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# A heterotrophic nitrification-aerobic denitrification bacterium *Acinetobacter* sp. WZ-1: the superior stress resistance and the unconventional nitrogen metabolic pathways

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ABSTRACT

Heterotrophic nitrification-aerobic denitrification (HN-AD) bacteria are outstanding in nitrogen removal for wastewater treatment. In this study, *Acinetobacter* sp. WZ-1 and *Rhodococcus* sp. JY-5 were isolated and identified as HN-AD strains. The $^{15}N$ isotope experiments demonstrated that WZ-1 and JY-5 both converted inorganic nitrogen to $N_2$. Compared with JY-5, WZ-1 showed the superior ability on $NH_4^+$-N removal under environmental stress. WZ-1 removed 85.15% and 97.06% of $NH_4^+$-N at the initial pH values of 5 and 9, respectively. At the high temperature of 40 °C, $NH_4^+$-N removal efficiency by WZ-1 still reached to 80.66%. Moreover, high C/N ratios showed the significant inhibition on growth of WZ-1 and JY-5 under alkaline environment. WZ-1 still removed $NH_4^+$-N of 47.15–87.68% with the low C/N ratios of 4–8 at pH 9. It indicated the potential of WZ-1 to treat high-ammonia wastewater featured by the high pH values and low C/N ratios. The genome and intermediate products inferred the unconventional nitrification-denitrification pathway of WZ-1 strain ($NH_4^+ \rightarrow NH_2OH \rightarrow NO \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2 \rightarrow N_2$). Particularly, nitrate production is attributed to NO oxidation according the *hmp* gene. Traditional genes for denitrification (*narGH, nirKS, norBD* and *nosZ*) were not found in genome, while flavorubredoxins and NosD protein might involve in nitrite/NO reduction and $N_2O$ reduction, respectively. This study proved that certain unreported enzymes and proteins play important roles in nitrogen removal of the unconventional HN-AD bacteria.

## 1. Introduction

With the development of human production and living activities, a plenty of excessive ammonia wastewater is discharged, such as landfill leachate, animal wastewater and industrial wastewater (Jiang et al., 2021; Malhotra et al., 2022; Zhang et al., 2024). Biological process showed the advantages in cost-effectiveness, energy-consumption and environmental protection in nitrogen removal (Liu et al., 2023a). Traditional biological methods combining the aerobic nitrification and anoxic denitrification, have disadvantages in treatment efficiency, management, building cost and facility area due to the differences between nitrifiers and denitrifying bacteria on carbon source and dissolved oxygen (Song et al., 2021).

Heterotrophic nitrification-aerobic denitrification (HN-AD) bacteria can simultaneously achieve nitrification and denitrification in aerobic environment (Liu et al., 2021; Song et al., 2021). Furthermore, HN-AD bacteria can enhance inorganic nitrogen removal by denitrification when providing organic carbon as electron donors (Li et al., 2024). Therefore, the application of HN-AD bacteria is considered as the potential and easy-regulated approach to improve the efficiency and reduce costs during wastewater treatment. In recent years, several HN-AD strains have been isolated in various environment, involving in many genera of *Acinetobacter, Pseudomonas, Rhodococcus, Sphingopyxis, Alcaligenes* and *Bacillus*, and etc. (Chen et al., 2021b, 2012, 2022; Li

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et al., 2024). Most studies focused on the optimization of environmental factors to improve the nitrogen removal of HN-AD strains such as pH value, temperature, shaking speed and C/N ratio (Liu et al., 2021; Wu et al., 2022; Yao et al., 2013). However, the optimized condition usually is not matched in properties of real wastewater. It should be more important to evaluate the adaptability and resistance of HN-AD strains to stress conditions during wastewater treatment. Raw wastewater loading much ammonia generally is characterized by the high pH values (8–9) and strong alkalinity, especially in which originated from anerobic digestate (Fuldauer et al., 2018; Liu et al., 2023a). The tolerance to alkaline environment is an important factor to screen the promising HN-AD strains. Furthermore, the alkaline environment of wastewater significantly affects the bacterial growth and metabolism (Liu et al., 2019). HN-AD bacteria are heterotroph, so it is necessary to investigate the effects of C/N ratio on HN-AD strains growth and nitrogen removal at alkaline condition. Besides, it is common that the treatment efficiency of biological process significantly declines in summer and winter (Lan et al., 2025; Li et al., 2021), indicating the importance of HN-AD strains on temperature adaptability. Therefore, it is necessary to evaluate the adaptability and resistance to extreme conditions such as pH and temperature for the outstanding HN-AD strains screening.

To date, the nitrogen metabolism pathways of HN-AD bacteria have been investigated by intermediates, key genes amplification and genome sequencing. The traditional HN-AD pathways involve in many conventional genes (Li et al., 2024; Hu et al., 2023), including ammonia monooxygenase (*amo*), hydroxylamine dehydrogenase (*hao*), nitrite oxidase/nitrate reductases (*nxrAB/narGH*), nitrate reductase (*nap*), nitrite reductases (*nirKS*), nitric oxide reductase (*norBD*) and nitrous oxide reductase (*nosZ*). Many studies confirmed the traditional HN-AD pathways in isolated HN-AD strains by gene amplification, such as *Acinetobacter* sp. ND7 (*hao, napA* and *nirKS*), *Pseudomonas* sp. DM02 (*napA, nirK* and *nosZ), Acinetobacter* sp. C-13 (*napA* and *nirS*) and *Rhizobium* sp. WS7 (*amoA, napA, nirK, norB* and *nosZ*) (Chen et al., 2021a; Deng et al., 2021; Wei et al., 2022; Xia et al., 2020). In addition, the whole genome analysis was also used for the nitrogen metabolism pathways in recent years. It was found that certain HN-AD strains (*Cupriavidus metallidurans* TX6, *Pseudomonas* sp. G16 and *Acinetobacter oleivorans* AHP123) performed the HN-AD process with the absence of conventional genes like *amo, hao, nirKS, nor* and *nosZ* in genomes (Gao et al., 2023; Liu et al., 2024b; Zhou et al., 2023). It indicated that certain unreported genes and proteins participate in nitrogen removal in these unconventional HN-AD strains. Furthermore, certain HN-AD strains showed the superior resistances to environmental stress. It was reported that *Cupriavidus metallidurans* TX6 efficiently removed $NH_4^+$-N/$NH_3$—N under stress conditions of high temperature (40 °C), the extreme C/N ratios (5–50), pH values (4.5–12) and $Cu^{2+}$ stress (Liu, et al., 2024b). *Pseudomonas* sp. G16 still grew well and removed $NH_4^+$-N at the low C/N ratios of 3–5 (Gao et al., 2023). Therefore, it is meaningful to figure out the nitrogen mechanism of the unconventional HN-AD strains with the superior resistances.

In this research, two HN-AD strains were isolated and identified, and their performances were compared at different stress conditions. The study obtained a superior HN-AD strain (*Acinetobacter* sp. WZ-1) with the great adaptability and nitrogen removal. Subsequently, the nitrogen metabolism pathway of WZ-1 was figured out by the intermediate products and whole genome analysis.

## 2. Materials and methods

### 2.1. Mediums

The bacterial enrichment (BE) medium consisted of the following the components (g/L): 2 g $(NH_4)_2SO_4$, 5 g sodium citrate, 0.3 g NaCl, 0.03 g $FeSO_4 \bullet 7H_2O$, 0.03 g $MgSO_4 \bullet 7H_2O$, 1 g $KH_2PO_4 \bullet 3H_2O$ and pH 8.0. The HN-AD medium consisted of the following components (g/L): 0.47 g $(NH_4)_2SO_4$, 3.50 g sodium citrate, 0.3 g NaCl, 0.03 g $FeSO_4 \bullet 7H_2O$, 0.03 g $MgSO_4 \bullet 7H_2O$, 1 g $KH_2PO_4 \bullet H_2O$ and pH 7.0.

### 2.2. The isolation and identification of HN-AD strains

A simultaneous nitrification and denitrification bioreactor contained the activated sludge from a municipal wastewater plant, and was continuously cultivated by BE medium for 30 days with hydraulic retention time (HRT) of 48 h. The sediment sludge was added into 100 mL of HN-AD medium, and shaken at 150 rpm and 30 °C for one week. The suspension was diluted into gradient diluents ($10^{-2}$ to $10^{-6}$) by sterilized water. 50 μL diluents of $10^{-4}$ and $10^{-6}$ were spread on to HN-AD ager plates and incubated at 30 °C until visible colonies appeared. The colonies were picked up, and individually cultivated in HN-AD medium containing 50 mg/L $NaNO_2$ at 30 °C for 48 h with 180 rpm. Strains simultaneously removing $NH_4^+$-N and $NO_2^-$-N were purified by repeated streaking on HN-AD plates for five times. Subsequently, the isolated strains were measured for nitrogen removal (Section 2.3). The strains capable of $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N removal were considered as HN-AD bacteria.

The 16S rRNA gene of isolated strains was amplified using bacterial universal primers 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′), and sequenced by Sangon company (Shanghai, China). Sequence alignment was performed by nucleotide Basic Local Alignment Search Tool (BLST) on the National Center for Biotechnology Information (NCBI) website. A phylogenetic tree was constructed by MEGA 10 using neighbor-joining method.

### 2.3. Nitrogen removal by HN-AD strains

To evaluate the nitrogen removal and pathway of the HN-AD strains, three nitrogen compounds ($NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N) of 100 mg/L were used as the sole nitrogen source in HN-AD medium, respectively. 1 mL of bacterial suspensions with $OD_{600}$ value of 1.0 were added into 100 mL of HN-AD mediums, and the initial $OD_{600}$ value of HN-AD mediums after inoculation was close to 0.03. The cultivation was conducted at 30 °C for 48 h with 150 rpm, nitrogen conversion and bacterial growth were determined within 48 h.

The biomass nitrogen (bio-N) was calculated by the total nitrogen (TN) difference between centrifuged samples (10,000 rpm for 5 min) and uncentrifuged samples (Zhou et al., 2023). Gas nitrogen (gas-N) was calculated by TN, total inorganic nitrogen (TIN) and bio-N.

### 2.4. The $^{15}N$ isotope experiments

WZ-1 and JY-5 were cultivated in $^{15}N$-labeled solution of $NH_4^+$-N and $NO_2^-$-N, respectively. The cultivation was conducted in closed serum bottles (total volume: 200 mL, work volume: 100 mL), and the setting of bacterial inoculation, temperature and shaken speed were same with Section 2.3. After 48 h, gas of serum bottles was collected for $N_2$ determination. The $\delta^{15}N$-$N_2$ was measured according to the reported method (Denk et al., 2017).

### 2.5. Effects of environmental factors on $NH_4^+$-N removal performance

The isolated HN-AD strains were inoculated in sterile HN-AD medium, and cultivated at 150 rpm and 30 °C for 48 h. The bacterial suspension ($OD_{600}$: 1.0) of 1 mL was inoculated into 100 mL HN-AD medium, to investigated the effects of initial pH (4–10), temperature (10–40 °C) and initial C/N ratio (1–30) on $NH_4^+$-N removal. Particularly, the effects of C/N ratio were conducted at neutral condition (pH=7) and alkaline condition (pH=9). The pH values were adjusted by 1 M HCl and 1 M NaOH, the C/N ratios were changed by sodium citrate doage. The temperature was controlled by cultivation in a constant temperature incubator.

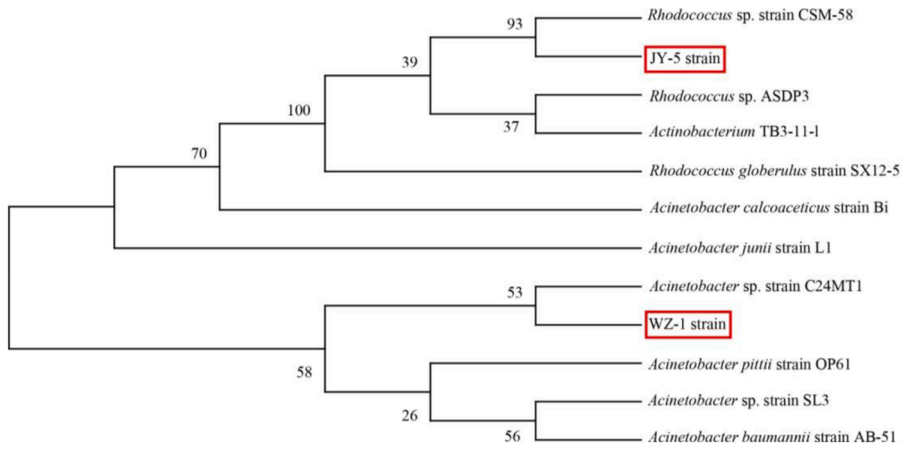


**Fig. 1.** The phylogenetic tree of the two HN-AD strains.

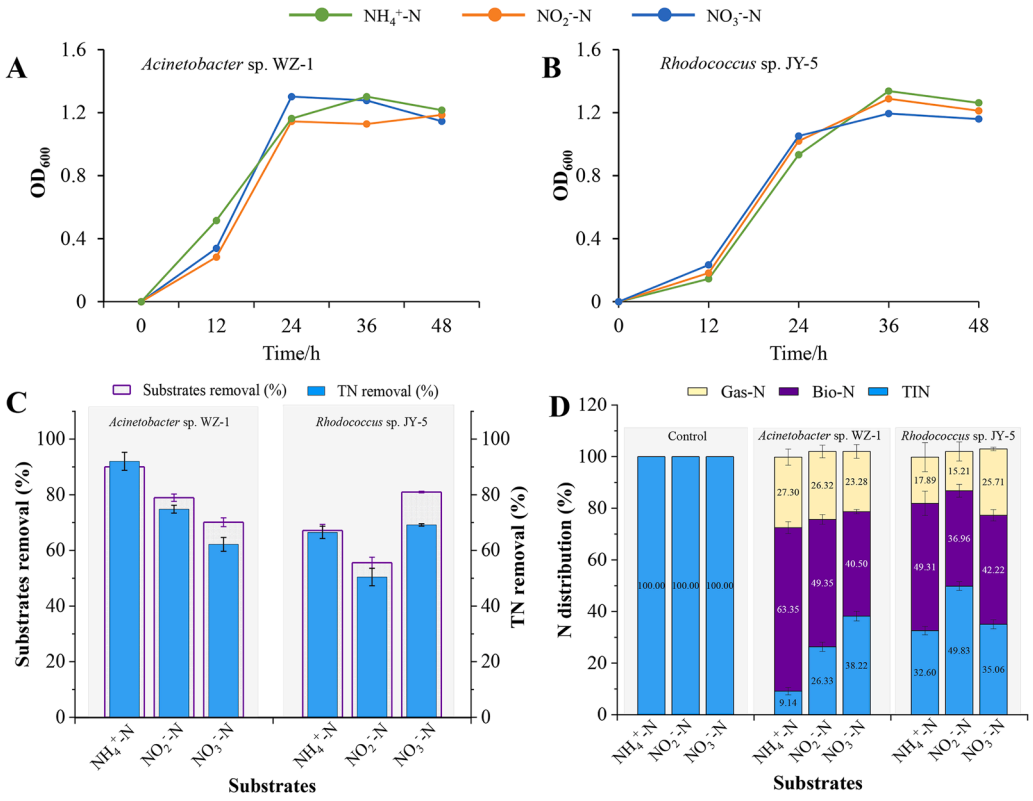


**Fig. 2.** The growth within 48 h (A, B), and nitrogen removal (C) and distribution (D) at 48 h with $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N as the sole nitrogen source, respectively.

### 2.6. Complete genome sequencing and annotation of WZ-1

The complete genome sequencing was performed by Majorbio technologies (Shanghai, China). The genome of WZ-1 was sequenced with combination of Illumina and PacBio sequencing technologies, and assembled by the unicycler software. The completeness of assembled genome was 100 % by CheckM evaluation. The whole genome data of WZ-1 has been submitted to NCBI database (accession number: PRJNA1223009). Gene annotation and functional prediction were conducted using 6 databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Non-redundant Protein Database (NR), Pfam Database, Clusters of Orthologous Groups (COG) and Swiss-Prot.

### 2.7. Analytical methods and statistical analysis

The growth of isolated strains was measured by spectrophotometry at 600 nm ($OD_{600}$). The concentrations of $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N were determined by AA3 analyzer (Bran +Luebbe), and the lowest limit of them were 0.003, 0.003 and 0.001 mg/L, respectively. Results were shown as mean ± standard deviation. TN was detected according to the protocol using HACH DR6000.

## 3. Results and discussion

### 3.1. Strains isolation and identification

Two HN-AD strains were obtained, their fragments of 16S rRNA were compared in NCBI website using nucleotide BLAST, indicating that the

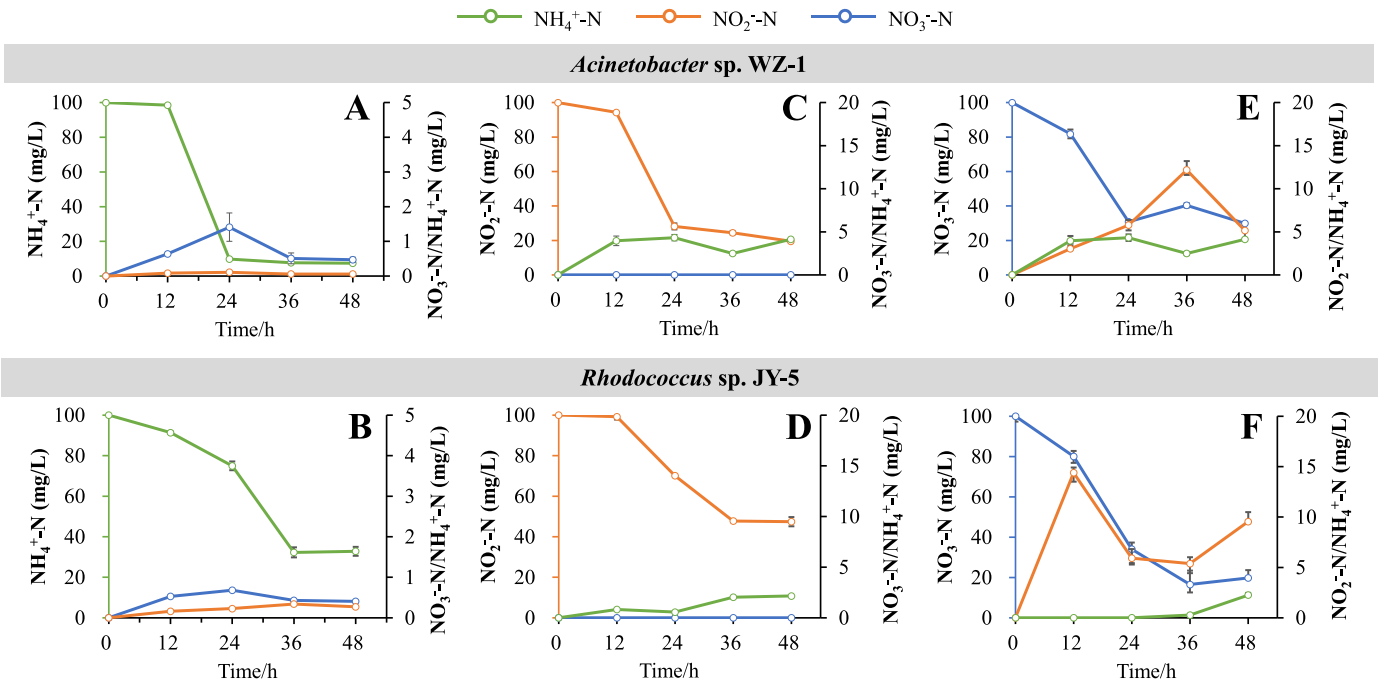


**Fig. 3.** Nitrogen conversion of WZ-1 and JY-5 in mediums of $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N as sole nitrogen source, respectively. (A, B: $NH_4^+$-N removal; C, D: $NO_2^-$-N removal; E, F: $NO_3^-$-N removal).

two strains were related to genera *Acinetobacter* and *Rhodococcus*. According to the 16S rRNA gene sequences, a phylogenetic tree was constructed in Fig. 1. It indicated that WZ-1 and JY-5 were clustered in the members of *Acinetobacter* sp. and *Rhodococcus* sp., respectively. The isolated HN-AD strains were identified and named as *Acinetobacter* sp. WZ-1 and *Rhodococcus* sp. JY-5. Further, two HN-AD strains were briefly described as WZ-1 and JY-5 in this study.

### 3.2. Nitrogen removal

Fig. 2A and B displayed that WZ-1 and JY-5 grew well in $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N as sole nitrogen source, respectively. For nitrogen removal in Fig. 2C, $NH_4^+$-N removal was almost equal to TN removal, which of WZ-1 and JY-5 were 90.03 % and 67.16 %, respectively. Differently, removal of $NO_2^-$-N and $NO_3^-$-N both was higher than TN removal, especially $NO_3^-$-N. For WZ-1, 78.95 % of $NO_2^-$-N and 70.12 % of $NO_3^-$-N were removed with TN removal of 74.82 % and 62.16 %, respectively. For JY-5, $NO_2^-$-N (55.59 %) removal and TN removal (50.43 %) were approximative, while $NO_3^-$-N removal (80.97 %) significantly exceeded TN removal (69.17 %). These results indicated that WZ-1 was more outstanding for $NH_4^+$-N and $NO_2^-$-N removal than JY-5.

As shown in Fig. 2D, nitrogen distribution results showed that WZ-1 and JY-5 removed nitrogen in the form of gas-N (15–27 %) and bio-N (36–63 %). It was consistent with several reported HN-AD bacteria. *Rhizobium* sp. WS7, *Acinetobacter indicus* ZJB20129, *Acinetobacter oleivorans* AHP123 and *Pseudomonas* sp. XF-4 where bio-N accounted for 47–89 %, and removed inorganic nitrogen in the form of gas-N only accounted for 12–28 % (Ke et al., 2022; Liu et al., 2024a; Wei et al., 2022; Zhou et al., 2023). Both this study and previous reports demonstrated that biological assimilation was the vital approach for nitrogen removal by HN-AD bacteria.

Fig. 3 showed the nitrogen conversion by two HN-AD stains in mediums of $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N as sole nitrogen source, respectively. In $NH_4^+$-N mediums, $NH_4^+$-N removal efficiencies reached to the maximums in WZ-1 and JY-5 cultivations at 24 h and 36 h, respectively. The similar phenomenon was also in $NO_2^-$-N and $NO_3^-$-N mediums. It was caused by the superior reproductive ability of WZ-1 than JY-5 (Fig. 2A and B). Besides, a small amount of $NO_2^-$-N and $NO_3^-$-N produced in $NH_4^+$-N mediums (Fig. 3A and B), indicating the nitrification process in WZ-1 and JY-5. In $NO_2^-$-N mediums, about 4 mg/L and 2 mg/L $NH_4^+$-N produced in WZ-1 and JY-5 cultivations, respectively (Fig. 3C and D). In $NO_3^-$-N mediums, $NO_2^-$-N and $NH_4^+$-N also accumulated during cultivation with the decreasing of $NO_3^-$-N concentrations (Fig. 3E and F). These results implied that WZ-1 and JY-5 were active in $NO_3^-$-N reduction and $NO_2^-$-N reduction. Meanwhile, $^{15}N$ experiments showed that $^{15}NH_4^+$-N and $^{15}NO_2^-$-N were converted to $^{15}N_2$—N by WZ-1 and JY-5 (Table 1), These results together implied the denitrification by WZ-1 and JY-5. Therefore, there were the three pathways of nitrogen transformation by WZ-1 and JY-5, including assimilation, nitrification and denitrification.

**Table 1**
The $N_2$ production of HN-AD strains for 48 h cultivation.

| Substrates | HA-ND strain | $\delta^{15}N$-$N_2$ | $N_2$ ( %) |
|---|---|---|---|
| $NH_4^+-{}^{15}N$ | WZ-1 | 30.46 | 85.59 |
| $NO_2^-{}^{15}N$ | WZ-1 | 937.03 | 84.81 |
| $NH_4^+-{}^{15}N$ | JY-5 | 65.45 | 84.03 |
| $NO_2^-{}^{15}N$ | JY-5 | 1560.50 | 88.43 |
| Air | | 0.36 | 78.08 |

$\delta^{15}N = R_{sample}/R_{standard} - 1$, R denotes the isotope ratio $^{15}N/^{14}N$. The $\delta^{15}N$ value of a sample is therefore the deviation of the sample's $^{15}N/^{14}N$ isotope ratio from the respective isotope ratio of the reference material (AIR-$N_2$ for N).

### 3.3. The resistance of WZ-1 and JY-5 to environmental stress

#### 3.3.1. pH value

As shown in Fig. 4A and B, two strains performed different abilities to $NH_4^+$-N removal under the different pH values from 4 to 10. JY-5 only adapted the narrow range from pH 6 to 9, resulting in $NH_4^+$-N removal efficiency of 64–78 %. WZ-1 grew well and exhibited efficient $NH_4^+$-N removal abilities from pH 5 to 9, with above 85 % of $NH_4^+$-N removal. Particularly, WZ-1 still was active and removed $NH_4^+$-N with 97.06 % and 85.14 % at pH 9 and pH 5, respectively. The capacity of WZ-1 was more excellent to resist the acidic (pH=5) and alkaline (pH=9)

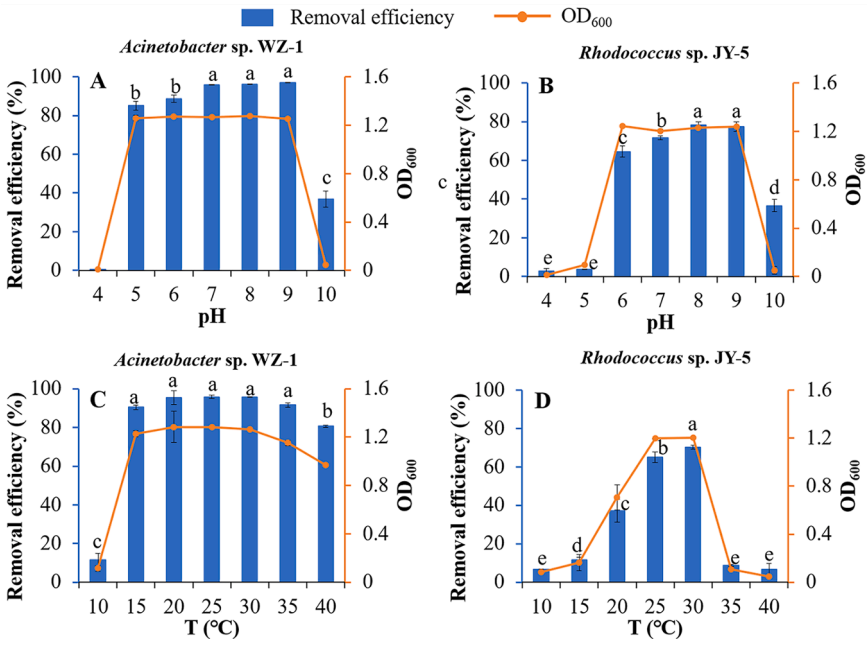


**Fig. 4.** The effects of pH and temperature on bacterial growth and $NH_4^+$-N removal (The small letters show the significance of removal efficiency at different conditions).

environments according to the significant advantages in $NH_4^+$-N removal ($p < 0.05$).

Many previous studies have demonstrated that acidic and alkaline environment both show the negative effects on nitrogen conversion bacteria. High-ammonia wastewater is characterized by massive free ammonia (FA) due to the high pH values. FA is inhibitory to ammonium and nitrite oxidation during nitrification. Many studies reported that nitrite oxidation bacteria (NOB) were more sensitive than ammonia oxidation bacteria (AOB) when suffering FA (Ma et al., 2023). It was observed that NOB activity was completely inhibited at FA concentrations of 10–15 mg/L (Sun et al., 2021). Furthermore, AOB activity was inhibited at the wide range of 10–150 mg/L FA (Liu et al., 2019). In this study, WZ-1 was active and achieve the efficient nitrogen removal at an FA concentration of 54.64 mg/L (pH=9), indicating the potential of WZ-1 to resist FA in raw wastewater treatment. In addition, acidification is inevitable during partial nitrification and denitrification (Yue et al., 2023). Many studies have found that AOB was completely inhibited with the pH decrease to 5.0–6.0 (Fumasoli et al., 2017; Le et al., 2019). Meanwhile, acidification also caused the $N_2O$ enrichment by inhibiting the reductase activity during denitrification (Ma et al.,

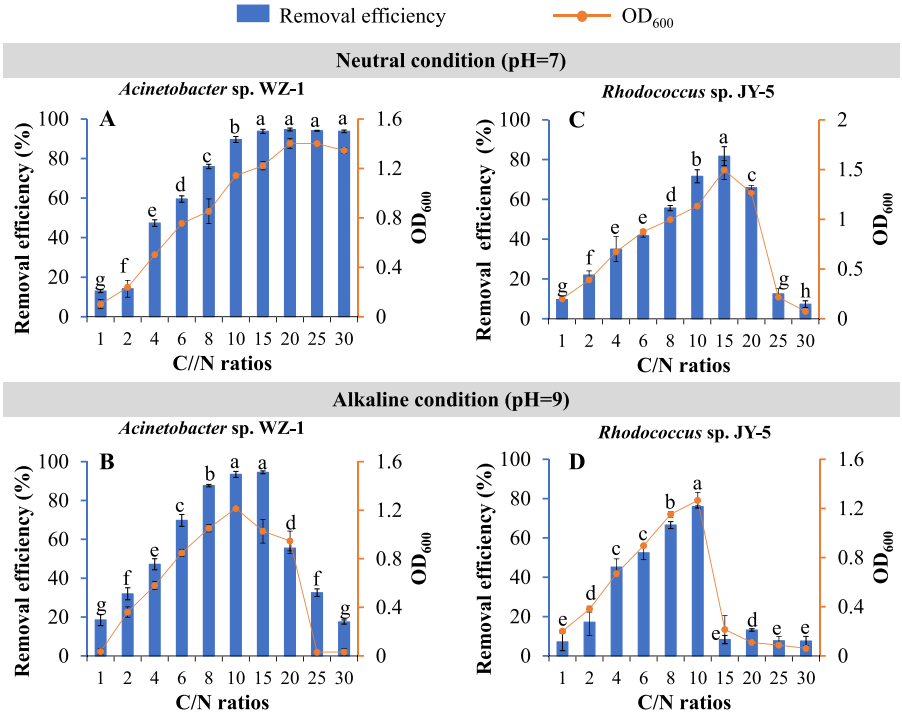


**Fig. 5.** The effects of C/N ratios on bacterial growth and $NH_4^+$-N removal under neutral and alkaline conditions (The small letters show the significance of removal efficiency at different conditions).

2023; Zhuge et al., 2020). This study showed that WZ-1 strain still was active at pH 5.0 according to the $NH_4^+$-N removal efficiency of 85.14 %, indicating the potential of WZ-1 strain for nitrogen removal.

### 3.3.2. Temperature

The effects of temperature on $NH_4^+$-N removal at neutral condition were showed in Fig. 4C and D. WZ-1 showed the excellent ability for $NH_4^+$-N removal from 15 to 40 °C. The efficiencies of $NH_4^+$-N removal exceeded 95 % in the range of 20–30 °C, and stabilized about 90 % at 15 °C and 35 °C. Particularly, WZ-1 strain still was active and removed $NH_4^+$-N of 80.66 % at 40 °C, indicating the strong adaptivity of WZ-1 to hot environment. In contrast, the growth and activity of JY-5 strain were significantly affected by temperature. JY-5 only grew well at 25–30 °C, and was significantly inhibited at 20 °C, further did not grow at 10–15 °C and 35–40 °C. Similarly, $NH_4^+$-N removal efficiency by JY-5 declined to 37.24 % at 20 °C, and was lower than 10 % at 10–15 °C and 35–40 °C. These results demonstrated the poor adaptivity of JY-5 to temperature change. Compared with JY-5, WZ-1 performed the strong adaptivity and kept the great activities in a wide range of temperature.

HN-AD process is mainly carried out under mesophilic conditions of 30–37 °C, and significantly affected by temperature variation in summer and winter (Hu et al., 2023). The resistance of WZ-1 strain for high temperature condition was superior to the most of HN-AD bacteria reported before. *Serratia marcescens* W5 and *Rhizobium* WS7 did not grow when temperature was over 30 °C (Li et al., 2024; Wang et al., 2016). Some HN-AD bacteria of *Acinetobacter* genus were able to grow at high temperature in previous research. *Acinetobacter* sp. ND7, *Acinetobacter indicus* CZH-5, *Acinetobacter* sp. C-13 and *Acinetobacter junii* YB normally grew at 30–37 °C, and conducted the efficient removal of $NH_4^+$-N (Chen et al., 2021a, 2024; Ren et al., 2014; Xia et al., 2020). It was reported that only few *Acinetobacter* strains still grew and kept activities at 40 °C. The $NH_4^+$-N removal by *Acinetobacter* sp. ND7 and *Acinetobacter indicus* ZJB20129 reduced from 93.23 % and 98.94 % to 50.73 % and 66.44 % when temperature raising from 35 to 40 °C, respectively (Xia et al., 2020; Ke et al., 2022). *Acinetobacter* sp. Z1 only removed $NH_4^+$-N of 38.6 % at 40 °C while that of 98.30 % at 30 °C (Zhou et al., 2022). Particularly, *Acinetobacter* sp. JR1 was tolerant to high temperature of 40 °C, resulting in only 20 % discount of bacterial growth and $NH_4^+$-N removal compared with that of 37 °C (Yang et al., 2019). Besides, bacteria also suffered the stronger FA stress due to high temperature in summer. In this study, WZ-1 performed the superior resistance to high temperature and FA, indicating the application potential of which for nitrogen removal in summer.

### 3.3.2. C/N ratios

Carbon source is necessary to support denitrifiers growth, provide energy and serve as electron donors during denitrification process (Huang et al., 2022; Yang et al., 2019; Zhou et al., 2022). Furthermore, the variation of C/N ratio significantly influences on bacterial growth and nitrogen removal efficiency. In this study, the effects of C/N ratios on WZ-1 and JY-5 were displayed in Fig. 5. With the C/N ratios increasing from 1 to 10, $NH_4^+$-N removal efficiencies of WZ-1 and JY-5 gradually increased at neutral and alkaline conditions. Under the high C/N ratios of 15–30, $NH_4^+$-N removal efficiencies by WZ-1 exceeded 90 % at neutral condition (Fig. 5A), while significantly decreased at alkaline condition (Fig. 5B). Moreover, JY-5 showed the poor adaptive ability to high C/N ratios, resulting in tolerance limits of 20 and 10 at neutral and alkaline condition, respectively (Fig. 5C and D), respectively. These results demonstrated that high C/N ratios have negative effects on strains growth and $NH_4^+$-N removal, especially in alkaline environment. WZ-1 showed the great performance in $NH_4^+$-N removal at the low C/N ratios under alkaline environment.

Previous studies investigated the effects of C/N ratios on HN-AD bacteria at neutral condition. Most of HN-AD bacteria showed the great performance with C/N ratios of 10–20, such as *Pseudomonas aeruginosa* SNDPR-01 (Huang et al., 2022), *Acinetobacter indicus* ZJB20129 (Ke et al., 2022), *Barnettozyma californica* K1 (Fang et al., 2021) and *Acinetobacter* sp. TAC-1 (Zhao et al., 2021). Only few HN-AD strains can grow and remove $NH_4^+$-N within the wide C/N ratios. *Acinetobacter* sp. Z1 (Zhou et al., 2022) and *Serratia marcescens* W5 (Wang et al., 2016) showed the efficient activity with C/N ratios of 8–20 and 6–20, respectively. *Acinetobacter* sp. JR1 was tolerant to the high C/N ratio of 24, and nearly removed all of $NH_4^+$-N (Yang et al., 2019). In this study, WZ-1 showed the efficient growth and $NH_4^+$-N removal in the range of C/N ratios from10 to 30. In addition, WZ-1 strain still grew and achieved 47.15–87.68 % with C/N ratios of 4–8. Therefore, WZ-1 showed the strong adaptive performance with the wide range of C/N ratios at neutral condition. However, actual high-ammonia wastewater usually is featured by the high pH values of 8–9 and low C/N ratios. Therefore, it is meaningful to investigate the effects of C/N ratios on $NH_4^+$-N removal at alkaline condition. In this study, WZ-1 and JY-5 suffered growth inhibition at the high C/N ratios under pH 9. Compared to JY-5, WZ-1 was more adaptive and removed more ammonium at low C/N ratios under alkaline environment (Fig. 5B).

In this study, WZ-1 strain showed the adaptability and resistance to stress conditions such as acidic and alkaline environment, high temperature and C/N ratios. All results suggested the potential of WZ-1 for high-ammonia wastewater treatment.

**Table 2**
Gene information of nitrogen metabolism in genome of WZ-1 strain.

| Gene number | Gene name/ COG ID | Functional description | Database |
|---|---|---|---|
| Gene1448 | *nasC* | Nitrate reductase [EC:1.7.99.-] | KEGG |
| Gene1447 | *nasD* | Nitrite reductase [EC:1.7.1.4] | KEGG |
| Gene1446 | *nirB* | Nitrite reductase [EC:1.7.1.15] | KEGG |
| Gene1091 | *glnA* | Glutamine synthetase [EC:6.3.1.2] | KEGG |
| Gene0328 | *gltB* | Glutamate synthase [EC:1.4.1.13] | KEGG |
| Gene0328 | *gltD* | Glutamate synthase [EC:1.4.1.13] | KEGG |
| Gene2607 | *gdhA* | Glutamate dehydrogenase [EC:1.4.1.4] | KEGG |
| Gene0380 | *gdhA* | Glutamate dehydrogenase [EC:1.4.1.3] | KEGG |
| Gene0431 | *hmp* | Nitric oxide dioxygenase [EC:1.14.12.17] | KEGG |
| Gene0973 | *npd* | Nitronate monooxygenase [EC:1.13.12.16] | KEGG |
| Gene1116 | *narK* | Nitrate/nitrite transporter | KEGG |
| Gene0175 | *narL* | Nitrate/nitrite response regulator | KEGG |
| Gene1650 | COG3420 | Nitrous oxide reductase accessory protein nosD | COG |
| Gene0157 | COG0426 | Flavorubredoxin | COG |
| Gene2639 | COG1773 | Flavorubredoxin | COG |

### 3.4. Nitrogen metabolism pathway of strain WZ-1

In this study, WZ-1 showed the great performance on nitrogen removal and stress resistance. To further figure out the nitrogen removal pathways, the genome sequencing, assembly and annotation were conducted. The whole genome data of WZ-1 has been submitted to NCBI database (accession number: PRJNA1223009). The genome of WZ-1 is only composed of one chromosome with 3858,230 bp, genes related nitrogen metabolism were automatically annotated in KEGG database and COG database (Table 2).

WZ-1 was propertied with simultaneous nitrification and denitrification functions, but no conventional nitrification-denitrification genes were identified such as *amoABC, hao, narGHI, nirKS* and *nosZ*. In traditional nitrogen removal pathway (KO00910), *nasC* and *nirBD* genes were discovered and responsible for nitrate assimilatory reduction and nitrite dissimilatory reduction to $NH_4^+$. However, WZ-1 still removed $NO_2^-$-N in the form of $N_2$ although lacking denitrification genes of *nirKS* and *nosZ* (Table 1). Similarly, the absent of genes in denitrification processes was reported in HN-AD strains. *nirKS* and *nosZ* genes were not matched in genomes of *Pseudomonas* sp. G16 and *Acinetobacter oleivorans* AHP123,

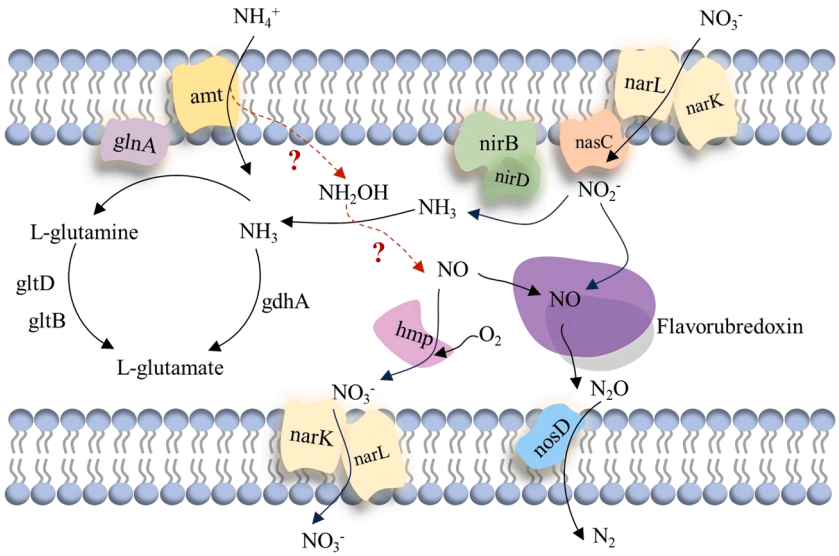


**Fig. 6.** The nitrogen metabolism of WZ-1 according to intermediate products and KEGG and COG prediction.

although $NO_2^-$-N and $NO_3^-$-N were removed in the form of gas-N (Gao et al., 2023; Zhou et al., 2023). Therefore, HN-AD strains might achieve the reduction of nitrite, NO and $N_2O$ by other oxidoreductases. Particularly, flavorubredoxins (gene0157 and gene2639) were annotated in WZ-1 via COG database. It was reported that flavorubredoxin is a new type of cytoplasmic reductase, and responsible for reduction of nitrite and NO relying on NADH as electron donors (Da Costa et al., 2003; Jin et al., 2019). Besides, the function of gene1650 was annotated as nitrous oxide reductase accessory protein NosD via COG database. Therefore, nitrite reduction to $N_2$ in WZ-1 possibly depends on the unconventional enzymes such as flavorubredoxins and NosD protein. The denitrification pathway of WZ-1 was speculated as follows: $NO_3^-\rightarrow NO_2^-\rightarrow NO\rightarrow N_2O\rightarrow N_2$.

For nitrification pathway, traditional nitrification genes (*amoABC* and *hao*) responsible for $NH_4^+$-N oxidation to $NO_2^-$-N, were not detected in WZ-1. The similar results also found in reported HN-AD bacteria, such as *Acinetobacter oleivorans* AHP123 (Zhou et al., 2023), *Sneathiella aquimaris* 216LB-ZA1–12$^T$ (Li et al., 2023), *Klebsiella* sp. KSND (Jin et al., 2019) and *Klebsiella pneumoniae* EGD-HPI9-C (Pal et al., 2015). Moreover, previous research revealed that *hao* gene is commonly lacking in most HN-AD bacteria (Li et al., 2023). In this study, a small amount of $NO_3^-$-N production (1–2 mg/L) was accompanied with few $NO_2^-$-N (< 0.1 mg/L) in $NH_4^+$-N medium by WZ-1 (Fig. 3A). It indicated that WZ-1 was able to achieve the nitrification according to the conversion of $NH_4^+$-N to $NO_3^-$-N although the missing of *amo* and *hao* genes. There might be other enzymes to achieve $NH_4^+$-N oxidation to $NH_2OH$ in WZ-1. For nitrite oxidation, previous studies reported that $NO_3^-$-N concentrations and the expression of *narGH/nxrAB* genes for nitrate reduction/nitrite oxidation significantly increased in $NO_2^-$-N medium by many HN-AD bacteria, including *Achromobacter* sp. strain HNDS-1 (Liu et al., 2023b), *Enterobacter* sp. strain HNDS-6 (Liu et al., 2023b), *Acinetobacter indicus* CZH-5 (Chen et al., 2024) and *Acinetobacter oleivorans* AHP123 (Zhou et al., 2023). However, $NO_3^-$-N was not detected in $NO_2^-$-N medium during WZ-1 cultivation (Fig. 3C), implying that WZ-1 also was not capable of nitrite oxidation in traditional nitrification. Moreover, *narGH/nxrAB* genes were not annotated according to the genome data, while *hmp* gene for NO oxidation to nitrate was found in WZ-1 genome (Table 2). Therefore, nitrate production for WZ-1 should be attributed to NO oxidation rather than traditional nitrite oxidation. Besides, few $NO_2^-$-N produced via $NO_3^-$-N reduction relying on *nasC* gene of WZ-1 in $NH_4^+$-N medium in this study. Therefore, we inferred the nitrification pathway of WZ-1 was speculated as follows: $NH_4^+\rightarrow NH_2OH\rightarrow NO\rightarrow NO_3^-$. The reported HN-AD bacteria *Acinetobacter johnsonii* ZHL01 and *Sneathiella aquimaris* 216LB-ZA1–12$^T$, were also featured by the same pathway ($NH_4^+\rightarrow NH_2OH\rightarrow NO$) with WZ-1. It requires more exploration to figure out the corresponding enzymes and genes for this bioprocess.

In summary, the HN-AD removal pathway of WZ-1 based on genome and intermediate products as follows (Fig. 6): $NH_4^+\rightarrow NH_2OH\rightarrow NO\rightarrow NO_3^-\rightarrow NO_2^-\rightarrow NO\rightarrow N_2O\rightarrow N_2$. In addition to nitrification and denitrification process, $NH_4^+$-N assimilation is also an important pathway for nitrogen removal. Genome results reveal that $NH_4^+$-N is assimilated to glutamate according to *glnA* and *gltBD* genes, and also directly converted to glutamate depending on *gdhA* genes (Table 2 and Fig. 6). The efficient assimilation benefits to improve the tolerance of HN-AD strain to the high contents of free $NH_3$ and $NH_4^+$-N in wastewater.

## 4. Conclusion

Two HN-AD strains were isolated from the active sludge for treating the municipal wastewater. *Acinetobacter* sp. WZ-1 showed the superior resistance to environmental stress than *Rhodococcus* sp. JY-5, especially in acidic and alkaline conditions, high temperature and low C/N ratios. It indicated the potential of WZ-1 strain to treat the low C/N ratio wastewater. Furthermore, the whole genome and intermediate products revealed the unconventional nitrogen removal pathways of WZ-1. Flavorubredoxin might contribute to nitrite and NO reduction, and NosD protein might be participate in $N_2O$ reduction. Besides, the unique nitrification via NO oxidation to nitrate was found in WZ-1. The inferred HN-AD removal pathway of WZ-1 was $NH_4^+\rightarrow NH_2OH\rightarrow NO\rightarrow NO_3^-\rightarrow NO_2^-\rightarrow NO\rightarrow N_2O\rightarrow N_2$.

## CRediT authorship contribution statement

**Hong Wang:** Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Peike Wu:** Investigation, Writing – original draft, Writing – review & editing. **Li Chen:** Investigation. **Lan Wang:** Supervision, Writing – review & editing. **Dan Zheng:** Supervision, Writing – review & editing. **Wenguo Wang:** Supervision, Writing – review & editing. **Liangwei Deng:** Supervision, Writing – review & editing, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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