at C/N ratio of 10 and 15, but the consumption of NH\(_4^+\)–N stopped at 46.61 mg/L at C/N 2 and 17.88 mg/L at C/N 5. The residual of NH\(_4^+\)–N at low C/N ratios was mainly due to the exhaustion of the carbon source (Yang et al., 2011; Guo et al., 2013). During the tests, the nitrification rate increased with the increasing of C/N ratio. The maximum NH\(_4^+\)–N removal rates at C/N ratios ranging from 2 to 15 were 4.04, 7.25, 8.98, 10.09 mg/L/h, respectively. The growth of the isolate increased with the removal of ammonium, and then leveled off at the termination of NH\(_4^+\)–N removal. The peaks of OD\textsubscript{600} at the four C/N ratios were 0.41, 0.83, 1.36 and 1.34, respectively. Meanwhile, the maximum specific growth rates were calculated to be about 0.24–0.31 h\(^{-1}\) under different C/N ratios. This growth rate was much higher than the rate of 0.03–0.05 h\(^{-1}\) by Nitrosomonas europaea (Gupta and Gupta, 2001), but comparable with other previous reported heterotrophs, e.g., Thiosphaera pantotropha; 0.28–0.45 h\(^{-1}\) (Robertson et al., 1988). Bacillus MS30; 0.3 h\(^{-1}\) (Mevel and Prieur, 2000) and A. faecalis No. 4; 0.2 h\(^{-1}\) (Joo et al., 2005). Furthermore, the nitrogen balance showed that the amount of ammonium consumed due to cell synthesis at higher C/N ratios (54.15% at C/N 10 and 62.55% at C/N 15) were larger than that at lower C/N ratios (41.71% at C/N 2 and 48.37% at C/N 5). The result indicated that cell synthesis was preferentially proceeding at high C/N ratios. This phenomenon was also observed with A. faecalis No. 4 (Joo et al., 2005). During the whole process of the oxidation of ammonium, almost all pH increased from 7.5 to 9.0 under different C/N ratios.

The accumulation of the nitrification intermediates hydroxylamine, nitrite and nitrate was observed throughout the culture period. The formation of nitrite and nitrate was minimal; nitrite was barely detectable and nitrate was only detected at the decline phase of low C/N ratios. As a crucial intermediate, hydroxylamine was detected at the logarithmic phases of all C/N ratios. The accumulation of hydroxylamine appeared earlier and degraded quickly at higher C/N ratios compared with the lower C/N ratios. As the formation of nitrification products was insignificant and removed simultaneously, the pattern of TN removal was consistent with the removal of NH\(_4^+\)–N at all C/N ratios. TN removal efficiencies at C/N ratios ranging from 2 to 15 within 24 h were 53.1%, 82.12%, 98.7% and 99.1%, respectively. The results indicated that strain YB had a high capability for ammonium removal in the presence of high organic load, and C/N ratio higher than 10 was required to achieve efficient heterotrophic nitrification.

3.2.3. Ammonium concentration

The heterotrophic nitrification of strain YB under low (50 mg/L), intermediate (200 mg/L) and high (500 and 1000 mg/L) ammonium concentrations are shown in Fig. 4. The maximum removal efficiencies of NH\(_4^+\)–N under low, intermediate and high ammonium loads were 99.91%, 98.72%, 58.77% and 56.39%, respectively, and the corresponding maximum removal rates were 5.56, 13.35, 17.42 and 24.54 mg/L/h, respectively. The maximum
removal rate of NH₄⁺–N under high ammonium concentrations was equal to that of *A. faecalis* No. 4 (Joo et al., 2005), but much faster than those of *Bacillus subtilis* A1 (Yang et al., 2011) and *Bacillus methylotrophicus* strain L7 (Zhang et al., 2012). The growth of strain YB was accompanied by the removal of ammonium, and the peaks of OD₆₀₀ at four cases were 0.723, 1.81, 1.91 and 1.66, respectively, indicating that strain YB was able to grow at a broad range of ammonium concentrations. The nitrogen balance showed that intracellular nitrogen concentrations accounted for about 52–56% of removed NH₄⁺–N.

Although a desired nitrification rate and cellular growth were observed at high ammonium loads, strain YB could not efficiently remove NH₄⁺–N after a long period of cultivation. This phenomenon might be due to the inhibition of high free ammonia caused by the increased pH, which strongly influenced the activities of autotrophs (Kim et al., 2006). It was observed that the activities of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) were inhibited by free ammonia in the range of 10–150 and 0.1–1.0 mg/L, respectively (Anthonisen et al., 1976). Vadivelu et al. (2007) reported that *Nitrobacter* likely ceased to grow at free ammonia concentrations of 6–9 mg/L. During the oxidation of ammonium, pH continued to increase from 7.10 to 8.45 under high ammonium loads. According to the free ammonia equilibrium relationship from Anthonisen et al. (1976), it was found that the concentrations of free ammonia increased over time and the maximums were 62.33 and 99.22 mg/L at high ammonium loads respectively. Therefore, it could be speculated that the high free ammonia might also severely inhibit the heterotrophic nitrification activity, but not the growth of strain YB.

The formation of nitrification intermediates were observed during the culture period. The accumulation of hydroxylamine was observed at the rapid ammonium removal phase, and then decreased gradually. The maximum NH₂OH concentrations reached in 6, 12, 18 and 18 h, respectively, and the peaks were 2.37, 5.32, 31.08 and 49.09 mg/L under the low, intermediate and high ammonium loads, respectively. It was previously reported that NH₂OH would also accumulate in autotrophic nitrifying cultures (Yang and Alleman, 1992). NH₂OH was found to inhibit ammonia oxidation and have an irreversible inhibitory effect on the growth of NOB (Yang and Alleman, 1992; Xu et al., 2012). Contrary to autotrophic nitrification, the high concentrations of NH₂OH did not severely inhibit the growth and heterotrophic nitrification of YB. The transformation of nitrate was consistent with the change of hydroxylamine, and the maximum concentrations of nitrate were observed at the peaks of hydroxylamine. The accumulation of nitrate might be due to the inhibition of high concentrations of free ammonia, thus causing the decrease of aerobic denitrification activity of strain YB. In contrast to nitrate, no significant nitrite was accumulated in the process of heterotrophic nitrification, and only minimal concentrations of nitrite were detected at high ammonium loads. The low accumulation of nitrite of the isolate was superior to conventional high-strength ammonium wastewater treatment and some other heterotrophic nitrifying bacteria (Joo et al., 2005; Kim et al., 2006; Zhang et al., 2012). The utilization of NH₄⁺–N demonstrated that strain YB was able to tolerate high concentrations of ammonium, free ammonia and hydroxylamine. The high ammonium removal rate and low accumulation of nitrification products in the process of heterotrophic nitrification make this strain a promising candidate for application in the treatment of high-strength ammonium wastewater.

### 3.3 Aerobic metabolic pathway by *A. junii* YB

To clarify the denitrification and possible nitrogen removal pathway by strain YB, three nitrification intermediates, hydroxylamine, nitrite and nitrate were used as the sole N-source under...
the aerobic culture conditions. In many previous studies, hydroxylamine was regarded as a crucial intermediate, which was involved in a novel metabolic pathway of NH\textsubscript{4}\textsuperscript{+}→NH\textsubscript{2}OH→N\textsubscript{2}O→N\textsubscript{2} (Joo et al., 2005; Zhao et al., 2012). This novel metabolic pathway might also contribute to nitrogen removal of the newly isolated strain under aerobic conditions. Fig. 5a shows the result from when hydroxylamine was added as the sole N-source, whereby hydroxylamine decreased significantly within 15 h and then remained stable at 7.5 mg/L. The OD\textsubscript{600} also showed a significant increase, implying that hydroxylamine was able to promote the growth of strain YB. This ability to use hydroxylamine as N-source for growth differed from A. faecalis No. 4 (Joo et al., 2005), Acinetobacter calcoaceticus HNR (Zhao et al., 2010) and A. faecalis strain NR (Zhao et al., 2012), but was consistent with T. pantotropha (Robertson et al., 1988). During the whole process, minimal amounts of nitrite and nitrate were accumulated at the logarithmic growth phase, and the pH increased from 7.51 to 8.45. The analysis of nitrogen balance demonstrated that 48.5% of consumed hydroxylamine was converted to intracellular nitrogen, and 51.5% was speculated to be denitrified.

When 200 mg/L of nitrite and nitrate were added as N-source, a significant decrease of them was observed by 60 h, with 85.67% and 99.05% removal efficiency (Fig. 5b). The maximum removal rate of nitrate and nitrite were 9.92 and 6.17 mg/L/h, respectively. The removal of nitrite and nitrate were observed during the rapid growth phase. Both the OD\textsubscript{600} and intracellular nitrogen reached their peaks at 48 h simultaneously. During the decline growth phase, the OD\textsubscript{600} decreased from 1.6 to 1.0 and the NH\textsubscript{4}\textsuperscript{+} accumulated to about 30 mg/L. The aerobic denitrification ability of strain YB was similar to P. stutzeri YB-001 (Zhang et al., 2011) and K. pneumoniae CF-S9 strain (Padhi et al., 2013). Nonetheless, some other heterotrophic nitrifying bacteria can utilize neither nitrite nor nitrate as the nitrogen source for growth nor as the energy source for denitrification (Joo et al., 2005; Zhao et al., 2010, 2012). During the whole process of the reduction of nitrite and nitrate, pH increased from 7.5 to 9.3. When nitrate was added as the sole N-source, no obvious accumulation of nitrate was found. However, the nitrite increased continuously and accumulated to 50.6 mg/L during the nitrate removal process. The results showed that not only ammonium could be oxidized with trace accumulation of hydroxylamine, nitrite and nitrate, but also hydroxylamine, nitrite and nitrate could be utilized by strain YB under aerobic conditions.

In order to further understand the possible pathway involved in this heterotrophic nitrogen removal process, the activities of three potential enzymes, HAO, NR and NiR, were conducted under aerobic conditions. The specific activities of HAO, NR and NiR were 0.0195, 0.0095 and 0.0386 U/mg proteins, respectively. Enzyme HAO has been proposed to be involved in the oxidation of hydroxylamine to nitrite. The specific activity of HAO was in the same order of magnitude as that of 0.016 U/mg proteins in A. faecalis strain NR (Zhao et al., 2012) and 0.03 U/mg proteins in Acinetobacter sp. Y16 (Huang et al., 2013). NR and NiR are two key enzymes related to denitrification process. Periplasmic NR and cd1-type NiR have been proposed to be responsible for the aerobic conversion of nitrate to nitrite and nitrite to nitrogenous gas, respectively (Richardson and Watmough, 1999). In the present experiment, the NiR activity was comparable to those in other experiment (Huang et al., 2013; Shi et al., 2013). The NR activity was similar to that of K. pneumoniae CF-S9 strain (Padhi et al., 2013), but less than the activities in Acinetobacter sp. Y16 (Huang et al., 2013) and Paracoccus versutus LYM (Shi et al., 2013). By the contrast of HAO and NR, NiR with higher activity could quickly reduce nitrite, which was also reflected by the few accumulations of nitrite in the process of ammonium removal. The presence of HAO, NR and NiR provided additional evidence of heterotrophic nitrification and aerobic denitrification by strain YB.

Based on the utilization of different nitrogen sources and the detection of potential enzymes in this study, the nitrogen removal pathway of strain YB is possibly achieved through heterotrophic nitrification coupled with aerobic denitrification (NH\textsubscript{4}\textsuperscript{+}→NH\textsubscript{2}OH→NO\textsubscript{2}→NO\textsubscript{2}→N\textsubscript{2}O→N\textsubscript{2}). This possible pathway of strain YB is largely consistent with that of some previously reported strains (Chen and Ni, 2012; Huang et al., 2013; Zhang et al., 2012). To date, the nitrogen removal pathway of heterotrophic nitrifying bacteria under aerobic conditions is not fully clear, and further research is still required to elucidate it. The present work has provided some further findings to improve understanding of the nitrogen removal pathway of heterotrophic nitrifying and aerobic denitrifying bacteria.

3.4. The flocculation and hydrophobicity tests of the isolate

The variations of visual aggregation degree, FA and RH within 48 h in BM and DM1&2 are shown in Fig. 6. The auto-aggregation experiment (Fig. 6a) demonstrated that the degree of aggregation
was increased during the cultivation. Small uniform aggregates (score-1) and easily visible aggregates in turbid suspensions (score-2) were observed at 16 h and 48 h in DM1&2, respectively. By contrast, the aggregation ability was higher in BM than DM1&2. Clearly visible aggregates after settling (score-3) were observed in BM after 48 h cultivation. The superior auto-aggregation ability in the BM was different from K. pneumoniae CF-S9 strain (Padhi et al., 2013), in which only small uniform aggregates were observed after 24 h cultivation.

Fig 6b shows that the RH and FA in the BM increased more quickly than DM-1&2, which was similar to the cell growth patterns cultivated under the three different nitrogen sources. The FA reached their peaks at the beginning of cells rapid growth phase, but decreased considerably during the logarithmic phase. The variation in trends of RH was consistent with the bacterial population, which reached the highest points successively when biomass reached the peaks, and then decreased gradually during the decline phase. The peak values of FA of the isolate were 30.94%, 27.24% and 18.28%, whereas the peak values of RH were 68.54%, 81.32% and 83.84% in BM, DM-1 and DM-2, respectively. These RH values were much higher than those observed in K. pneumoniae CF-S9 strain (Padhi et al., 2013). The ability of flocculation and hydrophobicity suggested that the new isolate was efficient in biomass enrichment in practical applications. So far, the capability for aggregation suggested that the strain was efficient in biomass enrichment, and further demonstrated the strain had great potential for practical applications.

4. Conclusions

A. junii YB is a newly isolated bacterium from activated sludge. It was found to exhibit efficient heterotrophic nitrification–aerobic denitrification ability at a wide range of ammonium loads. The preferred conditions for heterotrophic nitrification were succinate as the carbon source, C/N 15, pH 7.5, 37 °C and 200 rpm. Meanwhile, hydroxylamine, nitrite and nitrate could be metabolized by strain YB. The putative nitrogen pathway of the isolate could be heterotrophic nitrification coupled with aerobic denitrification. Moreover, the capability for aggregation suggested that the strain was efficient in biomass enrichment, and further demonstrated the strain had great potential for practical applications.

Acknowledgements

This study is supported by the National Natural Science Foundation of China (Grant No. 50878179), and the Scientific Research Foundation of XAUAT (Grant No. RC0736).

References
