Heterotrophic nitrification and aerobic denitrification by *Pseudomonas tolaasii* Y-11 without nitrite accumulation during nitrogen conversion

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**HIGHLIGHTS**

- *P. tolaasii* Y-11 has excellent heterotrophic nitrifying–aerobic denitrifying ability.
- High removal rates of inorganic nitrogen without nitrite accumulation at 15 °C.
- It tolerates to high concentrations of ammonium, nitrate and nitrite nitrogen.
- It could effectively remove NH₄⁺ in simultaneous nitrification and denitrification.

**ABSTRACT**

A hypothermia aerobic nitrite-denitrifying bacterium, *Pseudomonas tolaasii* strain Y-11, was found to display high removal capabilities for heterotrophic nitrification with ammonium and for aerobic denitrification with nitrate or nitrite nitrogen. When strain Y-11 was cultivated for 4 days at 15 °C with the initial ammonium, nitrate and nitrite nitrogen concentrations of 209.62, 204.61 and 204.33 mg/L (pH 7.2), the ammonium, nitrate and nitrite removal efficiencies were 93.6%, 93.5% and 81.9% without nitrite accumulation, and the corresponding removal rates reached as high as 2.04, 1.99 and 1.74 mg/L/h, respectively. Additionally, ammonium was removed mainly during the simultaneous nitrification and denitrification process. All results demonstrate that *P. tolaasii* strain Y-11 has the particularity to remove ammonium, nitrate and nitrite nitrogen at low temperatures, which guarantees it for future application in winter wastewater treatment.

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**1. Introduction**

Biotechnology is a cost-effective, high efficient and easy to implement method for the nitrogen removal from wastewater. According to traditional biological nitrogen removal strategies, it requires autotrophic nitrifiers and heterotrophic denitrifiers which worked in separate strict aerobic and anaerobic conditions, making it relatively expensive (Khardenavis et al., 2007). A unique wastewater treatment way that one species of bacteria could perform nitrification and denitrification aerobically in the same reaction vessel was generated until the aerobic denitrifier *Thiosphaera pantotropha* was reported (Lesley and Robertson, 1989). Up to now, a large number of aerobic denitrifiers have been reported, such as Paracoccus pantotrophus (Yang et al., 2008), *Rhodococcus pyridinivorans* (Chen et al., 2009), *Pseudomonas stutzeri* (Ji et al., 2014), *Providencia rettgeri*, *Bacillus subtilis* (Yang et al., 2011), *Klebsiella pneumonia* (Padhi et al., 2013) and so on. These special aerobic denitrifying bacteria could use various carbon sources and convert progressively different inorganic nitrogen, such as ammonium, nitrate and nitrite nitrogen, into nitrogenous gas (Zhao et al., 2012; Zhu et al., 2012). Meanwhile, the acidity generated during heterotrophic nitrification with ammonium could be compensated by alkalinity caused via aerobic denitrification, which would reduce the extra cost of pH-adjusting. Therefore, nitrogen removal by heterotrophic nitrification and aerobic denitrification bacteria has attracted increasing attention in wastewater treatment.

However, there are several great challenges during biological wastewater treatment process, such as low temperature, sensitive to dissolve oxygen, nitrite accumulation and so on. Previous studies demonstrate that both nitrification and denitrification would be inhibited strongly at the temperature lower than 20 °C (Hendrickx et al., 2012; Zheng et al., 2011; Yao et al., 2013), resulting in low nitrogen removal efficiency. Up to date, limited reports are available on a bacterium which could conduct nitrification and denitrification aerobically at low temperature. Additionally, most
studies indicate that nitrite accumulation is inevitable during biological treatment of wastewater (Zhao et al., 2010; Liang et al., 2013; Zou et al., 2014), which would reduce the removal efficiency of total nitrogen because the nitrite nitrogen is the end-product instead of gaseous nitrogen. Some researches indicate that membrane-bound nitrate reductase (NAR) and nitrite reductase (NIR) are sensitive to dissolved oxygen inhibition (Bell et al., 1990; Körner and Zumft, 1989), which would decrease the nitrogen removal efficiency also.

In our previous study, *Pseudomonas tolaasii* strain Y-11 was confirmed to be a hypothermia aerobic nitrite-denitrifying bacterium, which exhibited an excellent performance on nitrite and total nitrogen reduction at 15 °C (He et al., 2015). More important, dissolved oxygen with high concentration could well support the nitrite and total nitrogen removal, which suggested that strain Y-11 was not sensitive to oxygen inhibition. But it remains unclear about more information on strain Y-11, such as ammonium, nitrate and organic nitrogen removal ability and transformation relationships between different inorganic nitrogen. Therefore, it is worthwhile to explore the nitrogen removal ability such as heterotrophic nitrification, aerobic denitrification and simultaneous nitrification and denitrification with nitrogen source. In this study, we focused on the removal abilities of strain Y-11 on high concentrations of ammonium, nitrate, nitrite and organic nitrogen, special under aerobic condition and low temperature (15 °C). Meanwhile the intermediate products were analyzed during the nitrification and denitrification process. Additionally, the simultaneous nitrification and denitrification performance were also investigated in this research. All above attempts would be applied to elucidate the nitrogen removal ability and inorganic nitrogen transformation relationships in heterotrophic nitrification and aerobic denitrification by *P. tolaasii* Y-11.

2. Methods

2.1. Strain and culture media

Strain Y-11 was isolated from the long-term flooded paddy soil and successfully identified as *P. tolaasii* (Genbank No. KP410741) (He et al., 2015), and deposited in China center for type culture collection (CGMCC NO.10537).

Nitrification medium (NM) (Pal et al., 2015) per liter comprised 7.0 g K2HPO4, 3.0 g KH2PO4, 0.1 g MgSO4·7H2O, 1.0 g (NH4)2SO4, 0.05 g FeSO4·7H2O and 10 g CH3COONa. NM was used to determine the ammonium removal ability of strain Y-11.

Denitrification medium (DM) per liter contained 7.0 g K2HPO4, 3.0 g KH2PO4, 0.1 g MgSO4·7H2O, 1.8 g KNO3 (DM-1) or 0.986 g NaNO2 (DM-2), 0.05 g FeSO4·7H2O and 10 g CH3COONa. NM was used to determine the ammonium removal ability of strain Y-11.

Simultaneous nitrification and denitrification medium (SND) per liter contained 7.0 g K2HPO4, 3.0 g KH2PO4, 0.1 g MgSO4·7H2O, 1.0 g (NH4)2SO4, 1.8 g KNO3 (SND-1) or 0.986 g NaNO2 (SND-2), 0.05 g FeSO4·7H2O and 10 g CH3COONa. Two kinds of the DM were used to determine the denitrification ability of strain Y-11 for nitrate or nitrite.

Simultaneous nitrification and denitrification medium (SND) per liter contained 7.0 g K2HPO4, 3.0 g KH2PO4, 0.1 g MgSO4·7H2O, 1.0 g (NH4)2SO4, 1.8 g KNO3 (SND-1) or 0.986 g NaNO2 (SND-2), 0.05 g FeSO4·7H2O and 10 g CH3COONa. Two kinds of the SND were used to assess simultaneous nitrification and aerobic performance of strain Y-11 with ammonium and nitrate (or nitrite).

In order to evaluate the denitrification of strain Y-11 with organic nitrogen, nitrogen source of the DM was replaced by 1.575 g/L tryptone. The composition of Luria–Bertani medium (LB) was as follows (g/L): tryptone 10, yeast extract 5, NaCl 10.

Initial pH of all medium was set at 7.2 and all the above mediums were sterilized for 30 min at 0.11 MPa, 121 °C. Conical flasks (250 ml capacity) containing 100 ml medium were used after sterilized in all the experiments, then incubated at 15 °C in an orbital shaker at 150 r/min.

2.2. Estimation the nitrogen removal capacity of strain Y-11

Single colony of Y-11 strain was inoculated into 100 ml LB broth medium and cultured at 150 r/min and 15 °C for 36 h. 8 ml of pre-culture strain Y-11 was centrifuged at 4000 r/min and washed once with sterilized pure water before inoculating into 100 ml NM, DM-1, DM-2, organic medium, SND-1 or SND-2 respectively, which were incubated at 15 °C under aerobic conditions of 150 r/min. Different medium samples were taken and measured to investigate the cell density, ammonium, nitrate, nitrite and total nitrogen concentrations at 24 h intervals. All experiments were conducted in triplicate. The ammonium, nitrate, nitrite and total nitrogen removal efficiencies were calculated by the equation: \[ R = \frac{(T_2 - T_1)}{T_1} \times 100\% \] to assess the nitrification and denitrification ability of strain Y-11. Note that \( R \), \( T_1 \) and \( T_2 \) represent ammonium (nitrate, nitrite or total nitrogen) removal efficiency, the initial concentration of ammonium (nitrate, nitrite or total nitrogen) in medium and the final concentration of ammonium (nitrate, nitrite or total nitrogen), respectively.

2.3. Analytical methods

The cell density was monitored OD600 using a spectrophotometer (DU800, BECKMAN COULTER, U.S.A). Total nitrogen was calculated by the absorbance value at 220 nm subtracting the two times background absorbance value at 275 nm after using alkaline potassium persulfate digestion. Ammonium, nitrate and nitrite were detected using the supernatant after samples centrifuged at 8000 rpm for 5 min. Ammonium nitrogen was analyzed by indophenols blue method. Nitrate was calculated by the absorbance value at 220 nm subtracting the two times background absorbance value at 275 nm. Nitrite nitrogen was determined at wavelengths of 540 nm after adding 1 ml chromogenic reagent including (per liter) 100 mL phosphoric acid, 2 g N-(1-naphthyl)-1,2-diaminoethane dihydrochloride and 40 g sulfurilamide.

2.4. Statistical analysis and graphical work

Statistical analysis and graphical work were carried out by using Excel, SPSS Statistics and Origin 8.6. The results were presented as means ± SD (standard deviation of means).

3. Results and discussions

3.1. Assessment of ammonium nitrogen removal performance

To confirm the heterotrophic nitrification by *P. tolaasii* Y-11, the ammonium [(NH4)2SO4] was used as sole nitrogen source in nitrification medium. The cell growth and ammonium nitrogen removal performance by *P. tolaasii* Y-11 were showed in Fig. 1. The cells were largely reproduced from 1 to 2 d and reached a stationary phase after 3 d cultivation while OD600 increased from 0.11 to 1.68 in nitrification medium. The ammonium nitrogen decreased dramatically from 209.62 mg/L to 13.47 mg/L and the removal efficiency reached 93.6% after 4 d of incubation, and the nitrification rate was 2.04 mg NH4+-N/L/h, which was similar to that of *Bacillus methylotrophicus* (2.14 mg NH4+-N/L/h) (Zhang et al., 2012), but higher than that of *Pseudomonas alcaligenes* AS-1 (1.15 mg NH4+-N/L/h) (Su et al., 2006), *Pseudomonas* sp. (1.38 mg NH4+-N/L/h) (Jin et al., 2015) and *Bacillus* sp. LY (0.43 mg NH4+-N/L/h) (Zhao et al., 2010). Nitrification products, including nitrate and nitrite nitrogen, were not detected during the experiments, which were consistent with *Microbacterium* sp. strain SFA13 (Zhang et al., 2013), but inconsistent with the reports that nitrite accumulation is inevitable (Zhang et al., 2014; Kundu et al., 2014). It merits our
attention that several lineages of the genus *Pseudomonas*, such as *P. stutzeri* C3 (Ji et al., 2015), *P. stutzeri* X31 (Ji et al., 2014) and *P. stutzeri* HS-03 (Ping et al., 2006), have been reported incapable of heterotrophic nitrification, which might be due to lack of certain enzymes, for example the AmoA. However, strain Y-11 has been found to adapt well to the nitrification medium containing high concentration of ammonium and remove ammonium effectively. Meanwhile, total nitrogen decreased from 217.42 mg/L to 151.14 mg/L, and the removal efficiency was 30.5%. These experimental results indicated that strain Y-11 could conduct heterotrophic nitrification without accumulating of nitrification products at low temperature under aerobic condition, and a part of ammonium nitrogen should be converted to gaseous nitrogen during nitrification process.

3.2. Denitrification performance of strain Y-11 with nitrate nitrogen

Characteristics of the cultures growth tendency, nitrate nitrogen removal efficiency and ammonium nitrogen accumulation of *P. tolaasii* Y-11 were shown in Fig. 2. Cells growth kept slowly at 1 d, and OD<sub>600</sub> reached only 0.26, but the OD<sub>600</sub> increased quickly from 0.26 to 1.77 between 1 d and 3 d, and decreased to 1.64 at 4 d. Excellent nitrate nitrogen removal efficiency was observed for strain Y-11 at 4 d. The concentration of nitrate nitrogen decreased from 204.61 mg/L to 13.37 mg/L with nitrate removal efficiency of 93.5% that was consisted with the former ammonium nitrogen removal efficiency of 93.6% approximately. The nitrate removal rate was 1.99 NO<sub>3</sub>-N mg/L/h, which was higher than that of *Rhodococcus* sp. CPZ24 with nitrate removal rate of 0.93 NO<sub>3</sub>-N mg/L/h at 30 °C (Chen et al., 2012). It demonstrated that strain Y-11 could conduct heterotrophic nitrification with ammonium and aerobic denitrification with nitrate nitrogen. Total nitrogen removal efficiency (211.1 mg/L initial total nitrogen) reached 46.9% after 4 d of cultivation, which was higher than that of 30.5% in nitrification medium. These results suggested that strain Y-11 could converted more nitrate than ammonium nitrogen to gaseous nitrogen. A small amount of nitrite and ammonium nitrogen, were detected during the experiment. The concentration of nitrite nitrogen was accumulated only about 0.65 mg/L at 2 day after inoculation and removed completely at 4 day. However, ammonium nitrogen increased gradually to 10.28 mg/L at the end of incubation, which might be due to the decomposition of the death cells so that the ammonium containing in the cell and the organic nitrogen of death cell being converted to the ammonium could release into the denitrification medium. Similar results were also reported in previous studies (Jin et al., 2015; Li et al., 2015).

3.3. Denitrification performance of strain Y-11 with nitrite nitrogen

Nitrite nitrogen has a well-documented toxicity to many species such as goldfish, anuran larvae and swiss albino mice, even some bacteria. Our previous study suggested that Y-11 could perform denitrification with low concentration of nitrite nitrogen (He et al., 2015). However, the way of nitrite nitrogen conversion and the nitrogen accumulation are still unclear during denitrification with high concentration of nitrite nitrogen source. Fig. 3 depicted the performances of cells growth and nitrite nitrogen conversion during denitrification in presence of NaNO<sub>2</sub> as sole nitrogen source. The nitrite nitrogen was found to decrease from 204.33 mg/L to 37.07 mg/L, corresponding to a denitrification efficiency of 81.9% without nitrate nitrogen accumulation. The nitrite removal rate was 41.82 NO<sub>3</sub>-N mg/L/d, and significantly higher than that of 18.20 mg/L/d by of *Pseudomonas* sp. yy7 even at the moderate temperature of 25 °C (Wan et al., 2011). Additionally, Patureau et al. (2000) reported that high amount of nitrite nitrogen could inhibit the bacteria growth and thus repress their nitrification and denitrification activities. This phenomenon was also found in *Pseudomonas* sp. yy7 with an initial nitrite concentration of 50 mg/L (Wan et al., 2011). However, the bacterial growth and nitrogen removal capacity of strain Y-11 was not affected even when the concentration of nitrite nitrogen was more than 200 mg/L. Meanwhile, the higher growth rate and more cell yield were obtained with nitrite than nitrate as sole nitrogen at the initial two days. The similar results were reported by Zhao et al. (2010) and Yao et al. (2013). The Y-11 strain grew into decline phase and OD600 decreased slightly from 1.71 to 1.59 from 3 d to 4 d cultivation, which could explain the phenomenon that the ammonium nitrogen slightly increased because of some cell decomposition in the meantime. During 4 days incubation, total nitrogen decrease trends matched well with the concentration of nitrite nitrogen and the increase of bacterial optical density.
A significant decrease of total nitrogen from 215.15 mg/L to 123.48 mg/L was observed at the end of 4 d inoculation at 15 ℃ and the removal efficiency reached 42.6% which indicated that 91.76 mg/L of the initial nitrogen was lost. It should be removed in the form of nitrogen gases, such as NO₂, N₂O, NO and N₂. All of these results were different from the report that Paracoccus versutus LYM could not reduce any nitrogen when nitrite was sole nitrogen source even if C/N ratio reached as high as 30 (Zhang et al., 2015).

3.4. Denitrification performance of strain Y-11 with organic nitrogen

Organic nitrogen accounted for 14–90% of total nitrogen in all over the world river (Seitzinger and Sanders, 1997), and it would stimulate the algae growth, leading to the eutrophication of water bodies. The performances of organic nitrogen conversion and the change curves of bacterial growth in the organic nitrogen medium were shown in Fig. 4. Tryptone as the organic nitrogen contained some inorganic nitrogen, such as ammonium and nitrate nitrogen. There was a little nitrate nitrogen accumulated in the dis- pose. The P. tolaasii Y-11 grew very well and did not reach to the stationary phase until 4 d, which demonstrated that the organic nitrogen was helpful for the bacterial growth. Total nitrogen decreased slowly from 218.02 mg/L to 194.42 mg/L and removed only 23.60 mg/L nitrogen without nitrite nitrogen accumulation. All of these results indicated that strain Y-11 could easily convert the organic nitrogen into biomass nitrogen, but hardly convert it into nitrogenous gas.

3.5. Assessment of simultaneous nitrification and denitrification performance with ammonium and nitrate nitrogen

Simultaneous nitrification and denitrification is an attractive strategy to treat the nitrogen polluted wastewater (Jin et al.,
Up to date, few reports are about the simultaneous nitrification and denitrification bacteria (Zhang et al., 2015). P. tolaasii Y-11 could perform heterotrophic nitrification with ammonium and aerobic denitrification nitrate and nitrite separately. However, the nitrogen removal ability of the strain Y-11 was still unclear when two nitrogen sources were present in the same medium. The simultaneous nitrification and denitrification of Y-11 was shown in Fig. 5. The initial concentrations of ammonium and nitrate nitrogen were about 204.44 mg/L and 206.48 mg/L in SND-1 medium. High ammonium removal efficiency appeared in the bacterial growth of logarithmic phase from 1 d to 3 d, which was consistent with the conclusion of Zhang et al. (2015). Ammonium nitrogen was completely removed at 4 d, and the removal efficiency of ammonium nitrogen was up to 100% under 15 °C. The removing rate was 2.13 mg NH₄⁺–N mg/L/h, which was higher than the ammonium as sole nitrogen source. However, the concentration of nitrate nitrogen decreased gradually to 157.02 mg/L and the removal efficiency was only 24.0%. The corresponding removal rate was 0.52 NO₃⁻–N mg/L/h, which was lower than that of the nitrate as sole nitrogen source. These results suggested that strain Y-11 was more efficient in nitrification with ammonium than that in denitrification with nitrate, which was inconsistent with the report that P. versutus LYM was more efficient with NO₃⁻–N than NH₄⁺–N with C/N 30 at 30 °C (Zhang et al., 2015). At a total nitrogen content of 419.17 mg/L, the removal efficiency was only 20.6%. Nitrite nitrogen, as the intermediate product in the nitrogen removal process, was not detected during the experiments. Above results indicated that strain Y-11 preferred to ammonium nitrogen in SND-1 medium and could perform simultaneous heterotrophic nitrification and aerobic denitrification with the temperature as low as 15 °C, although the nitrate nitrogen removal rate was only 0.52 mg NO₃⁻–N/L/h.
3.6. Assessment of simultaneous nitrification and denitrification performance with ammonium and nitrite nitrogen

Ammonium and nitrite nitrogen were much more poisonous than other nitrogenous compounds. Fig. 6 illustrated the bacterial growth and the nitrogen removal performance of Y-11 in SND-2 medium. Cell grew very slowly during the first 3 days and then the OD<sub>600</sub> increased quickly from 0.48 to 1.57 within the fourth day. Ammonium nitrogen decreased from 201.54 mg/L to 75.84 mg/L and the removal efficiency was 62.4% in the experiment, which correlated well with the cell growth characteristic. An insignificant decrease of nitrite nitrogen by Y-11 was observed. Approximately 6.6% of the nitrite nitrogen was removed during 4 d. These results suggested that the simultaneous heterotrophic nitrification and aerobic denitrification of strain Y-11 were inhibited when concentrations of ammonium and nitrite nitrogen were high. The heterotrophic nitrification and aerobic denitrification characteristics of strain Y-11 were inconsistent with the report that <i>P. versutus</i> LYM had the ability to remove nitrogen compounds thoroughly in presence of ammonium and nitrite nitrogen with sufficient carbon source, but nitrite could not be reduced when it served as sole nitrogen source in the medium even if C/N ratio reached as high as 30 (Zhang et al., 2015). The abilities of strain Y-11 for nitrification with ammonium and denitrification with nitrite may be improved by adding more carbon source. The total nitrogen decreased only from 412.12 mg/L to 395.43 mg/L within 4 d cultivation, which suggested that high concentration of ammonium and nitrite nitrogen was harmful for cell growth and nitrogen removal performance.

4. Conclusion

At 15 °C, <i>P. tolaasii</i> Y-11 could aerobically remove 93.6% ammonium at initial concentration of 209.62 mg/L, 93.5% nitrate at initial concentration of 204.61 mg/L and 81.9% nitrite at initial concentration of 204.33 mg/L after 4 d cultivation respectively. There was no nitrite nitrogen accumulation in the biological nitrogen removal process. The results indicated that <i>P. tolaasii</i> Y-11 was capable of both heterotrophic nitrification and aerobic denitrification. Additionally <i>P. tolaasii</i> Y-11 could efficiently remove ammonium during the simultaneous nitrification and denitrification process.

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Fig. 6. Simultaneous nitrification and denitrification characteristics of strain Y-11 with ammonium and nitrite nitrogen.


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