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Isolation and identification of cold-tolerance aerobic denitrifier *Pseudomonas plecoglossicida* Y-1

Shu-di Xiang, Zhen-lun Li*, Qing Ye, Teng-xia He, Yi Xu, Xue-jiao Huang, Mi Feng

Chongqing Key Laboratory of Soil Multiscale Interfacial Progress, College of Resource and Environments, Southwest University, Chongqing 400715, China, emails: lizhlun4740@sina.com (Z.-l. Li), xiangshudi123@163.com (S.-d. Xiang), yq1992xd@email.swu.edu.cn (Q. Ye), hetengxia5153@163.com (T.-x. He), xy1992xd@163.com (Y. Xu), 1548217611@qq.com (X.-j. Huang), 183773603@qq.com (M. Feng)

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ABSTRACT

Aerobic denitrification is one of the main biological methods for removing nitrate–nitrogen from water. At present, the optimum temperature for most aerobic denitrifying bacteria is between 25°C and 35°C, and there are few reports regarding the performances of nitrate aerobic denitrifiers at low temperatures. In this experiment, we isolated an aerobic denitrifier Y-1 that can effectively remove nitrate–nitrogen at 15°C and has strong alkali resistance. Characterizing this strain revealed that the bacterium is *Pseudomonas plecoglossicida* and this is the first report on the aerobic denitrification of this bacterium. The optimal denitrification conditions for strain Y-1 were: temperature 15°C, pH 7, shaking speed 150 rpm, C/N 15, inoculation amount 1.5×10^8 CFU, and the most suitable carbon source was glucose. At pH 11 or 12, strain Y-1 could still remove nitrate–nitrogen effectively. Furthermore, the removal efficiency and transformation pathways of single nitrogen sources (NO_3^-–N, NO_2^-–N, and NH_4^+–N) by strain Y-1 were explored revealing that strain Y-1 can effectively reduce nitrate–nitrogen by denitrification but cannot use high concentrations of nitrite nitrogen, Interestingly, the removal of ammonium nitrogen is achieved by the assimilating ammonia. Strain *P. plecoglossicida* Y-1 has application value in the treatment of low temperature and alkaline wastewater.

Keywords: Low temperature; Aerobic; Alkali resistance; Denitrification; Ammonia assimilation

1. Introduction

Nitrogen is an important factor to lead to eutrophication of water bodies, and nitrate nitrogen is one of the important sources of nitrogen pollution. Nitrate nitrogen is the most stable form of nitrogenous compounds in aerobic environments and is the final product of the decomposition of the nitrogenous organic compound during an organization. Currently, global concentrations of nitrate–nitrogen in drinking water and surface water have exceeded safety levels. As early as the 1960 s, the World Health Organization revealed that nitrate pollution in surface water is caused by the overuse of nitrogen fertilizers in Europe and the United States. With the development of the industrial economy, in some western European countries, the concentration of nitrate in surface water was as high as 40–50 mg N/L [1]. Nitrate pollution is very serious in the northern China cities [2], such as Changchun and Xi'an, where the nitrate–nitrogen content in surface water can reach several hundred milligrams per litre [3,4]. Although high-concentration of nitrate–nitrogen does not directly harm humans and animals, it easily converts into toxic nitrite nitrogen under hypoxic conditions, thus indirectly threatening the health of many creatures. Also, a high concentration of nitrate–nitrogen affect the crop yield,

* Corresponding author.

quality, and nutritional value, prompting global attention to the phenomenon of high nitrate–nitrogen pollution in water. There is an urgent need to develop methods effectively reduce levels or remove nitrate–nitrogen from water.

Traditionally, microbial nitrogen removal includes aerobic nitrification and anaerobic denitrification [5,6], but the two methods are difficult to achieve the unity of time and space, so they are considered to be energy-consuming and time-consuming [7,8]. Robertson et al. [9] first reported the isolation of aerobic denitrifiers, revealing a new method for denitrification that did not rely on anaerobic conditions. Most reports on aerobic denitrifying bacteria primarily concentrated on *Pseudomonas* species, including *Pseudomonas stutzeri* [10], *Pseudomonas aeruginosa* [11], *Pseudomonas tolaasii* [12], and *Pseudomonas putida* [13], but there is no report about *Pseudomonas plecoglossicida*.

The denitrification capacity of aerobic denitrifiers is affected by various environmental factors, with temperature serving especially a vital role. Studies have shown that the optimal temperature for most aerobic denitrifiers is 25°C–35°C. At temperatures lower than 25°C, the denitrification activity of most strains are inhibited. For example, Zhu et al. [14] reported that the removal rate of nitrate–nitrogen by *Pseudomonas mendocina* 3–7 was 97.7% at 30°C, but this activity was strongly inhibited at 10°C. Denitrifying bacterial C3 can removal 95.69% of the nitrate–nitrogen from contaminated water when incubated for 12 h at 30°C, but the removal rate was only 29.32% at 20°C [15]. Zhang et al. [16] reported the nitrate removal using *P. stutzeri* YZN-001 at 30°C, but strains of these bacteria have not been investigated for nitrogen removal at low temperatures. Liang et al. [17] revealed that the nitrification and denitrification activity of the heterotrophic nitrification-aerobic denitrifying bacteria *Pseudomonas mendocina* 3–7 was severely inhibited at 10°C, with a removal rate of nitrate–nitrogen of only 9.2%. Denitrification is strongly inhibited at low temperatures, and cold-tolerant strains need to be identified for the practical application of aerobic denitrifiers. pH is also an important factor that affects nitrogen removal ability. Enzymatic activity of the microorganism is reduced or completely inhibited outside of standard pH ranges [18]. The optimal pH of the strain investigated by Gao et al. [19] was 7.5, as it would barely grow at pH > 9. Huang et al. [20] isolated strain A14, and the optimal denitrification pH was 7, while the strain was strongly inhibited at pH > 10. In this study, we studied the denitrification ability of strain Y-1 at pH > 10, discovering that the nitrate removal rate can still achieve 50%, suggesting that strain Y-1 may have advantages when compared to other strains.

In this experiment, the isolated strain Y-1 was identified via morphology, phospholipid fatty acid (PLFA), and 16S rRNA sequence analysis. The optimum growth conditions (temperature, shaking speed, pH, inoculation amount, carbon source, and C/N) for the strain were investigated. The nitrogen removal activity of the strain was measured using NO_3^-–N, NO_2^-–N, or NH_4^+–N as the sole nitrogen source, revealing stark differences in activity based on the nitrogen source [21]. We further explored the denitrification characteristics and transformation pathways of strain Y-1 regarding each nitrogen source, to provide a theoretical basis for the treatment of actual wastewater.

2. Materials and methods

2.1. Materials

2.1.1. Strain source

Source of hypothermia and aerobic nitrite denitrifying bacterium strain Y-1 was isolated in our laboratory.

2.1.2. Medium

- Enrichment medium: $C_6H_{12}O_6$ 5 g/L, NaCl 1 g/L, $NaNO_3$ 2 g/L, $MgSO_4$ 0.2 g/L, KH_2PO_4 4 g/L, K_2HPO_4 6 g/L, pH 7.2.
- The bromothymol blue medium (BTB): $NaNO_3$ 1 g/L, KH_2PO_4 1 g/L, $FeCl_2{\cdot}6H_2O$ 0.5 g/L, $CaCl_2{\cdot}7H_2O$ 0.2 g/L, $MgSO_4{\cdot}7H_2O$ 0.1 g/L, sodium succinate 8.5 g/L, BTB reagent 1 ml (1.5% in ethanol), 2% agar, pH 7.2.
- Luria-Bertani medium (LB): Tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.2.
- Inorganic nitrogen medium included nitrification medium (NM), denitrification medium (DM-1, DM-2 and DM-3) [22], the components of each medium are shown in Table 1.

2.2. Methods

2.2.1. Bacterial enrichment, isolation and purification

The bacteria were inoculated in 100 mL enrichment medium, and cultured at 15°C, shaking at 150 rpm for 2 d. Then 5 ml of the culture was used to inoculated 100 mL of fresh enriched medium, repeating this operation three times. Three different enrichment cultures (20, 30, and 40 μL) were spread on BTB agar plates and incubated for 2, 3 d at 15°C. Single colonies that turned the BTB agar blue were streaked onto new BTB agar plates and isolated another 3–5 times to obtain a purified denitrifying strain. The obtained purified strain was inoculated into LB medium, cultured for 1 d at 15°C, and stored at 4°C until use.

2.2.2. Morphological identification

The morphological identification of the strains was performed by gram staining and electron microscopy [23].

2.2.3. Identification of PLFAs

PLFAs were extracted using about 40 mg pure culture of strain Y-1. Each type of PLFAs was analyzed by Agilent 6850, USA.

2.2.4. 16S rRNA gene sequence analysis

The nearly full-length sequence of the 16S rRNA gene was amplified using genomic DNA as a template, which was extracted by a genomic DNA purification kit (Thermo Scientific, USA). 16S rRNA polymerase chain reaction amplification was performed using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGT TACCTTGTTACGACTT-3′). The cloned products were sent to the sequencing company for sequencing. The 16S rRNA gene sequences were compared in the GenBank nucleic acid sequence database, and the phylogenetic tree was constructed using the Neighbor-Joining method [24].

Table 1
Ingredients of inorganic medium in 1 L ultrapure water

Medium	$NaNO_3$	$NaNO_2$	$(NH_4)_2SO_4$	CH_3COONa	$C_6H_{12}O_6$	KH_2PO_4	Na_2HPO_4	$MgSO_4{\cdot}7H_2O$	$FeSO_4{\cdot}7H_2O$
	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)
DM-1[a]	0.31	–	–	2.56	–	1.5	0.42	0.1	0.05
DM-2	0.607	–	–	–	3.75	1.5	0.42	0.1	0.05
DM-3	–	0.493	–	–	3.75	1.5	0.42	0.1	0.05
NM[a]	–	–	0.707	–	5.625	1.5	0.42	0.1	0.05

[a]NM and DM stands for the nitrification medium and denitrification medium, respectively.

2.2.5. Denitrification characterization

The effect of six factors on the denitrification capacity was explored by changing single variables, including temperature (5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C), shaking speed (0, 50, 100, 150, and 200 rpm), initial pH (6, 6.5, 7, 8, 9, 10, 11, and 12), inoculation amount ((0.5×10^8, 1.0×10^8, 1.5×10^8, 2.0×10^8, and 2.5×10^8 CFU/100 ml of medium), carbon source (sodium citrate, sodium succinate, glucose, sucrose, and sodium acetate), and C/N (0, 5, 10, 15, 20, and 25). After a 48 h incubation, the nitrogen concentration of the sample was measured to analyze the effects of the above factors on the denitrification activity of strain Y-1.

2.2.6. Denitrification characteristics and transformation pathways of single nitrogen sources by strain Y-1

To study how strain Y-1 utilizes different single nitrogen sources (NO_3^-–N, NO_2^-–N, and NH_4^+–N), pre-cultured bacteria were inoculated into 250 mL flasks (three replicates per treatment). Each medium contained 100 mL of culture medium and was continuously cultured under optimum conditions. The non-inoculated medium was used as a blank, with the OD_{600} value, and pH value, as well as the NO_3^-–N, NO_2^-–N, NH_4^+–N, and total nitrogen (TN) concentrations were measured every 6 h to calculate the denitrification rate of strain Y-1. These data were used to examine the denitrification activity of strain Y-1, exploring its nitrogen conversion pathway by analyzing the intermediate products.

2.2.7. Analytical methods

In this experiment, NO_3^-–N was determined by UV spectrophotometry (HJ/T346–2007); determination of NO_2^-–N was performed by an N-(1-naphthyl)-ethylenediamine photometric method [25]; determination of NH_4^+–N was performed by indole blue colorimetry [26]; determination of TN was performed using alkaline potassium persulfate digestion UV spectrophotometry [27] (GB 11894–1989); the pH value was measured with a pH agent; the amount of cell growth was determined by measuring the OD_{600} using a photometric method.

2.2.8. Data processing and analysis

DNAMAN software was used for DNA sequence analysis and splicing. MEGA (4.0) was used for homology comparison and phylogenetic analysis. Data statistical analysis and mapping were performed using Excel, and Origin. The nitrogen removal rate was calculated by the equation:

$$\mathrm{Rv} = \frac{\left(T_1 - T_2\right)}{T_1} \times 100\%$$

where Rv is the rate of nitrogen removal, R_1 is the initial nitrogen concentration, and R_2 is the final nitrogen concentration.

3. Results

3.1. Morphological features

Strain Y-1 was streaked on BTB plates and cultured at 15°C. After 48 h, colonies were observed to be milky yellow with a smooth surface, neat edges, and a slight central elevation. Scanning electron microscopy revealed that strain Y-1 was a short rod, shaped, with non-spores, and lacked flagellum (Fig. 1).

3.2. Fatty acid identification from phospholipids

Phospholipids are important components of organisms, and specific fatty acids phospholipids differ between

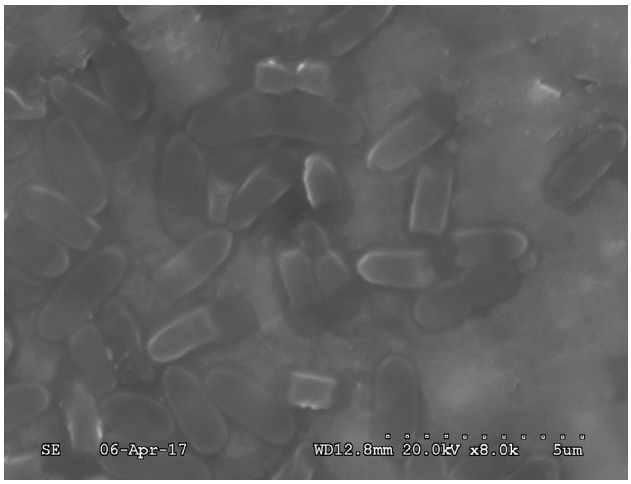

Fig. 1. Strain Y-1 as visualized under a scanning electron microscope.

different microbial groups but are relatively constant between microbes that are closely related. PLFA has microbial specificity and structural diversity, generating specific fatty acid profiles. The PLFA profile for strain Y-1 were identified, revealing a similarity index between the strain Y-1 and *Pseudomonas putida*-biotype A of 0.163, which can auxiliary proof the 16S rRNA gene identification results that strain Y-1 is a *Pseudomonas* species (Table 4).

3.3. 16S rRNA sequence and phylogenetic analysis

The 16S rRNA of strain Y-1 was amplified by PCR, revealing that the 16S rRNA was 1,070 bp, with the sequence ID KY927413. Blast comparison analysis found that strain Y-1 was similar to *P. plecoglossicida*. The corresponding strains of this species were selected from the GenBank database, and a phylogenetic tree was constructed using the proximity method (Fig. 2).

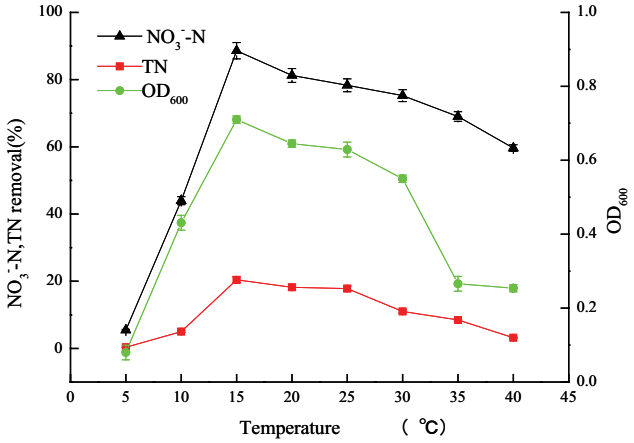

Fig. 3. Effect of temperature on the denitrification activity of strain Y-1.

3.4. Influence of temperature on nitrogen removal activity

Temperature is an important factor that affects bacterial growth and enzymatic activity. Denitrifying bacteria typically only exhibit high denitrification activity at a certain temperature, and the nitrogen removal activity of strain Y-1 initially increases with temperature to a maximum at 15°C, then decreasing, with removal rates of 88.59% for nitrate–nitrogen and 20.42% for TN (Fig. 3). This suggests that the optimum growth temperature for strain Y-1 is 15°C. At temperature >15°C, the nitrogen removal rate gradually decreased, potentially due to denaturation/inhibition of the enzyme, thus affecting the denitrification rate. When compared to the effects of high temperature, low temperature had a stronger inhibitory effect on the denitrification rate. Strain Y-1 can survive between 10°C and 35°C, indicating that it is a psychrophilic aerobic denitrifying bacteria.

3.5. Effect of shaking speed on nitrogen removal activity

Next, the effect of dissolved oxygen on the growth and denitrification rate of strain Y-1 was examined by adjusting the shaking speed. Without shaking, the NO_3^-–N and TN removal rates were very low and the strain grew poorly, indicating that low concentrations of dissolved oxygen inhibited the growth rate and denitrification ability of strain Y-1. The OD_{600} value gradually increased with the greater shaking speed, and the removal rates of NO_3^-–N and TN were also increased. When the shaking speed was 150 rpm the removal rate reached the maximal values of 88.50% for NO_3^-–N and 19.28% for TN (Fig. 4). The removal rate slightly decreased above 150 rpm, which was probably because of the high concentration of dissolved oxygen inhibited the synthesis and activity of the microbial denitrification enzymes, thereby inhibiting denitrification. These data suggest that, within a certain range, increased dissolved oxygen concentration contributes to the growth and the denitrification activity of strain Y-1.

3.6. Effect of initial pH on nitrogen removal activity

pH can affect the rate of denitrification by impacting cellular and enzymatic activity. At pH 6, the denitrification activity of the bacteria was almost completely suppressed, with NO_3^-–N and TN removal of only 3.23% and 0.25%,

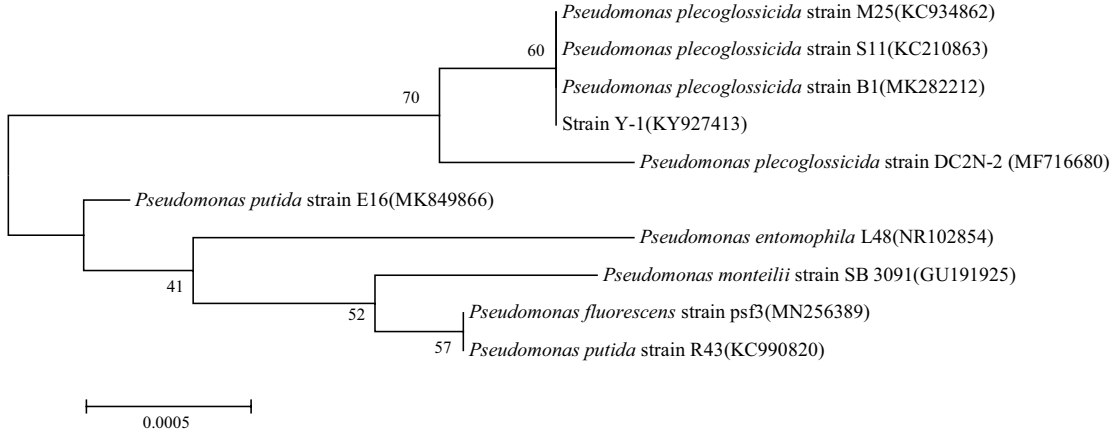

Fig. 2. Phylogenetic tree of the strain. Numbers in parentheses represent the sequences in GenBank. The number at each branch point is percentage supported by bootstrap (1,000 resamplings).

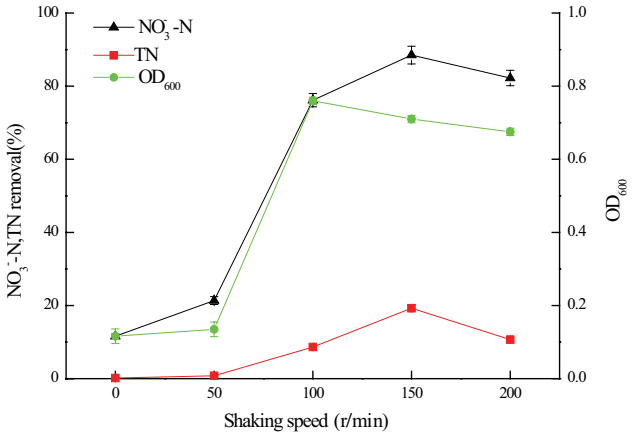

Fig. 4. Effect of shaking speed on the denitrification activity of strain Y-1.

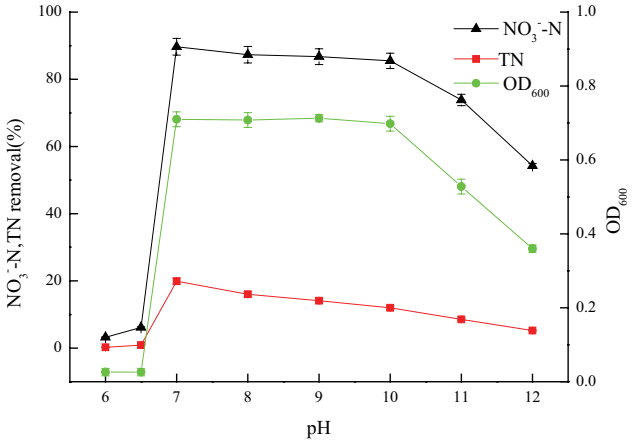

Fig. 5. Effect of pH on the denitrification activity of strain Y-1.

respectively (Fig. 5). Removal rates of NO_3^-–N and TN reached maximum values (89.70% and 19.91%, respectively) at pH 7. The removal rate for NO_3^-–N changed only slightly as the pH value increased from 7 to 10, while the TN removal rate diminished slightly. Even at pH 11–12, there was still a high removal rate for NO_3^-–N (>50%). These results indicate that the optimum pH of strain Y-1 was 7, as acidic conditions strongly inhibited denitrification, but NO_3^-–N could still be removed under even strong alkali conditions.

3.7. Effect of inoculation amount on nitrogen removal activity

Aerobic denitrifying bacteria require NO_3^-–N for growth and synthesize, several reductases that reduce the NO_3^-–N to other forms of nitrogen [28]. Therefore, the absolute rate of removal is closely related to the amount of aerobic denitrifying bacteria that are present. By studying the effects of different inoculation amounts on nitrate removal, the optimal inoculation amount of strain Y-1 was determined. When the inoculation amount was 0.5 × 10^8 CFU, the TN removal rate was only 2.88% (Fig. 6). Increasing the inoculation amount to 1.5 × 10^8 CFU, the removal rates of NO_3^-–N and TN reached the maximum values of 89.42% and 19.26%, respectively.

Table 2
Phospholipid fatty acid profile and similarity index

Library	Sim index	Entry name
TSBA6	0.115	*Paucimonas lemoignei*
	0.108	*Aeromonas ichthiosmia* A/hydrophila
	0.104	*Aeromonas veronii*-GC subgroup B (biogroup sobria)
CLIN6.20	0.163	*Pseudomonas putida*-biotype A

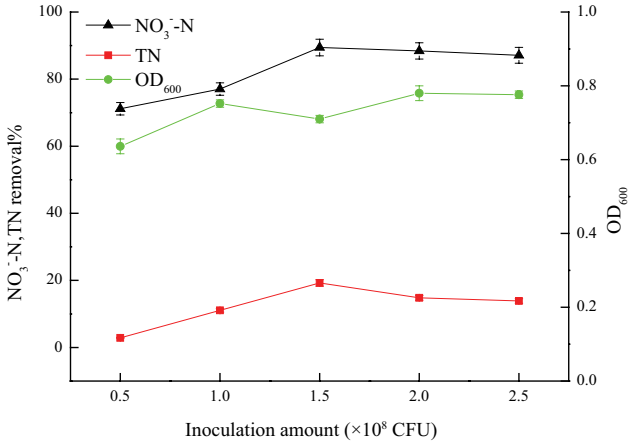

Fig. 6. Effect of the inoculation amount on the denitrification activity of strain Y-1.

The removal rate for NO_3^-–N changed only slightly when the inoculation amount was increased to 2.5 × 10^8 CFU, while the TN removal rate slightly decreased. The experiment revealed that the optimal inoculation amount of strain Y-1 was 1.5 × 10^8 CFU.

3.8. Effect of carbon sources on nitrogen removal activity

The carbon source indirectly affects the denitrification rate by impacting the growth and metabolism of the bacteria. Strain Y-1 grew well when sodium citrate, sodium succinate, glucose, and ammonium acetate were used as the carbon source, while sucrose supplementation led to virtually no growth (Fig. 7). The effect of carbon source on the denitrification activity of strain Y-1 was similar to the effect of growth. When glucose was used as the carbon source, the removal rates for NO_3^-–N and TN were 98.88% and 20.08%, respectively. When sodium butyrate and sodium citrate were used as carbon sources, the removal rate of NO_3^-–N was >97%, while the removal rate of NO_3^-–N was 2.81% when using sucrose. This revealed that the optimal carbon source of strain Y-1 was glucose, and that the carbon source affected the denitrification rate, likely by affecting the rate of bacterial growth.

3.9. Effect of C/N ratio on nitrogen removal activity

The C/N ratio strongly influences the denitrification process, as denitrifying bacteria are heterotrophic facultative

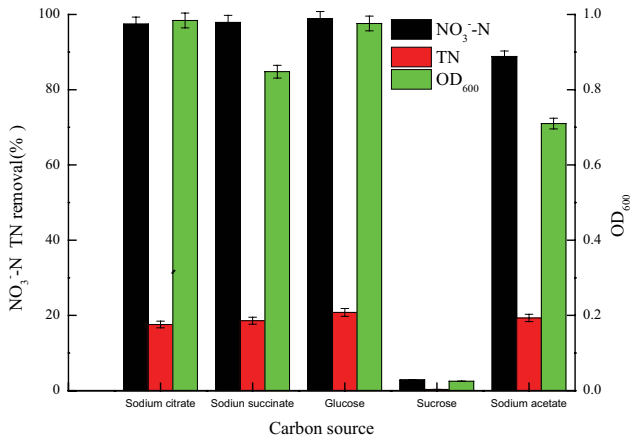

Fig. 7. Effect of different carbon sources on the denitrification activity of strain Y-1.

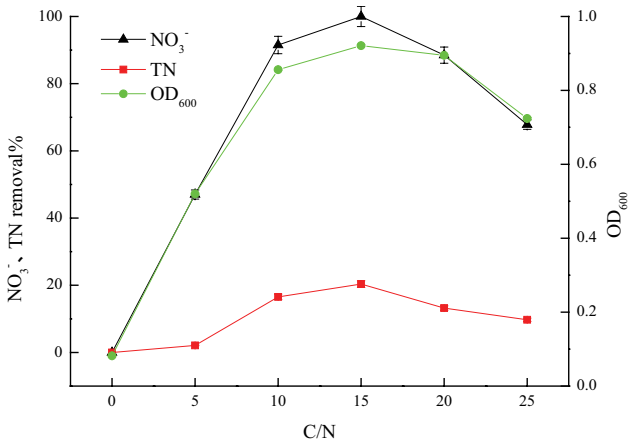

Fig. 8. Effect of different C/N ratio on the denitrification activity of strain Y-1.

anaerobic bacteria that reduce the electron acceptors in energy metabolism (N^{5+} and N^{3+} of nitrate and nitrite, respectively) under anaerobic conditions, while organics provide energy and act as an electron donor. As the C/N ratio increased, the removal rate of NO_3^-–N and TN gradually increased and reached a maximum at 15 C/N (Fig. 8); at this time, the removal rate of NO_3^-–N was 100.00% and the TN removal rate was 20.35%. When the C/N ratio increased further, the removal rate decreased slightly. This could be due to sufficient energy required for cell growth and denitrification, and that the carbon and nitrogen source was non-limiting factors, the growth, and denitrification activities becoming stable. This optimum C/N ratio was 15, similar to *Vibrio halophiles* Y1–15 screened from the ocean by Li et al. [29].

3.10. Denitrification characteristics of strain Y-1 under optimum conditions

According to the analysis above, the optimal denitrification conditions of strain Y-1 were: temperature 15°C, pH 7, shaking speed 150 rpm, C/N 15, inoculation amount 1.5×10^8 CFU, and using glucose as the carbon source. Strain Y-1 was cultured under these conditions for 48 h and the denitrification activities measured. The nitrate concentration decreased from 51.140 to 0.570 mg/L with a removal ratio of 98.89% over this time interval (Table 3). The initial and final concentration of TN before centrifugation was 55.530 and 43.977 mg/L, respectively, decreasing by 11.553 mg/L, while the initial and final concentration of TN after centrifugation decreased from 54.833 to 4.091 mg/L, respectively, with a removal ratio of 92.54%, 11.553 mg/L (removal ratio of 21.07%) converted into gas from water, 39.189 mg/L (removal ratio of 71.47%) was used by cell and converted to biomass nitrogen. Throughout the process, there was no accumulation of nitrate–nitrogen.

3.11. Denitrification activity of strain Y-1 using different single nitrogen sources and determining the denitrification pathway

When NO_3^-–N was used as the sole nitrogen source, the NO_3^-–N concentration decreased from 100.71 to 32.00 mg/L within 24 h, the removal rate reached 68.23%, and the concentration decreased to 0 mg/L after 36 h, achieving complete reduction (Fig. 9). A small amount of NO_2^-–N (3.128 mg/L) was detected throughout the whole process, but the NO_2^-–N was removed completely. The logarithmic growth phase was from 12 to 30 h, reaching a maximum OD_{600} value of 1.727. Because the nitrate reduction reaction consumes H^+ [30], the pH of the medium increased from 7.21 to 8.27 throughout the experiment. The use of NO_3^-–N, accumulation of intermediate products, and the change in pH suggests that strain Y-1 removes NO_3^-–N through denitrification.

When 100 mg/L NO_2^-–N was used as the sole nitrogen source, strain Y-1 did not grow and the OD_{600} value decreased from 0.080 to 0.056, probably due to the toxic effects of nitrite (Fig. 10). During the 36 h, the concentration of NO_2^-–N decreased from 105.23 to 98.20 mg/L, and the removal rate was negligible. NH_4^+–N and NO_3^-–N was not detected during the experiment and TN remained constant. The pH of the medium decreased from 7.17 to 6.13, probably due to the release of NH_4^+–N after the death of the bacteria, and strain

Table 3
Denitrification characteristics of strain Y-1 under optimum conditions

Project	NO_3^-N (mg/L)	NO_2^-N (mg/L)	TN after centrifugation (mg/L)	TN before centrifugation (mg/L)	Biomass nitrogen (mg/L)	OD_{600}
Initial value	51.140	0.000	54.833	55.530	0.697	0.012
Final value	0.570	0.000	4.091	43.977	39.886	1.176

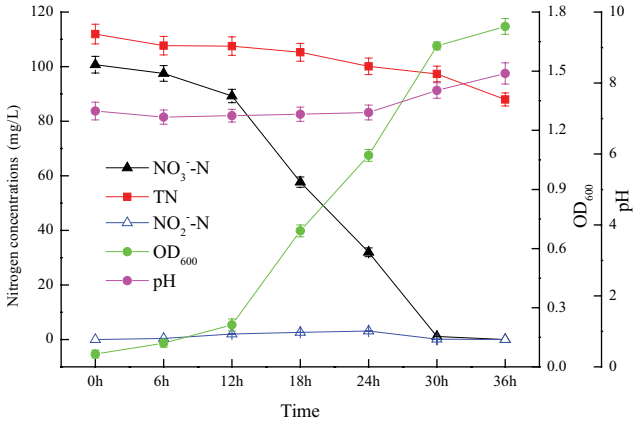

Fig. 9. Denitrification activity of strain Y-1 using nitrate–nitrogen as the sole nitrogen source.

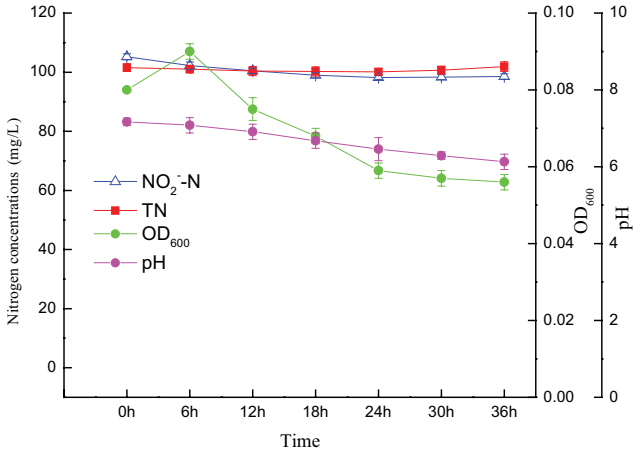

Fig. 10. Denitrification activity of strain Y-1 using nitrite–nitrogen as the sole nitrogen source.

Y-1 could not remove the high concentration of NO_2^-–N when it was supplied as the sole nitrogen source.

When NH_4^+–N was used as the sole nitrogen source, the OD_{600} value increased from 0.068 to 1.180, reaching the plateau at 30 h, with the logarithmic growth phase occurring from 6 h to 18 h (Fig. 11). The NH_4^+–N concentration decreased from 150.48 to 81.78 mg/L by 30 h, and the removal rate was 45.65%. The TN removal rate was zero throughout the entire process, and no accumulation of either NO_2^-–N or NO_3^-–N was detected. Based on analyzing the data strain Y-1 uses NH_4^+–N by assimilation rather than nitrification.

4. Discussion

The discovery of aerobic denitrification overcame the limitation of oxygen inhibition, providing a new biological nitrogen removal pathway. At present, a large number of aerobic denitrifier strains that have strong nitrogen removal activity have been reported. For example, Chen et al. [31] screened and isolated *Rhodococcus pyogenes* from livestock and poultry breeding wastewater, which could remove 66.74% of the TN and 64.27% of NO_3^-–N present (initial concentration of 50 mg/L) by aerobic denitrification. Gao et al. [19] isolated an aerobic denitrifier *Pseudomonas* sp. 2–8, which removes 92% of NO_3^-–N present within 48 h. Li et al. [29] screened and isolated a strain *Vibrio halophiles* Y1–15 from surface sediments in Jiaozhou Bay, which could remove NH_4^+–N and NO_3^-–N simultaneously, and the removal rate of NO_3^-–N was 98% within 48 h. However, most aerobic denitrifying bacteria that have been investigated are *Pseudomonas stutzeri* low, *P. aeruginosa* and *P. putida*, while there have been few reports on the aerobic denitrification ability of *P. plecoglossicida*. Most aerobic denitrifying bacteria have poor tolerance to low temperatures and high pH environments. In this study, we investigated strain *P. plecoglossicida* Y-1, which has aerobic denitrification activity and can remove nitrogen at both low temperatures and in strong alkaline water environments.

Temperature strongly affects cell growth and reproduction, thereby impacting the denitrification capacity of bacteria, and low temperature is a key factor in inhibiting the denitrification rate [32]. The optimum temperature of the highly efficient denitrifying phosphate-accumulating bacteria N-11, selected by Sun et al. [33], is 30°C. *P. stutzeri* YZN-001 was discovered by Zhang et al. [16] and could remove NH_4^+–N between 4–37°C, but NO_3^--N could be effectively removed only at 30°C. Chen et al. [15] isolated *Pseudomonas* C3 from an estuarine reed wetland, which could remove nitrate–nitrogen by 97.17% within 36 h at 30°C, but this dropped to 29.32% at 20°C. Most identified strains have high optimum temperatures that are not suitable for low-temperature water bodies. This study revealed that the optimum temperature for strain Y-1 was 15°C and that the removal rate of NO_3^-–N reached 88.59% at this temperature. *P. plecoglossicida* Y-1 is a denitrifying strain with cold-tolerant properties and is superior to other strains in removing nitrogen from low-temperature water.

pH affects the membrane charge of cells, thereby impacting the balance of electrolytes in the cell, and the concentration of nutrients or inhibitory substances in solution, ultimately dictating how the microorganisms absorb nutrients [34] and indirectly or directly affecting bacterial activity [35]. pH can also affect the enzyme activity of microorganisms, altering metabolism [33]. At extreme pH, the enzymatic activity of the microorganisms can be strongly inhibited, which can be deleterious to cellular growth. Most aerobic denitrifying bacteria have optimal activity at neutral or slightly alkaline conditions, while nitrogen removal efficiency is significantly reduced under strongly alkaline conditions. For example, the optimum nitrogen removal pH of a strain found by Gao et al. [19] was 7.5 and barely grew when pH > 9. Huang et al. [20] isolated strain A14, which had maximal nitrogen removal at pH 7. Sun et al. [36] screened strain *Klebsiella* sps.y5 and found that the nitrate–nitrogen removal rate was drastically decreased when pH > 8. *Pseudomonas* sp. C3 had minimal denitrification activity when pH = 9 [15]. The experimental strain Y-1 has high efficiency for nitrogen removal in neutral or weakly alkaline environments and is similar to strain L7 [37] (when the initial pH = 7, the denitrification efficiency is higher) and strain SF16 [38] (the optimal pH range is 7.5–9.5). However, stain Y-1 has greater denitrification activity at pH 11–12 in comparison to other

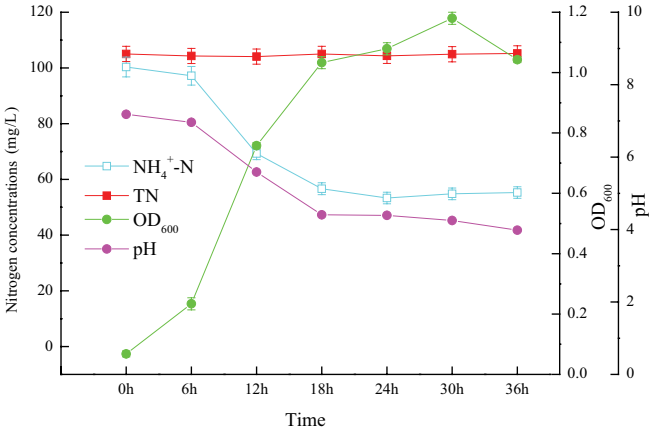

Fig. 11. Denitrification activity of strain Y-1 using ammonium nitrogen as the sole nitrogen source.

strains, suggesting that strain Y-1 can be used in alkaline water treatment.

Previous studies have suggested that most aerobic denitrifying bacteria have both denitrification and nitrification capabilities, such as *P. putida* Y-12 reported by Ye et al. [39], and NH_4^+–N removal inferred to be achieved by nitrification, while ammonia assimilation is often overlooked. The process by which ammonia is assimilated is an important component in nitrogen metabolism, where it serves as the main biosynthetic donor for all other nitrogen-containing cellular components. Studies have demonstrated that ammonia assimilation is directly related to bacterial growth and affects the rate of aerobic denitrification, indicating that this process is essential for many organisms that use ammonia as a nitrogen source. In the 1970s, Tempest et al. [40] discovered the pathway for ammonia assimilation in Enterobacter (*Klebsiella*). Additionally, Yang et al. [41] discovered and characterized three bacterial strains that perform ammonia assimilation from livestock and poultry manure compost samples. For the first time, strain Y-1 was demonstrated to remove ammonium nitrogen via ammonia assimilation.

5. Conclusion

- The strain Y-1 was identified as *P. plecoglossicida*, which was not previously reported to have denitrification activity. The discovery of strain Y-1 adds to the list of aerobic denitrifying species, while also providing more information on the role of *P. plecoglossicida* in nature;
- Strain Y-1 is an aerobic denitrifying bacteria that is cold-tolerant with strong alkali resistance. It can effectively remove nitrate–nitrogen at 15°C and pH 11–12. The tolerance of strain Y-1 to low temperatures and alkali environment is stronger than other reported strains;
- Strain Y-1 can effectively remove nitrate–nitrogen, but does not have activity at high concentrations (100 mg/L) of nitrite nitrogen;
- *Pseudomonas plecoglossicida* Y-1 can remove ammonium nitrogen by assimilation rather than nitrification, which differs from most denitrifying bacteria.

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