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ORIGINAL PAPER

Heterotrophic nitrogen removal by *Providencia rettgeri* strain YL

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Abstract *Providencia rettgeri* strain YL was found to be efficient in heterotrophic nitrogen removal under aerobic conditions. Maximum removal of NH_4^+–N occurred under the conditions of pH 7 and supplemented with glucose as the carbon source. Inorganic ions such as Mg^{2+}, Mn^{2+}, and Zn^{2+} largely influenced the growth and nitrogen removal efficiency. A quantitative detection of nitrogen gas by gas chromatography was conducted to evaluate the nitrogen removal by strain YL. From the nitrogen balance during heterotrophic growth with 180 mg/l of NH_4^+–N, 44.5% of NH_4^+–N was in the form of N_2 and 49.7% was found in biomass, with only a trace amount of either nitrite or nitrate. The utilization of nitrite and nitrate during the ammonium removal process demonstrated that the nitrogen removal pathway by strain YL was heterotrophic nitrification-aerobic denitrification. A further enzyme assay of nitrate reductase and nitrite reductase activity under the aerobic condition confirmed this nitrogen removal pathway.

Keywords Heterotrophic nitrification-aerobic denitrification · *Providencia rettgeri* · Nitrogen removal · Nitrate reductase · Nitrite reductase

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Introduction

Nitrogen removal by conventional biological means has been widely adopted for wastewater treatment due to its effectiveness and economic benefits. The process is normally carried out by the oxidation of NH_4^+–N into NO_2^-–N, and NO_3^-–N (nitrification) under aerobic conditions by autotrophic nitrifiers and followed the reduction of NO_2^-–N and NO_3^-–N into N_2 (denitrification) under anoxic conditions by heterotrophic denitrifiers. Thus, nitrogen removal in this process is fulfilled either by a spatial separation of aerobic and anoxic in different reactors or by temporal division of the conditions in a sequencing batch reactor [25].

Various processes have been reported periodically based on conventional biological nitrification and denitrification, such as anaerobic ammonium oxidation (Anammox) [22, 28], completely autotrophic nitrogen removal over nitrite (Canon) process [21], oxygen-limited autotrophic nitrification–denitrification (Oland) process [9], and short-cut nitrification and denitrification [15, 20]. Also, nitrogen removal by conversion of ammonium to nitrogen gas by individual heterotrophic bacteria under aerobic conditions is being identified. Moreover, heterotrophic nitrification and aerobic denitrification have usually been accepted as the nitrogen removal pathway by this kind of bacteria. Certain groups of heterotrophic bacteria such as *Pseudomonas stutzeri*, *Thiosphaera pantotropha*, *Alcaligenes faecalis*, *Pseudomonas putida*, *Bacillus* sp., *Comamonas* sp., and *Diaphorobacter* sp. have been shown to possess the capability of simultaneous heterotrophic nitrification and aerobic denitrification [3, 5, 6, 8, 14, 19, 23]. The nitrogen removal completed under aerobic conditions by an individual heterotrophic microorganism was identified here as a single process of heterotrophic nitrogen removal. This discovery may create

treatment processes that do not require redox cycling on complex community structures.

A novel bacterium belonging to the genus *Providencia rettgeri* showed high ammonium removal abilities under various ammonium concentrations in previous study, and the factors (C/N ratios, shaking speeds, temperatures, and ammonium concentrations) affecting the optimum performance of *P. rettgeri* strain YL have been reported [26]. However, the total nitrogen removal (TN) by strain YL needs to be further investigated. The TN removal efficiency through heterotrophic nitrification and aerobic denitrification by strain YL was unclear, and the assumed ammonium removal pathway was not confirmed. Very few reports are available on the nitrogen removal capability of genus *Providencia*. Considering the rarity of this genus in the biologic nitrogen removal process, this strain merits detailed study. In this study, further work was conducted to investigate the TN removal efficiency by strain YL under the aerobic condition. Also, the suggested ammonium removal pathway of heterotrophic nitrification-aerobic denitrification in previous research [26] was proved by intermediates investigation and enzyme assay under aerobic conditions.

Materials and methods

Microorganism

Providencia rettgeri strain YL was stocked in 25% glycerol solution at −80°C [26].

Basal medium

A basal medium (BM) in Taylor's research [26] was used in the present study and modified as follows (per liter): NH_4Cl, 0.31 g; glucose, 2 g; NaCl, 4 g; Na_2HPO_4, 2.66 g; KH_2PO_4, 1 g; trace elements solution, 3 ml. The trace elements solution contained (g/l): $MgSO_4{\cdot}7H_2O$, 3; $MnSO_4$, 3; $ZnSO_4{\cdot}7H_2O$, 3; H_3BO_3, 1.12; $FeSO_4{\cdot}7H_2O$, 0.3; $CaCl_2{\cdot}2H_2O$, 0.6. BM was autoclaved for 15 min at 121°C. The chemicals were purchased from China National Medicines Corporation Ltd. (Beijing, China).

Phylogenetic tree construction

The partial 16S rRNA sequences of *P. rettgeri* strain YL and type strains with heterotrophic nitrogen removal capability from other genera were used for phylogenetic tree construction. All sequences were aligned using the "Clustal X 1.18" program [27]. Thereafter, a phylogenetic tree was built in MEGA software version 4 using the neighbor-joining method [10]. The robustness of the tree was determined by bootstrap test with 1,000 replications [4].

Shake flask experiments

In flask experiments to study the growth and TN removal efficiency of strain YL at different organic carbon substrates, sucrose, citrate, acetate, and L-malate were used instead of glucose in BM. The medium without any organic carbon substrate was used as a control. To observe the growth and nitrogen removal efficiency of strain YL at different pH values, pH value was adjusted to 5, 6, 7, and 8 in BM, respectively. Fe^{3+}, Fe^{2+}, Ca^{2+}, BO_3^{3-}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (10 mg/l) were added to BM, respectively, instead of the trace elements solution to study the growth and TN removal efficiency by strain YL at different inorganic ions. The medium without addition of inorganic ions was used as a control. The bacterial suspension of strain YL was prepared by the method in Taylor's research [26]. In each BM (100 ml) for different experiments, a bacterial suspension (1 ml) of strain YL was inoculated and incubated at 30°C and 120 rpm for 72 h. Samples were drawn at regular intervals, and the growth and TN removal rate were estimated.

Aerated batch culture

Nitrogen balance and TN removal efficiency through heterotrophic nitrification–aerobic denitrification of strain YL were tested in a flask (1.2 l). Strain YL was inoculated into BM (600 ml) in which the NH_4^+–N concentration was changed to 180 mg/l. The flask was tightly sealed with a rubber septum and fully aerated with O_2–He (95:5) gas. The flask was incubated at 30°C and 120 rpm for 96 h. The system without the addition of strain YL was used as a control. Samples (2 ml) were drawn periodically to determine cell optical density (OD_{600}) and then centrifuged (4°C, 15 min, 3,600×*g*) to obtain supernatant for the determination of ammonium, hydroxylamine, nitrite, nitrate, TN, and TOC (total organic carbon). Meanwhile, gas samples (50 µl) were collected periodically using a gas-tight syringe to detect N_2, N_2O, and O_2 by gas chromatography.

Utilization of nitrite and nitrate

To observe the nitrogen removal pathway of strain YL, a bacterial suspension (1 ml) of strain YL and BM (100 ml) was incubated in the flasks (250 ml) at 30°C and 120 rpm for 48 h. Nitrite or nitrate (10 mg/l) was added to BM after 6 h of cultivation. The medium without the addition of nitrite or nitrate was used as a control. Cultures were sampled at regular intervals for chemical analysis.

Enzyme assay

Cells, for preparation of extracts, were harvested from BM (2 l) after 24 h of cultivation at 30°C. Cells were centrifuged

(4°C, 15 min, 3,600×*g*) and then suspended in a 0.01 M potassium phosphate buffer (pH = 7.4). Cell-free extracts were obtained by subjecting the bacterial suspensions to lysis by ultrasonication and then centrifuged (4°C, 10 min, 11,190×*g*) to separate whole cells and cell debris. The supernatant fraction was concentrated by fractional precipitation with $(NH_4)_2SO_4$ and dialysis to harvest the concentrated cell-free crude extracts. For assay of nitrate reductase (NR) activity, a reaction mixture (20 ml) containing enzyme extract, 0.2 mM NADH, and 10 mM potassium phosphate buffer (pH = 7.4) was prepared, and the reaction was stated by addition of $NaNO_3$. The reaction mixture was incubated at 30°C for 15 min, and the disappearance of NO_3^-–N from the reaction mixture in presence of enzyme extract was considered as a measure for NR activity. Similarly, when $NaNO_2$ was added to the reaction mixture instead of $NaNO_3$, the disappearance of NO_2^-–N from the reaction mixture in presence of enzyme extract was considered as a measure for nitrite reductase (NiR) activity. Protein concentration in the crude extracts was determined by the Bradford Reagent Kit (Sangon, Shanghai). One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the transformation of 1 μmol of substrate per minute. The specific activity (U/mg) was defined as the amount of enzyme units divided by the concentration of protein in mg.

Analytical methods

OD was determined by a spectrophotometer (uv-2800, Unico, Dayton, NJ) at a wavelength of 600 nm. Intracellular nitrogen was calculated from the relationship between nitrogen content in dry biomass and OD_{600}. The nitrogen content in dry biomass was obtained using an element analyzer (Vario EL III, Elementar, Germany). The dry biomass was prepared by drying the bacteria at 105°C overnight. Ammonium, hydroxylamine, nitrite, nitrate, TN, and TOC were determined using the same methods in Taylor's research [26]. Organic N in the medium was calculated by subtracting NH_4^+–N, hydroxylamine, nitrite, and nitrate from TN. N_2O was analyzed using a gas chromatography (6890N, Agilent, USA) with an electron capture detector (ECD) and a column (30 m × 0.32 mm × 20 μm) packed with Plot-Q. The column, injector, and detector temperatures were 40, 100, and 300°C, respectively. The carrier gas was N_2 at a flow rate of 30 ml/min. N_2 and O_2 were assayed by a gas chromatograph (GC-14B, Shimadzu, Japan) equipped with a thermal conductivity detector (TCD) and a 5A molecular sieve column (2 m × 0.22 mm × 25 μm). The carrier gas was Ar with a flow rate of 20 ml/min. Temperatures were as follows: column, 60°C; injector, 100°C; detector, 120°C. Morphological characteristic of the strain was observed by scanning electron microscopy (SEM) (JSM-5610LV, Jeol, Japan) after 12 h of incubation. Cell lysis was accomplished by an ultrasonic cell disruptor (JY92-2D, Scientz, China).

Results

Morphology of strain YL

The colony of strain YL on the plate was yellowish white, translucent, round, salient, had a smooth surface and regular edge, and its diameter was about 2 mm. In the early research, strain YL was considered to be non-motile [26]. However, by repeating the SEM, strain YL appeared as short rods (approximately 1.0∼1.3 × 0.4∼0.5 μm) with flagella. The flagella of strain YL can be observed clearly in Fig. 1. The observation of flagella in the present experiment might be due to the improvement of operation for SEM.

Phylogenetic relationship between *P. rettgeri* strain YL and other genera with heterotrophic nitrogen removal

A neighbor-joining phylogenetic tree based on partial 16S rRNA sequences was constructed. Partial sequences of *P. rettgeri* strain YL and type strains from other groups with heterotrophic nitrogen removal capability were included (Fig. 2). The tree showed a clear evolutionary divergence of *P. rettgeri* strain YL from type strains with nitrogen removal capability belonging to other genera. The results also suggest that *Pseudomonas*, *Acinetobacter*, and *Providencia* may have relatively close phylogenetic relationship comparing to *Paracoccus denitrificans*, *Bacillus*, *Rhodococcus*, and *Arthrobacter*. The continuing discovery of heterotrophic nitrogen removal in different genera indicates the extensive of heterotrophic nitrogen removal in the

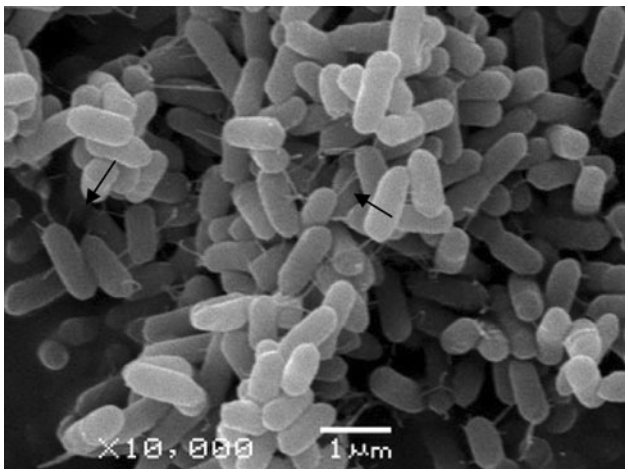

Fig. 1 Scanning electron micrograph of *P. rettgeri* strain YL after 12 h of cultivation

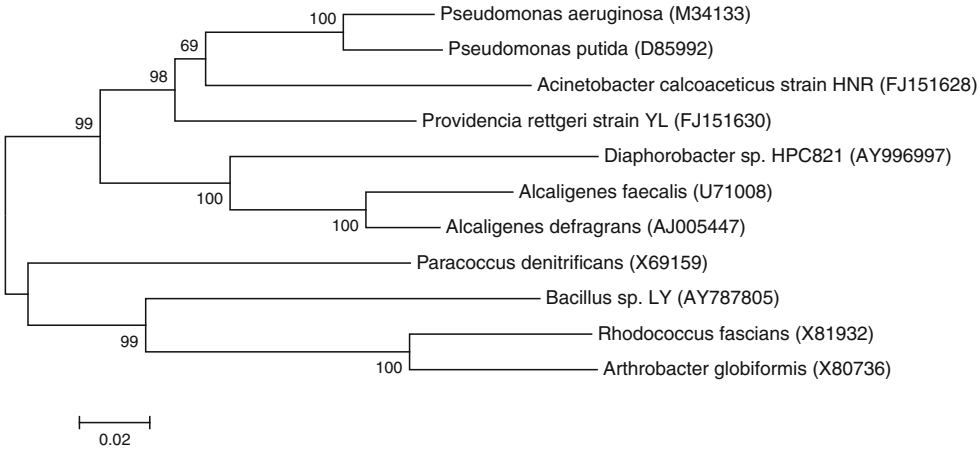

Fig. 2 Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences showing phylogenetic relationships between *P. rettgeri* strain YL and heterotrophic nitrogen removal strains from other groups. Names of the different groups along with the accession numbers are shown in the parentheses. Bootstrap values (1,000 replications) are indicated at the interior branches

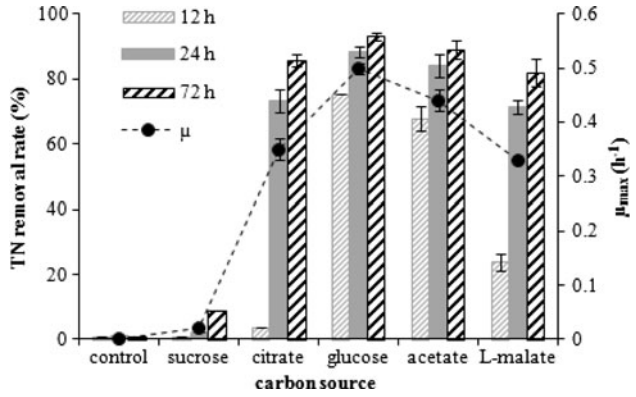

Fig. 3 Effects of various carbon sources on the growth and nitrogen removal by *P. rettgeri* strain YL. *Error bars* mean ± SD of two replicates

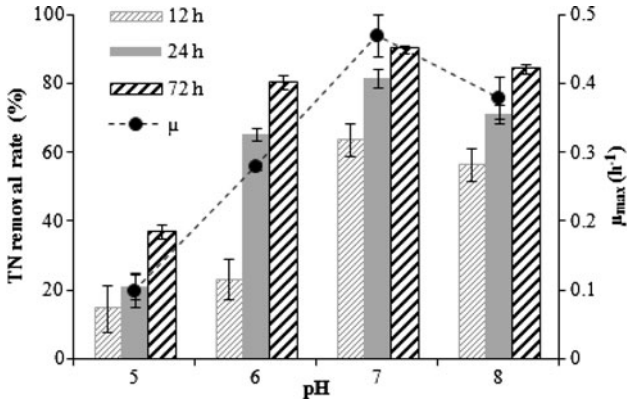

Fig. 4 Effects of various pH on the growth and nitrogen removal by *P. rettgeri* strain YL. *Error bars* mean ± SD of two replicates

nature. The reports on genus *Providencia* for nitrogen removal were quite rare. The 16S rRNA sequence of strain YL was deposited into the NCBI GenBank with an accession number of FJ151630.

Heterotrophic nitrogen removal by *P. rettgeri* strain YL at different organic carbon sources

Flask experiments for the study on growth and nitrogen removal capability of strain YL at various carbon sources (sucrose, citrate, glucose, acetate, and L-malate) were conducted (Fig. 3). In the control group, strain YL did not grow, and TN removal was not detected. In the case of sucrose, the growth and TN removal rates were extremely low. When L-malate or citrate was provided as the sole carbon source, TN removal rates were limited in the first 12 h. When glucose and acetate were provided as the sole carbon source, respectively, the nitrogen removal patterns were similar. The maximum growth and TN removal rate were found in BM supplemented with glucose followed by acetate.

Heterotrophic nitrogen removal by *P. rettgeri* strain YL at different pH values

The growth and nitrogen removal of strain YL at various pH values were investigated in BM (Fig. 4). The maximum growth and TN removal were observed in pH 7 followed by pH 8. In the case of pH 6 and 5, the growth was less and less, indicating the inhibition of growth under acidic conditions. Although a relatively high TN removal efficiency was still obtained in pH 6 at 72 h, the TN removal efficiency was much lower at 12 h than that in pH 7 and 8. The TN removal efficiency in pH 5 was low when compared to pH 7.

Heterotrophic nitrogen removal by *P. rettgeri* strain YL at different inorganic ions

As shown in Table 1, in the absence of inorganic ions (control), the growth and nitrogen removal rate of strain YL were extremely low. However, 10 mg/l inorganic ions of Zn^{2+}, Mg^{2+}, and Mn^{2+} largely stimulated the growth and

Table 1 Effects of various ions on the growth and nitrogen removal by *P. rettgeri* strain YL

Measurement	Control	Fe^{3+}	Fe^{2+}	Ca^{2+}	BO_3^{3-}	Mg^{2+}	Mn^{2+}	Zn^{2+}
μ_{max} (h^{-1})	0.16 ± 0.02	0.17 ± 0.03	0.21 ± 0.01	0.23 ± 0.01	0.28 ± 0.01	0.55 ± 0	0.47 ± 0.01	0.46 ± 0.01
%TN removal	20.1 ± 1.5	21.4 ± 0.5	26.8 ± 0.8	22.5 ± 1.2	23.4 ± 0.8	86.8 ± 1.8	78.9 ± 1.5	69.9 ± 2.0

Values represent mean ± SD of two replicates

TN removal rate of strain YL. In the case of Fe^{2+}, the growth and TN removal rate were slightly stimulated when compared to the control. However, no significant stimulation of either the growth or the TN removal rate was observed when Fe^{3+} was added. Ca^{2+} and BO_3^{3-} had some effects only on the growth of strain YL, indicating that Ca^{2+} and BO_3^- only related to the assimilation of NH_4^+–N.

Nitrogen balance during heterotrophic nitrogen removal process by *P. rettgeri* strain YL

An aerated batch experiment to supply a sufficient amount of oxygen was carried out to calculate the nitrogen balance and evaluate the nitrogen removal efficiency. In the control group (without the addition of strain YL), the concentration of NH_4^+–N was almost stable, indicating that no additional bacteria existed in this system to decrease the NH_4^+–N (data not shown). When strain YL was added to the system, TN decreased dramatically, and 97.8% of high TN removal efficiency was obtained in 96 h. The corresponding conversion of nitrogen compounds in BM during heterotrophic growth is shown in Fig. 5a. NH_4^+–N decreased dramatically and reached a maximum removal rate of 18.7 mg-N/l/h. All the NH_4^+–N was removed in 96 h, in which 49.7% of NH_4^+–N was assimilated to intracellular N as biomass. The accumulation of hydroxylamine followed the decrease of NH_4^+–N, but was extremely low, with only a maximum of 2.1 mg/l at 12 h, and then decreased. The concentration of nitrite was close to the detection limit. Nitrate appeared to accumulate after 12 h and reached a maximum of 13.2 mg/l at 48 h, then decreased. The conversion of nitrogen compounds in BM indicated the occurrence of the nitrification process and the utilization of nitrification products by strain YL. Meanwhile, N_2 kept increasing with the decrease of NH_4^+–N and was basically stable in 96 h in the headspace (Fig. 5b), while N_2O was hard to detect. During the experimental period, a high concentration of O_2 always remained in the headspace. The results revealed that aerobic denitrification occurred, and N_2 was the final product during the nitrogen removal process by strain YL as well as intracellular N.

The nitrogen balance during the nitrogen removal process by *P. rettgeri* YL is tabulated in Table 2. NH_4^+–N was the sole N-source at the initial time. Finally, NH_4^+–N decreased to zero, and the increase in hydroxylamine, nitrate, organic N (mainly released from the bacterial decay or extracellular materials), intracellular N, and N_2 was observed. Comparing the initial and final total N mass, only 3.6% of initial N was lost. Considering the measurement errors, the nitrogen was balanced. From the nitrogen balance, 44.5% of NH_4^+–N was removed in the form of N_2, and 49.7% of NH_4^+–N was found in biomass. Only a trace amount of NH_4^+–N remained in the culture in the formations of hydroxylamine, nitrate, and organic N.

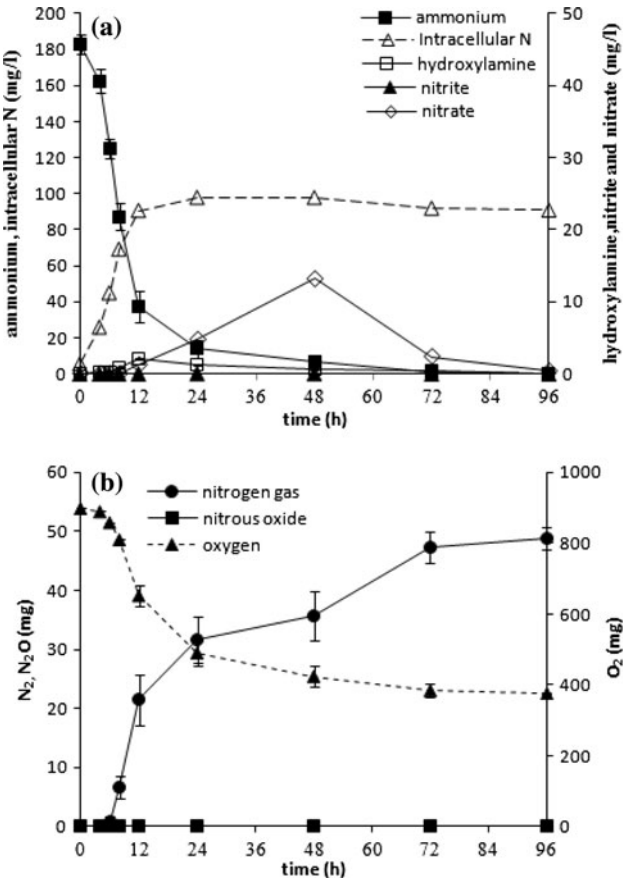

Fig. 5 Changes in nitrogen compounds in BM (**a**) and gaseous constituents in the headspace (**b**) during aerated batch culture of *P. rettgeri* strain YL. The unit in **b** refers to the total mass of O_2, N_2O, and N_2 in the gas phase volume. *Error bars* mean ± SD of two replicates

Utilization of nitrite and nitrate during heterotrophic nitrogen removal by *P. rettgeri* strain YL

In Fig. 5, heterotrophic nitrification-aerobic denitrification was found to contribute to the ammonium removal. To further

Table 2 Nitrogen balance during the nitrogen removal process by *P. rettgeri* YL (unit: mg)

Initial amt of N	Final amt of N							N lost[a]
	NH_4^+–N	NH_2OH–N	NO_2^-–N	NO_3^-–N	Organic N	Intracellular N	N_2	
109.6 ± 3.0	0 ± 0	0.1 ± 0	0 ± 0	0.2 ± 0	2.1 ± 0.6	54.5 ± 0.5	48.8 ± 1.9	3.6%

[a] % N lost = 100 × (initial amt of N − final amt of N)/initial amt of N

Values represent mean ± SD of two replicates

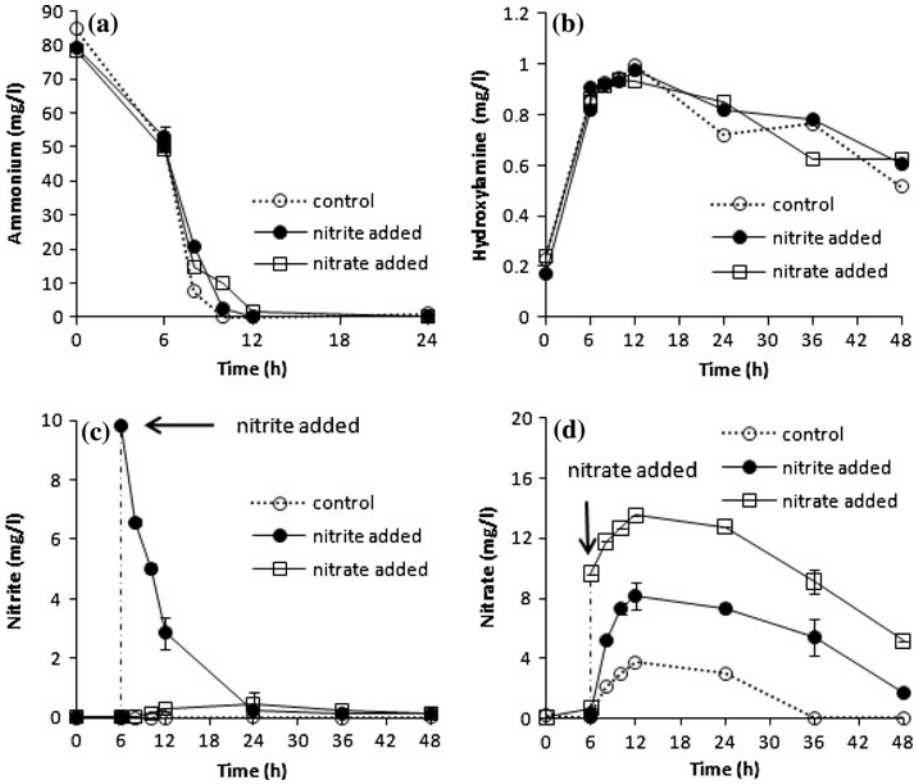

Fig. 6 Changes in ammonium (**a**), hydroxylamine (**b**), nitrite (**c**), and nitrate (**d**) in control group, nitrite added group, and nitrate added group. *Error bars* mean ± SD of two replicates

confirm that aerobic denitrification was involved in the nitrogen removal pathway, nitrite or nitrate (10 mg/l) was added to the ammonium removal process in BM under the aerobic condition after 6 h of cultivation (Fig. 6). Ammonium as the sole nitrogen source was used as a control group. In the control group, there was little accumulation of nitrite. The accumulation of nitrate was not very high, only 3.76 mg/l, and then reduced to zero at 36 h. In the nitrite and nitrate added group, the ammonium removal rates both decreased a little compared to the control group, indicating an inhibition of ammonium removal when an extra nitrite or nitrate was supplied. This inhibition might be related to the high oxidation state of N in nitrite and nitrate. The variation of hydroxylamine in each group was almost similar. When nitrite was added to the ammonium removal process, the added nitrite decreased and transferred to nitrate rapidly, and thus resulted in a high accumulation of nitrate (8.15 mg/l) at 12 h (Fig. 6c, d). However, the accumulated nitrate decreased gradually after 12 h. When nitrate was added to the ammonium removal process, the concentration of nitrate kept increasing because of nitrification and reached a concentration of 13.54 mg/l at 12 h, and then reduced after 12 h (Fig. 6d). Meanwhile, a slight increase in nitrite was observed (Fig. 6c). From these results, both nitrite and nitrate could be reduced by stain YL under aerobic conditions during the ammonium removal process. Moreover, nitrite could be used by strain YL in a more efficient way. Thus, aerobic denitrification indeed occurred during the ammonium removal process by strain YL.

Enzyme assay

A preliminary study on enzyme activities of NR and NiR of strain YL was conducted under the aerobic condition. The enzyme activity of NR was detected as 0.13 U with a specific activity of 0.031 U/mg protein. An activity of NR was also observed in strain HPC 856 with a specific activity of

0.075 U/mg protein, which was in the same order of magnitude as the result in current experiments [6]. Strain HPC 856 was reported as a heterotrophic bacterium belonging to *Diaphorobacter* sp. with the capability of simultaneous nitrification and denitrification. A 0.15 U activity of NiR in strain YL was observed with a specific activity of 0.041 U/mg protein, which was slightly higher than that of NR.

Discussion

Heterotrophic nitrogen removal was observed by strain YL at different levels when glucose, acetate, sucrose, citrate, and L-malate were used as organic carbon substrate, respectively. The maximum growth and TN removal rate by strain YL were obtained in BM supplemented with glucose. This was consistent with the early research in which glucose was used as the carbon source [26]. The reason why glucose exhibits the highest nitrogen removal rate in our experiment may be explained in that a monosaccharide is more easily utilized by our bacteria and the reducibility of aldehyde (–CHO) in glucose stimulates the reduction process of aerobic denitrification. Glucose was also used in the experiment of aerobic nitrification-denitrification by *Bacillus* sp. [8]. Moreover, acetate also exhibited a remarkable utilization by strain YL. Acetate was commonly used as an organic substrate for heterotrophic nitrification and aerobic denitrification by some heterotrophic microorganisms such as *Pseudomonas* sp. AS-1 and *Thiosphaera pantotropha* [19, 24]. However, the suitable carbon source for *Alcaligenes faecalis* no. 4 was citrate compared to acetate [5]. Thus, the utilization of carbon sources in different heterotrophic microorganisms was different.

It was obvious that the growth and nitrogen removal rate of strain YL were much higher in neutral and faintly alkaline conditions, while they were repressed under acidic conditions. The optimum pH for nitrogen removal was 7 by strain YL. Similar pH was used in some *Diaphorobacter* bacteria with nitrogen removal capability [6]. The faintly alkaline condition of pH 7.5 was also suitable for a thermophilic heterotrophic bacterium of *Bacillus* MS30 to achieve the maximum heterotrophic nitrification rate [11]. In another consortium of *Pseudomonas* sp. KW1 and *Bacillus* sp. YW4, the maximum of nitrate reduction was also observed at pH 7 [16]. The pH used in another heterotrophic bacterium of *Thiosphaera pantotropha* with nitrogen removal capability was 8 [19]. Thus, neutral and faintly alkaline conditions were more suitable for heterotrophic bacteria.

The inorganic ions experiment reveals that the addition of inorganic ions is necessary for the growth and nitrogen removal of strain YL. Remarkably, the addition of inorganic ions of Zn^{2+}, Mg^{2+}, and Mn^{2+} largely catalyzed the growth and nitrogen removal rate of strain YL. Fe^{2+} also slightly stimulated the growth and TN removal rate of strain YL. These phenomena can be explained in that Mg^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+} may be closely involved in the enzyme system of nitrogen removal process of strain YL. Mg^{2+} was known to be necessary to the ammonia oxygenase activity in the heterotrophic nitrifier of *Thiosphaera pantotropha* [18]. In Kim's research, Mg^{2+} also influenced the growth of *bacillus* PK15 and removal of NH_4^+–N, and Mn^{2+} significantly influenced the growth of PK15. However, Zn^{2+} did not show any effect on either the growth or the removal of ammonium of strain PK15, which was different from the effect of Zn^{2+} on strain YL [8]. Fe^{2+} was reported to be found in nitric-oxide reductase [13].

During the nitrogen removal process in Fig. 5, the production and conversion of nitrate and the simultaneous increase in N_2 indicated the nitrogen removal process of simultaneous heterotrophic nitrification and aerobic denitrification by strain YL. This was consistent with the assumption of a nitrogen removal pathway in early research [26]. The utilization of added nitrite and nitrate during the ammonium removal process by strain YL in Fig. 6 further demonstrates that the nitrogen removal is achieved by combining heterotrophic nitrification and the aerobic denitrification pathway, which is $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$ and simultaneous $NO_3^- \rightarrow NO_2^- \rightarrow N_2$. A study on enzyme activity of strain YL also showed that both NR and NiR activity were present in strain of YL. It is well known that NR and NiR are related to the denitrification process. Moreover, a periplasmic NR has been involved in the conversion of nitrate to nitrite under aerobic conditions [1, 2, 17]. A *cd*1-type NiR has also been reported to be related to the aerobic denitrification of nitrite by *Thiosphaera pantotropha* [12]. Thus, this enzyme assay provided added evidence of aerobic denitrification for nitrogen removal in our research. According to the enzyme assay, the NiR activity of strain YL was a little higher than the NR activity. This result was consistent with the result in Fig. 6 in which the added nitrite was reduced more efficiently than that of nitrate. These results well explained the phenomenon in Fig. 5a in which nitrate was accumulated in the nitrogen removal process while nitrite was hard to detect. Further study on enzymes is required to understand the pathway of strain YL comprehensively.

A high TN removal rate was obtained by strain YL under aerobic conditions during heterotrophic growth with 180 mg/l of NH_4^+–N. As discussed above, heterotrophic nitrification and aerobic denitrification were responsible for TN removal. The nitrogen balance revealed that assimilation also contributed to TN removal as well as N_2 production. For strain YL, about 44.5% of NH_4^+–N was removed through heterotrophic nitrification and aerobic denitrification, while 49.7% was converted to biomass. In

a culture of mixed *Bacillus* strains, about 33% of the N source was estimated to be removed by aerobic nitrification–denitrification [8]. In Joo's report, 40–50% of ammonium was denitrified by *Alcaligenes faecalis* no. 4 under aerobic conditions [5]. More than half of NH_4^+–N was estimated to be removed in the form of N_2 by *Pseudomonas alcaligenes* aerobically [24]. Thus, heterotrophic nitrification-aerobic denitrification made a significant contribution to nitrogen removal. On the other hand, it is hard to avoid a high assimilation ratio in heterotrophic nitrifiers. However, this large heterotrophic biomass may offset the low nitrification per unit of biomass [7]. Very few reports are available on the nitrogen removal capability of genus *P. rettgeri*. Considering the rarity of this genus having nitrogen removal capability, our study expands the variety of genera capable of nitrogen removal under aerobic conditions.

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References

1. Bedzyk L, Wang T, Ye RW (1999) The periplasmic nitrate reductase in *Pseudomonas* sp. strain G-179 catalyzes the first step of denitrification. J Bacteriol 181:2802–2806
2. Bell LC, Richardson DJ, Ferguson SJ (1990) Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*. The periplasmic enzyme catalyzes the first step in aerobic denitrification. FEBS Lett 265:85–87
3. Daum M, Zimmer W, Papen H, Kloos K, Nawrath K, Bothe H (1998) Physiological and molecular biological characterization of ammonia oxidation of the heterotrophic nitrifier *Pseudomonas putida*. Curr Microbiol 37:281–288
4. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791
5. Joo HS, Hirai M, Shoda M (2005) Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. J Biosci Bioeng 100:184–191
6. Khardenavis AA, Kapley A, Purohit HJ (2007) Simultaneous nitrification and denitrification by diverse *Diaphorobacter* sp. Appl Microbiol Biotechnol 77:403–409
7. Killham K (1986) Heterotrophic nitrification. In: Prosser JI (ed) Nitrification. IRL Press, Oxford, pp 117–126
8. Kim JK, Park KJ, Cho KS, Nam SW, Park TJ, Bajpai R (2005) Aerobic nitrification–denitrification by heterotrophic *Bacillus* strains. Bioresour Technol 96:1897–1906
9. Kuai L, Verstraete W (1998) Ammonium removal by the oxygen limited auto-trophic nitrification–denitrification (oland) system. Appl Environ Microbiol 64:4500–4506
10. Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequence. Brief Bioinform 9:299–306
11. Mevel G, Prieur D (2000) Heterotrophic nitrification by a thermophilic *Bacillus* species as influenced by different culture conditions. Can J Microbiol 46:465–473
12. Moir JWB, Baratta D, Richardson DJ, Ferguson SJ (1993) The purification of a cd1-type nitrite reductase from, and the absence of a copper-type nitrite reductase from, the aerobic denitrifier *Thiosphaera pantotropha*; the role of pseudoazurin as an electron donor. Eur J Biochem 212:377–385
13. Palmer T (1995) Understanding enzymes, 4th edn. Prentice Hall, London, pp 202–206
14. Patureau D, Bernet N, Moletta R (1997) Combined nitrification and denitrification in a single aerated reactor using the aerobic denitrifier *Commonas* sp. strain SGLY2. Water Res 31:1363–1370
15. Peng Y, Zhu G (2006) Biological nitrogen removal with nitrification and denitrification via nitrite pathway. Appl Environ Microbiol 73:15–26
16. Rajakumar S, Ayyasamy PM, Shanthi K, Thavamani P, Velmurugan P, Song YC, Lakshmanaperumalsamy P (2008) Nitrate removal efficiency of bacterial consortium (*Pseudomonas* sp. KW1 and *Bacillus* sp. YW4) in synthetic nitrate-rich water. J Hazard Mater 157:553–563
17. Richardson DJ, Ferguson SJ (1992) The influence of carbon substrate on the activity of the periplasmic nitrate reductase in aerobically grown *Thiosphaeva pantotropha*. Arch Microbiol 157:535–537
18. Robertson LA, Kuenen JG (1988) Heterotrophic nitrification in *Thiosphaera pantotropha*: oxygen uptake and enzyme studies. J Gen Microbiol 134:857–863
19. Robertson LA, Van Neil EWJ, Torremans RAM, Kuenen JG (1988) Simultaneous nitrification and denitrification in aerobic chemostat at cultures of *Thiosphaera pantotropha*. Appl Environ Microbiol 54:2812–2818
20. Ruiz G, Jeison D, Chamy R (2003) Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration. Water Res 37:1371–1377
21. Sliekers AO, Derwort N, Strous M, Kuenen JG, Jetten MSM (2002) Completely autotrophic nitrogen removal over nitrite in one single reactor. Water Res 36:2475–2482
22. Strous M, Van Gerven E, Zheng P, Kuenen JG, Jetten MSM (1997) Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (ANAMMOX) process in different reaction configurations. Water Res 31:1955–1962
23. Su JJ, Liu BY, Liu CY (2001) Comparison of aerobic denitrification under high oxygen atmosphere by *Thiosphaera pantotropha* ATCC 35512 and *Pseudomonas stutzeri* SU2 newly isolated from the activated sludge of a piggery wastewater treatment system. J Appl Microbiol 90:457–462
24. Su JJ, Yeh KS, Tseng PW (2006) A strain of *Pseudomonas* sp. isolated from piggery wastewater treatment systems with heterotrophic nitrification capability in Taiwan. Curr Microbiol 53:77–81
25. Szewczyk KW, Kuroń-Kulig A (2007) Simultaneous nitrification and denitrification in a new air-lift reactor. J Biotechnol 131:S148
26. Taylor SM, He YL, Zhao B, Huang J (2009) Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Providencia rettgeri* YL. J Environ Sci 21:336–1341
27. Thompson JD, Gibson TJ, Plewniak F (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882
28. van de Graaf AA, Mulder A, Slijkhuis H, Robertson LA, Kuenen JG (1990) Anozic ammonium oxidation. In: Proceedings of the 5th European congress on biotechnology, vol I, Copenhagen, Denmark, 8–13 July