

Article

A Newly Isolated *Rhodococcus* sp. S2 from Landfill Leachate Capable of Heterotrophic Nitrification and Aerobic Denitrification

Xianglan Chen ¹, Shuangfei Li ², Wenxuan Zhang ¹, Shaofeng Li ¹, Yurong Gu ¹ and Liao Ouyang ^{1,*}

¹ School of Materials and Environmental Engineering, Shenzhen Polytechnic University, Shenzhen 518055, China; cxlan12580@163.com (X.C.); wenxuan2727@163.com (W.Z.); lshaofeng@szpu.edu.cn (S.L.); gyr0809405018@szpu.edu.cn (Y.G.)

² Shenzhen Key Laboratory of Marine Bioresource & Eco-Environmental Sciences, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518071, China; sfli@szu.edu.cn

* Correspondence: ouyangl@szpu.edu.cn

Abstract: Nitrogen removal through heterotrophic nitrification–aerobic denitrification (HN–AD) has been acknowledged as one of the most efficient and cost-effective nitrogen removal processes. This study involved the isolation of a novel HN–AD bacterium (*Rhodococcus* sp. S2) from landfill leachate. *Rhodococcus* sp. S2 exhibited high nitrogen removal performance under aerobic conditions without the accumulation of nitrite as an intermediate. The maximum removal efficiencies for $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ were found to be $99.97 \pm 0.3\%$, $99.79 \pm 0.14\%$, and $83.53 \pm 0.31\%$, respectively. Additionally, *Rhodococcus* sp. S2 demonstrated simultaneous nitrifying and denitrifying capabilities and showed a preference for utilizing $\text{NH}_4^+\text{-N}$ in mixed nitrogen sources. The optimal nitrogen removal conditions for *Rhodococcus* sp. S2 were as follows: sodium acetate as a carbon source, a C/N ratio of 16, a shaking speed of 200 rpm, a pH of 9, and a temperature of 35 °C. Genome sequencing results revealed the presence of nitrate reductases (*NarG*), nitrate oxidoreductase (*NxrA*), and nitrite reductase (*NirBD*) in *Rhodococcus* sp. S2, providing further evidence of its HN–AD capability. In treating raw wastewater under the aforementioned experimental conditions, S2 achieved a maximum TN removal efficiency of $57.16 \pm 0.52\%$ with the addition of sodium acetate as a carbon source. These results suggest that *Rhodococcus* sp. S2 might be a promising candidate for wastewater nitrogen removal.

Keywords: *Rhodococcus* sp.; genome; heterotrophic nitrification; aerobic denitrification; wastewater



Citation: Chen, X.; Li, S.; Zhang, W.; Li, S.; Gu, Y.; Ouyang, L. A Newly Isolated *Rhodococcus* sp. S2 from Landfill Leachate Capable of Heterotrophic Nitrification and Aerobic Denitrification. *Water* **2024**, *16*, 431. <https://doi.org/10.3390/w16030431>

Academic Editor: Wojciech Czekala

Received: 4 January 2024

Revised: 24 January 2024

Accepted: 26 January 2024

Published: 29 January 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Nitrogen removal is essential for averting adverse environmental and human health impacts stemming from improper wastewater disposal. Biological nitrogen removal is widely employed in sewage treatment due to its advantages over physical and chemical treatment technologies, such as its efficient removal capacity, cost-effectiveness, and the absence of secondary pollution [1]. The conventional process for biological nitrogen removal involves aerobic nitrification and anoxic denitrification. Nitrifiers oxidize ammonium to nitrite or nitrate under aerobic conditions, while anoxic denitrifiers convert nitrate and nitrite into N_2 under anoxic conditions [2–4]. Performing these two processes requires different facilities and operating conditions, leading to increased facility costs, energy consumption, and time requirements. Heterotrophic nitrification–aerobic denitrification (HN–AD) bacteria have the ability to simultaneously carry out nitrification and denitrification under aerobic conditions [5]. They offer significant advantages owing to their faster growth rate, environmental adaptability, and capacity to utilize organic compounds. Consequently, research on HN–AD bacteria has garnered increasing attention in recent years.

At present, numerous HN–AD bacteria, such as *Klebsiella* sp. [6], *Pseudomonas* sp. [7], *Acinetobacter* sp. [8], *Chryseobacterium* sp. [9], and *Thauera* sp. [10], have been isolated and

studied. However, a study found that there was a significant build-up of nitrite in the biological processes involved in HN-AD when both ammonia and nitrate were used as nitrogen sources, ultimately resulting in the retention of total nitrogen (TN) [11–13]. The accumulation of nitrite in the reported HN-AD bacteria may be attributed to two factors: (a) the lack of specific genes encoding nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) or reductase damage [14] and (b) the impact of environmental factors, such as high dissolved oxygen (DO) (3 mg L^{-1}) or low-carbon sources ($\text{C/N} = 2.5$), on gene expression and enzyme activity, leading to incomplete metabolic processes [15]. Moreover, in other studies, even a low concentration of nitrite accumulation, such as 2.04 mg L^{-1} , was found to be toxic to certain environmental bacteria (such as *Paracoccus versutus* LYM and *Pseudomonas stutzeri* D6), with it inhibiting their ability to remove nitrogen and limiting their application in wastewater treatment [4,16]. Consequently, it is of great significance to explore new HN-AD bacteria that can address multiple nitrogen contaminants without accumulating nitrite.

It has been reported that some HN-AD bacteria exhibit effective nitrogen removal performance even under extreme conditions, including low pH (3–6), low temperature ($5\text{--}15^\circ\text{C}$), or high salinity ($30\text{--}50 \text{ g L}^{-1}$) [17]. Landfill leachate, one of the most complex forms of urban waste, has garnered attention due to its high concentrations of ammonium ($50\text{--}2200 \text{ mg/L}$), high salinity, and high chemical oxygen demand (COD) concentration (commonly $\leq 4000 \text{ mg L}^{-1}$) and the presence of heavy metals (0.01 to $1.0 \times 10^4 \text{ mg/kg}$) [18–20]. Landfill leachate may serve as an ideal source for salt/heavy metal-resistant HN-AD bacteria. Furthermore, HN-AD bacteria existing in landfill leachate may be able to adjust to high ammonium concentrations. In previous studies, HN-AD bacteria such as *Stenotrophomonas maltophilia* DQ01 [21] and *Alcaligenes faecalis* TF-1 [22] were isolated from landfill leachates and exhibited good performance in carrying out nitrogen removal. However, although these strains showed considerable nitrification ability, they only showed low denitrification ability. Furthermore, it remains unclear as to how a high initial $\text{NH}_4^+\text{-N}$ concentration affects HN-AD bacteria. On the other hand, functional genes in the nitrogen metabolism pathways of HN-AD are worth investigating, with such investigations being useful in understanding the HN-AD mechanism of different strains. Hence, the separation and screening of microbes with efficient nitrogen removal capacity from landfill leachate offer significant potential for application.

In this study, our aim is to investigate a high-performance HN-AD bacterium that can remove nitrogen without nitrite accumulation under aerobic conditions. In this experiment, *Rhodococcus* sp. S2 was isolated from landfill leachate collected from a wastewater treatment plant in Shenzhen, China. Complete genome sequencing was conducted to explore the key genes related to the HN-AD process and elucidate its nitrogen metabolism mechanism. Additionally, the nitrogen removal capacity of S2 was analyzed through the use of different nitrogen sources. The optimal growth conditions of S2 were investigated under various carbon sources, carbon-to-nitrogen ratio (C/N), dissolved oxygen (DO), pH, temperature, and initial $\text{NH}_4^+\text{-N}$ concentrations. Finally, S2 was introduced to actual sewage to verify its nitrogen removal capacity.

2. Materials and Methods

2.1. Media

During the bacterial screening process, Luria–Bertani (LB) media were utilized, and bromothymol blue (BTB) agar plates were employed to screen aerobic denitrifiers. The specific formulation of these two media matched that of Wei et al. [18]. The heterotrophic nitrification medium (HNM) was employed to measure nitrification capacity and consisted of the following composition per liter: 4.052 g sodium succinate, 0.47 g $(\text{NH}_4)_2\text{SO}_4$, 0.25 g K_2HPO_4 , 0.125 g NaCl , 0.125 g $\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$, 0.0025 g $\text{MnSO}_4 \cdot 4 \text{ H}_2\text{O}$, 0.0025 g $\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$, and pH 7.5

2.2. Isolation and Identification

Landfill leachate samples were collected from a wastewater treatment plant (Lao-hukeng) in Shenzhen, China, in November 2019. The initial $\text{NH}_4^+\text{-N}$ and COD concentrations were 1200 and 40,000 mg/L, respectively. A 10 mL sample was mixed with 90 mL sterilized 0.9% NaCl in a 250 mL flask and cultured at 30 °C at 150 rpm. After shaking for 8 h, a 10 mL suspension was added to 90 mL LB medium and incubated for 7 days. For subsequent selection, a 2% portion of the bacterial suspension was transferred to 150 mL sterilized HNM and incubated for 3 days. This procedure was repeated three times. The obtained supernatant (100 μL) was diluted 10^{-4} -, 10^{-5} -, 10^{-6} -, and 10^{-7} -fold by distilled water, and then, 200 μL diluent was applied to the HNM plate reproduction for 3 days at 30 °C. The selected isolates were cultured in HNM and then on BTB plates at 30 °C for 24 h. Colonies with blue circles were separated and kept in 25% glycerol solution at -80 °C.

The genomic DNA of strain S2 was extracted using a TIANamp Bacteria DNA Kit (TIANGEN Biotech, Beijing, China). The PCR product was obtained using a TaKaRa 16S rRNA Genetic Identification PCR Kit (TaKaRa, Japan) with universal primers 27F and 1492R. Subsequently, it was sent to BGI Company (Shenzhen, China) for sequencing. The corresponding phylogenetic tree was constructed using MEGA 7 software based on BLAST analysis.

2.3. Complete Genome Sequencing and Functional Annotation

S2 was cultured in LB media at 30 °C and shaken at 150 rpm for 16 h. The DNA was extracted as described previously. The concentration of extracts was detected using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA), and the purity and integrity were assessed using a Qubit fluorometer (Thermo Scientific, USA). The sequencing library was constructed by BGI Company (Shenzhen, China) using a Nanopore sequencing platform (Oxford Nanopore Technologies, Oxford, UK). The fragment size and library concentration were determined using an Agilent 2100 Bioanalyzer. Functional genes in the nitrogen metabolism pathways of S2 were investigated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.4. Factors Affecting N Removal Capacity

After being enriched with LB media for 15 h, S2 was centrifuged at 8000 rpm for 10 min to obtain bacterial pellets. The pellets were resuspended in sterilized NaCl (0.9%) to obtain the experimental bacterial suspensions ($\text{OD}_{600} = 0.8$). A 3 mL bacterial suspension was added to 150 mL HNM and cultured for up to 72 h at 30 °C and 150 rpm to detect the nitrification capacity. Samples were taken at different times (0 h, 4 h, 8 h, 12 h, 1 day, 2 days, and 3 days) to measure the OD_{600} value and nitrogen concentration ($\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$). Next, to determine the aerobic denitrification capacity of S2, experiments were conducted through S2 incubation in HNM media with $(\text{NH}_4)_2\text{SO}_4$ replaced by NaNO_2 or KNO_3 . S2 was also cultured in HNM with mixed nitrogen sources (with the addition of 100 mg L^{-1} of $(\text{NH}_4)_2\text{SO}_4$, NaNO_2 , and KNO_3) to detect the simultaneous nitrification and denitrification capacity. Additionally, experiments were performed to identify the factors affecting ammonium removal efficiency. Sodium succinate in HNM was substituted by glucose, sucrose, sodium acetate, and sodium citrate to explore the impact of the carbon sources on S2. Based on the HNM medium, the C/N ratio was set to 4, 8, 12, 16, and 20, while the shaking speeds were adjusted from 50 to 250 rpm to explore the effects of the C/N ratio and shaking speed. Furthermore, the initial pH was set at 5, 6, 7, 8, 9, and 10 to examine the effect of pH. The temperatures were varied from 20 to 40 °C to assess the effect of temperature. Lastly, the initial $\text{NH}_4^+\text{-N}$ concentrations were set to 5, 10, 20, 100, and 300 mg L^{-1} to explore the tolerance of S2 to high ammonium concentrations. The variables, other than those mentioned, were kept the same throughout the experiment.

2.5. Nitrogen Balance Experiment

During this experiment, 0.6 mL of bacterial suspension was inoculated into 30 mL HNM in 60 mL vials to prepare the samples for N_2 determination and nitrogen balance during the ammonium removal process. Sodium acetate was used as the sole carbon source, and the initial C/N ratio, pH, and DO concentration were 16, 9, and 6.2 mg L^{-1} , respectively. The initial NH_4^+-N concentration was set at 100 mg L^{-1} . The vial was sealed and incubated for 24 h at 35°C and 200 rpm. Approximately 1 mL of $ZnCl_2$ (50%, *w/v*) was added to the vial to end the reaction at 0 and 24 h, respectively. The N_2 production in the vials was determined using a gas chromatography (GC) system (Agilent 7890B, Agilent, Technologies, Lexington, MA, USA). Some parallel vials were prepared to determine the nitrogen balance in this reaction system, and cultures were collected at 0 h and 24 h to determine the TN concentration. Biomass nitrogen was calculated by deducting the TN of cultures following the filter ($0.22 \mu\text{m}$, Jinteng, China) from the TN of unfiltered cultures.

2.6. Nitrogen Removal Performance of S2 on Sewage

The sewage sample was taken from the Guanlan Sewage Treatment Plant in Shenzhen, China. To examine the nitrogen removal performance of S2 on sewage, the bacterium was cultured in a 1 L flask with a 500 mL sewage sample at a 2% inoculation ratio. The initial pH of the sewage was 7.44. Sodium acetate was added to achieve an initial C/N ratio of 16. The flasks were shaken at 200 rpm at 35°C for up to 18 days. Samples were collected at different time points and centrifuged, and the supernatants were analyzed for NH_4^+-N , NO_2^--N , NO_3^--N , and TN. Additionally, sewage samples (500 mL) without S2 inoculation, as well as sterilized sewage samples (500 mL) with S2 inoculation incubated under the same conditions, served as controls.

2.7. Analytical Methods

The growth trend of S2 was measured using a multifunctional microplate reader (Lx, BioTek, VT, USA) at 600 nm. The concentrations of NH_4^+-N , NO_2^--N , NO_3^--N , and TN were calculated with reference to APHA's methods by using Berthelot colorimetry, N-(1-naphthalene)-diaminoethane dihydrochloride, sulfamic acid, and alkaline potassium persulphate methods, respectively [19]. Biomass nitrogen was tested using Roun et al.'s method [20], deducting the TN of inoculated media following the filter ($0.22 \mu\text{m}$, Jinteng, China) from the TN of the unfiltered samples.

3. Results and Discussion

3.1. Isolation and Identification of Strain S2

Strain S2 appeared as a rod-shaped bacterium (Figure 1a) with reddish brown, circular-shaped colonies, a rough surface, and complete edges (Figure 1b). Moreover, a 1322 bp 16S rRNA gene sequence was amplified and obtained by means of PCR. The BLAST results indicated 100% homology between the strain S2 and *Rhodococcus* sp. S18 (MN859993.1), and a phylogenetic tree constructed using the neighbor-joining method confirmed the identification of strain S2 as *Rhodococcus* sp. (Figure 1c).

Some researchers have reported that *Rhodococcus* sp., a Gram-positive bacterium, could be used in bioremediate environmental pollutants [21–23]. It has been found to exhibit impressive tolerance to various environments such as coking wastewater [24], Antarctic sea ice [25], and polluted soil [26]. However, only a few studies have confirmed the HN–AD capability of *Rhodococcus* sp. Previously, *Rhodococcus erythropolis* strain Y10 isolated from surface flow constructed wetlands (SFCWs) was proven to have heterotrophic ammonium assimilation and aerobic denitrification capacity [27]. *Rhodococcus* sp. CPZ 24 isolated from swine wastewater also performed simultaneous nitrification and denitrification [28]. The isolation of *Rhodococcus* sp. S2 in this study extends the important role of *Rhodococcus* sp. in the nitrogen removal process.

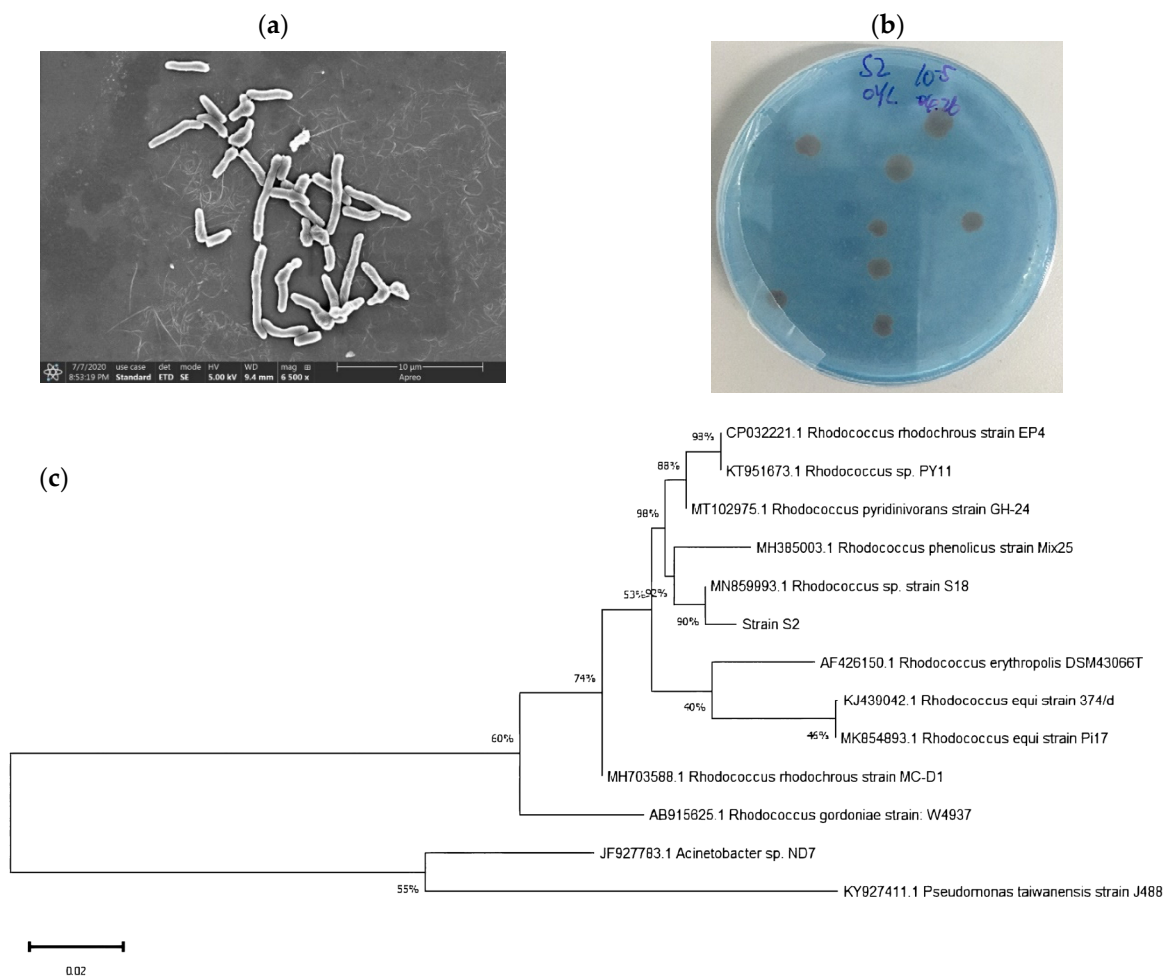


Figure 1. Scanning electron micrograph (6500×) (a), the colony on the BTB plate (b), and the phylogenetic characteristics of *Rhodococcus* sp. S2 (c).

3.2. Heterotrophic Nitrification and Aerobic Denitrification Capacity of S2

The heterotrophic nitrification capacity of strain S2 was studied using $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source. As depicted in Figure 2a, the ammonium-N removal capacity of S2 exhibited a close relationship with cell growth.

In the first 8 h, S2 exhibited a low ammonium-N removal rate of $1.62 \pm 0.07 \text{ mg L}^{-1}\text{h}^{-1}$. However, its removal rate increased significantly to a maximum of $2.08 \pm 0.03 \text{ mg L}^{-1}\text{h}^{-1}$ after 48 h of incubation, surpassing that of *Bacillus* sp. LY ($0.43 \text{ mg L}^{-1}\text{h}^{-1}$) [14], *Pseudomonas* sp. ADN-42 ($1.38 \text{ mg L}^{-1}\text{h}^{-1}$) [29], and *Pseudomonas tolaasii* Y-11 ($2.04 \text{ mg L}^{-1}\text{h}^{-1}$) [30]. Although the ammonium-N removal rate of S2 was lower than *Rhodococcus* sp. CPZ 24 ($3.10 \text{ mg L}^{-1}\text{h}^{-1}$) [28], notably, there was no significant accumulation of nitrite or nitrate during the NH_4^+ -N removal process, indicating the effective removal of nitrogen and demonstrating the potential denitrification capacity of S2. Similarly, *Ochrobactrum anthropic* LJ81 [12] and *Acinetobacter junii* YB [31] showed no intermediate product accumulation during the ammonium removal process, with the pH gradually rising from 7.68 to 9.69 at 72 h due to alkali production in the denitrification process. This shift in pH is supported by the findings of Zhu et al., who demonstrated that minimal pH fluctuations could create a favorable environment for bacterial growth [32]. These results indicate that the S2 strain possesses excellent heterotrophic nitrification ability; a high pH value ($\text{pH} > 8$) could lead to the conversion of ammonium to ammonia.

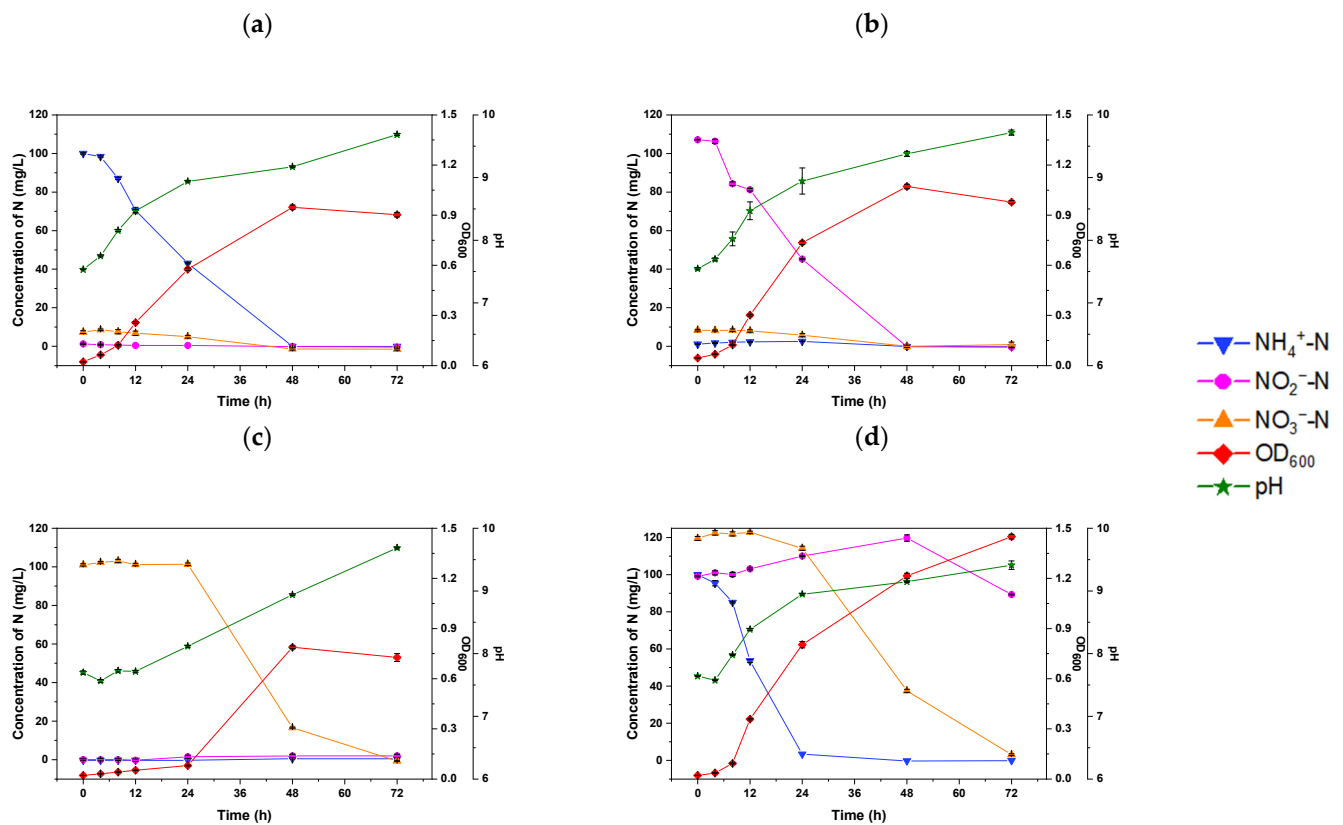


Figure 2. The changes in nitrogen concentration in HNM-cultured *Rhodococcus* sp. S2 with the addition of (a) 100 mg/L $\text{NH}_4^+\text{-N}$, (b) 100 mg/L $\text{NO}_2^-\text{-N}$, (c) 100 mg/L $\text{NO}_3^-\text{-N}$, and (d) a mixture of N sources (100 mg/L $\text{NH}_4^+\text{-N}$, 100 mg/L $\text{NO}_2^-\text{-N}$, and 100 mg/L $\text{NO}_3^-\text{-N}$).

To evaluate aerobic denitrification ability, the experiment was conducted using HNM, wherein $(\text{NH}_4)_2\text{SO}_4$ was replaced with NaNO_2 or NaNO_3 . As illustrated in Figure 2b,c, the bacteria in the two groups exhibited a similar growth phenomenon that was closely related to the nitrogen removal rates, albeit with significant differences between the two substrates. When S2 was cultured with nitrite as the sole nitrogen source, it displayed an OD₆₀₀ of 1.11 ± 0.01 and $99.95 \pm 0.14\%$ $\text{NO}_2^-\text{-N}$ removal efficiency (Figure 2b). Conversely, S2 cultured with NaNO_3 experienced a 24-h lag phase, reaching its maximum biomass (OD₆₀₀ = 0.79 ± 0.008) and $\text{NO}_3^-\text{-N}$ removal efficiency of $83.53 \pm 0.3\%$ after 48 h (Figure 2c). These results suggest that strain S2 can utilize nitrate as a nitrogen source, although it requires more time to adapt to this condition, resulting in an extended lag phase (Figure 2c). This phenomenon has been well-documented in various HN-AD strains [33–35].

Moreover, the removal capacity of S2 was tested in a mixed nitrogen medium containing $(\text{NH}_4)_2\text{SO}_4$, NaNO_2 , and NaNO_3 . In Figure 2d, the growth rate of S2 in the mixture media surpassed that of the medium with a singular nitrogen source, with it entering the logarithmic growth phase with a maximum OD₆₀₀ value of 1.45 ± 0.01 at 72 h, indicating the higher removal efficiency of ammonium-N at 24 h ($96.60 \pm 0.43\%$) (Table 1).

Table 1. Maximum nitrogen removal efficiencies and rates of *Rhodococcus* sp. S2 using different nitrogen sources (C/N = 12, shaking speed of 150 rpm, initial pH of 7.5, sodium acetate as the carbon source, and incubated at 30 °C).

N Source	NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N	NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N
	(%) ^a			(mg L ⁻¹ h ⁻¹) ^b		
NH ₄ ⁺ -N	99.97 ± 0.3 (48 h)			2.08 ± 0.03 (48 h)		
NO ₂ ⁻ -N		99.76 ± 0.14 (48 h)			1.77 ± 0.01 (48 h)	
NO ₃ ⁻ -N			83.53 ± 0.31 (48 h)			2.10 ± 0.02 (48 h)
Mixed nitrogen source	96.53 ± 0.05 (24 h)	8.39 ± 0.25 (72 h)	97.40 ± 0.37 (72 h)	4.02 ± 0.02 (24 h)	0.14 ± 0.003 (72 h)	3.53 ± 0.03 (72 h)

^a maximum removal efficiency; ^b maximum removal rate.

The NH₄⁺-N removal rate of $4.17 \pm 0.69 \text{ mg L}^{-1} \text{ h}^{-1}$ in the relevant condition was found to be higher than that in the medium containing ammonium ($2.46 \pm 0.13 \text{ mg L}^{-1} \text{ h}^{-1}$). Subsequently, S2 initiated the removal of NO₃⁻-N and achieved $97.40 \pm 0.37\%$ removal efficiency at 72 h. The decrease in NH₄⁺-N and NO₃⁻-N resulted in an increase in NO₂⁻-N from $99.10 \pm 0.28 \text{ mg L}^{-1}$ to $119.72 \pm 1.72 \text{ mg L}^{-1}$ at 48 h, followed by a gradual reduction to $89.30 \pm 0.25 \text{ mg L}^{-1}$ at 72 h. These results underline the efficient HN-AD capacity of S2 in utilizing the three nitrogen sources. An additional experiment using sealed vials was carried out to confirm the production of N₂ gas and the nitrogen balance during the NH₄⁺-N removal process. During this experiment, the TN concentration dropped from $88.77 \pm 2.69 \text{ mg L}^{-1}$ to $71.44 \pm 0.10 \text{ mg L}^{-1}$ between 0 and 24 h; however, only $2.21 \pm 0.41 \text{ mg L}^{-1}$ of biomass nitrogen accumulated (Table S1). These findings suggest that the majority of the N was removed as N₂ gas. The proportion of N₂ in the vial increased from $79.70 \pm 0.26\%$ to $89.30 \pm 0.64\%$, further confirming S2's aerobic denitrifying ability (Fig.S1). Overall, the results provide evidence that bacterial denitrification primarily drove ammonium removal, demonstrating S2's high nitrogen removal capacity.

3.3. Factors Affecting the Ammonium Removal Efficiency of the S2 Strain

3.3.1. Influence of Carbon Sources

Organic carbon is consistently considered an energy resource for microorganisms to proliferate, yet different species exhibit a distinct preference for organic substrates [36]. To identify the optimal carbon source for S2, experiments were conducted with various carbon sources at a C/N ratio of 12:1 (Figure 3a). Among these, the medium containing sodium acetate exhibited the highest NH₄⁺-N removal rate ($90.05 \pm 0.34\%$) and supported the most notable cell growth (with an OD600 peak of 0.79 ± 0.01) within 48 h.

It has been documented that carbon sources with a simple structure and low molecular mass may be more suitable for bacterial growth and as electron donors in the nitrification process due to their ability to readily participate in the straightforward biochemical pathway of carbon source utilization [4,17,31]. Acetate can be utilized by the majority of the known HN-AD bacteria. Considering the growth and NH₄⁺-N removal rate, sodium acetate was selected for the subsequent experiment.

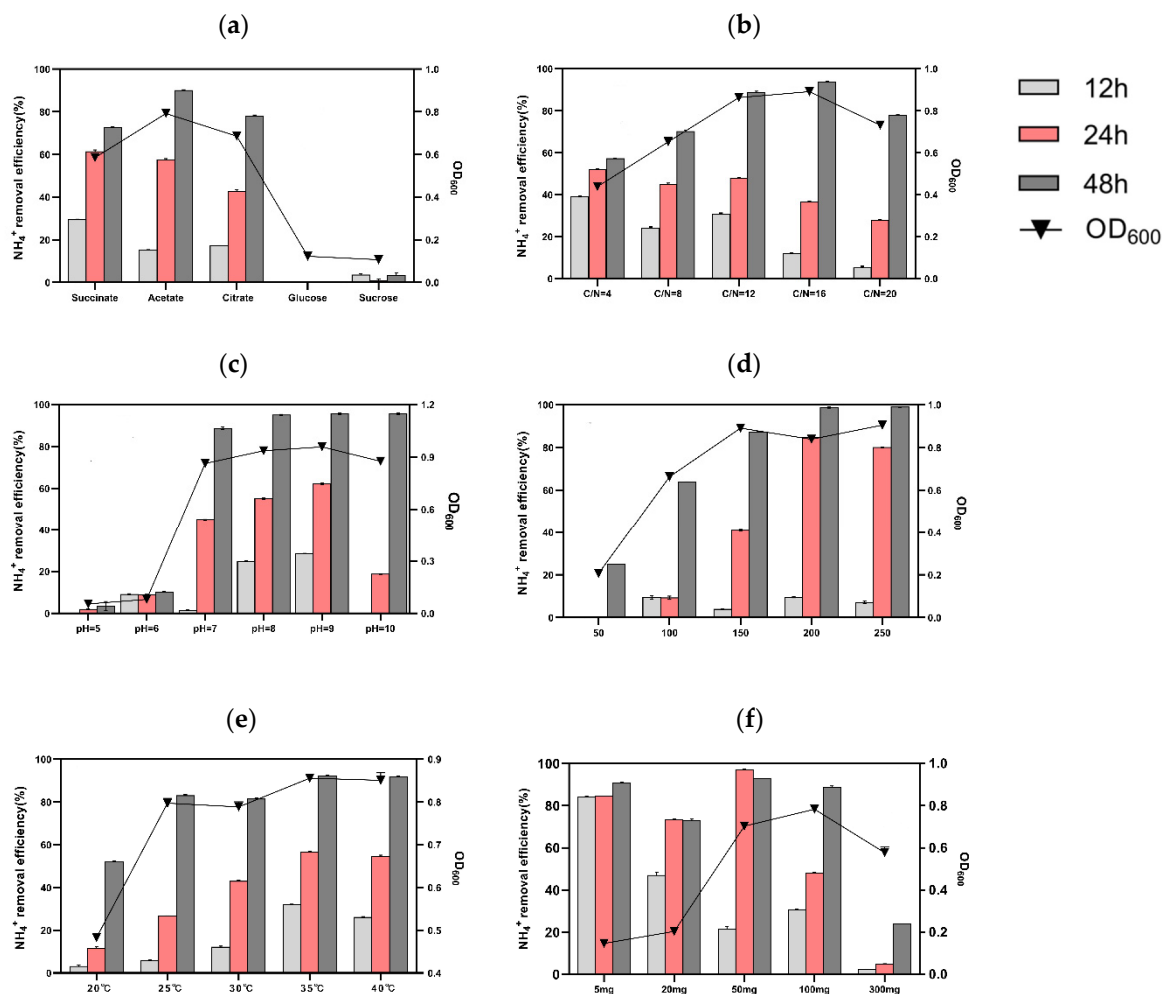


Figure 3. Influencing factors of (a) carbon source, (b) C/N ratio, (c) pH, (d) shaking speed, (e) temperature, and (f) initial NH_4^+ -N concentration on the growth and nitrogen removal capacity of *Rhodococcus* sp. S2 incubated for 12, 24, and 48 h.

3.3.2. Carbon–Nitrogen Ratio

It is generally reported that most HN–AD bacteria require a higher carbon–nitrogen ratio (C/N) to achieve high nitrogen removal efficiency, and a low C/N ratio results in poor HN–AD performance [36]. Therefore, the NH_4^+ -N removal efficiency of S2 was tested under different C/N ratios (Figure 3b). The results demonstrated an improvement in removal efficiency from $57.22 \pm 0.28\%$ to $93.85 \pm 0.2\%$ when the C/N ratio increased from 4 to 16. Additionally, the OD_{600} value rose to 0.89 ± 0.01 at a C/N ratio of 16. However, when the C/N ratio was adjusted to 20, the NH_4^+ -N removal efficiency slightly decreased. Therefore, the optimal C/N ratio for strain S2, considering energy savings, was 16. These findings align with the high NH_4^+ -N removal capacity exhibited by *Rhodococcus erythropolis* Y10 [27] and *Acinetobacter indicus* ZJB20129 [3] with a C/N ratio of 15.

3.3.3. Initial pH

The impact of pH on strain S2's growth and nitrogen removal capacity is illustrated in Figure 3c. The growth of strain S2 was poor at pH 5 and 6 (OD_{600} values of 0.05 and 0.08, respectively), resulting in removal efficiencies of only $3.45 \pm 1.31\%$ and $10.23 \pm 0.28\%$ within 48 h. In contrast, at pH levels between 7 and 10, the OD_{600} values were 0.86, 0.94, 0.96, and 0.86, with respective maximum removal efficiencies reaching up to $88.67 \pm 0.18\%$, $95.03 \pm 0.29\%$, $95.66 \pm 0.16\%$, and $95.65 \pm 0.56\%$ within 48 h. These results indicate that

strain S2 is an alkaline HN-AD bacteria, consistent with *Marinobacter* NNA5 [37]. However, a high pH value (>8) might result in the conversion of ammonium to ammonia.

3.3.4. Dissolved Oxygen

The influence of dissolved oxygen content on ammonium removal efficiency by S2 was investigated by varying the shaking speed, as depicted in Figure 3d. When the rotational speed was adjusted from 50 to 200 rpm, the nitrogen removal efficiency improved from $24.95 \pm 0.05\%$ to $98.76 \pm 0.37\%$. Furthermore, the OD₆₀₀ peak at 200 rpm reached 0.84 ± 0.01 . Notably, at a speed of 250 rpm, the NH₄⁺-N removal efficiency was consistent with that at 200 rpm, indicating that S2 could tolerate a high oxygen concentration, in line with findings on *Acinetobacter tandoii* MZ-5 [38] and *Pseudomonas putida* NP5 [39]. It has been reported that a higher or lower DO concentration has a negative impact on the nitrogen removal performance of HN-AD bacteria [40]. Hence, the shaking speed of 200 rpm was conducive to faster growth and a more efficient ammonium removal rate.

3.3.5. Temperature

The susceptibility of HN-AD bacteria to temperature variation, affecting cell membrane fluidity and enzyme activity, was reported in one example study [11]. In this study, we investigated the influence of temperature on strain S2, and the experimental results are presented in Figure 3e. It is evident that strain S2 exhibited a temperature-dependent response within the nitrogen removal range of 20–35 °C. At 20 °C, the maximum removal efficiency and OD₆₀₀ value were approximately $52.73\% \pm 0.18$ and 0.482 ± 0.01 within 48 h, demonstrating that the low temperature primarily slowed down microorganisms' propagation, leading to a reduction in ammonium removal efficiency. Conversely, at 35 °C, the OD₆₀₀ and maximum removal efficiency reached 0.86 ± 0.01 and $92.5\% \pm 0.18$, respectively. Interestingly, at 40 °C, the NH₄⁺-N removal capacity of S2 was lower than at 35 °C ($27.47\% \pm 0.31$ vs. $35.49\% \pm 0.18$ at 12 h). These results indicate that the optimal culture temperature of S2 was 35 °C, consistent with the mesophilic nature of microorganisms [17].

3.3.6. Initial NH₄⁺-N Concentration

The nitrogen removal capacity of strain S2 was assessed at initial NH₄⁺-N concentrations ranging from 5–300 mg/L, as shown in Figure 3f. The cell growth demonstrated a gradual increase with rising NH₄⁺-N concentration within the range of 5–100 mg L⁻¹. Notably, at 24 h, when the initial NH₄⁺-N concentrations were set to 5, 20, and 50 mg/L, the nitrogen removal efficiencies were $84.47\% \pm 0.12$, $73.36\% \pm 0.26$, and $97.05\% \pm 0.49$, respectively. Conversely, under the conditions of 100 and 300 mg/L NH₄⁺-N, the maximum removal efficiencies for 48 h were $88.67\% \pm 0.65$ and $23.93\% \pm 0.12$, with OD₆₀₀ values reaching 0.78 ± 0.01 and 0.58 ± 0.02 , respectively, suggesting that high concentrations of ammonium nitrogen inhibited cell growth and nitrogen removal performance. These findings align with previous reports indicating that ammonium becomes an inhibitor above a certain threshold, hindering microorganism growth during wastewater treatment [12,41]. Our results also concur with *Acinetobacter tandoii* MZ-5, which displayed robust growth at a wide range of NH₄⁺-N concentrations [38], suggesting the potential applicability of strain S2 in wastewater treatment.

3.4. Genome Sequencing and KEGG Pathway Analysis

The length of strain S2's genome is 5,295,328 bp, with a GC content of 68.14%, as shown in Figure 4. Through gene prediction, the total gene length was determined to be 5,495,886 bp, with a GC content of 68.02%. Of the 5822 annotated genes, 53 were tRNA genes, and 12 were rRNA genes. The number of genes annotated in different databases varied, yielding the following results: Swiss-Prot ($n = 2061$), NR ($n = 5516$), GO ($n = 3204$), COG ($n = 3947$), and KEGG ($n = 3092$).

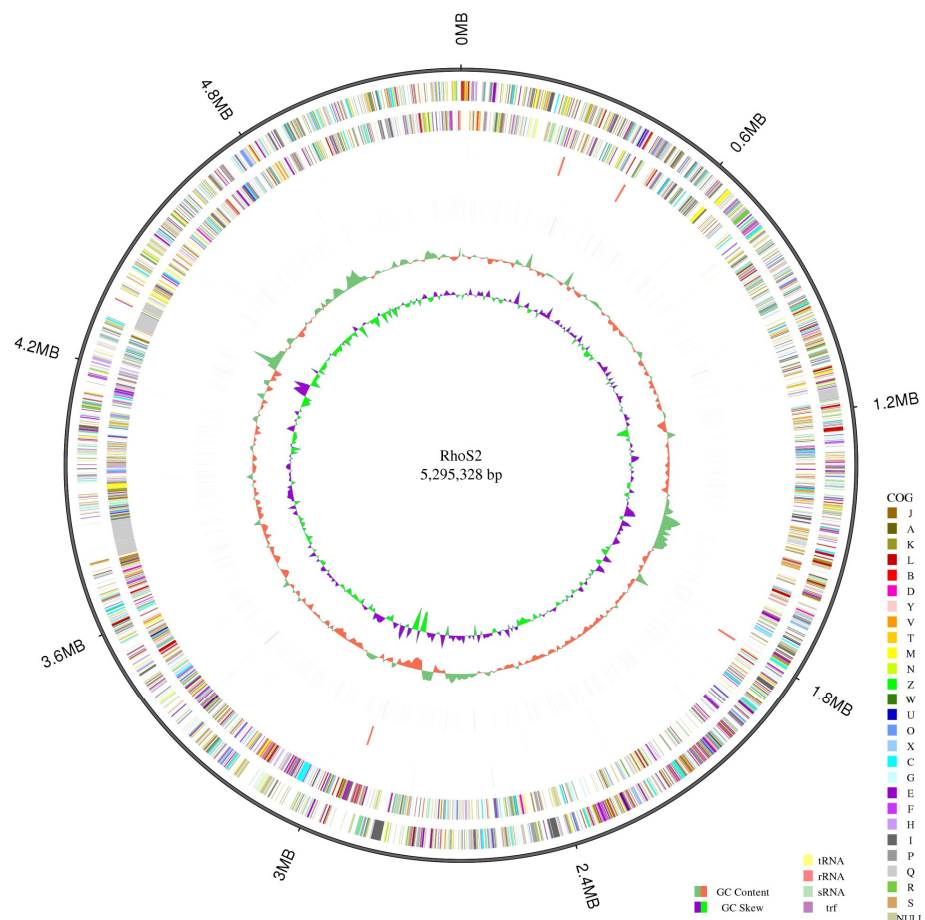


Figure 4. Schematic genome of the *Rhodococcus* sp. S2. Figure note: The first circle is the genome from the outside to the inside. The second and third circles of the circle diagram are the CDS on the positive or the negative chain, colored according to a cluster of orthologous groups (COG) classification. Different colors indicate different classifications of COG functions. The fourth circle corresponds to the repetition sequence. The fifth circle is the GC content information, and the last circle is the GC skew.

In the conventional nitrogen removal pathway for HN-AD bacteria, inorganic nitrogen compounds such as nitrite, ammonium, hydroxylamine, and nitrate are converted into nitrogen gas. Essential enzymes in the HN-AD process, including hydroxylamine oxidoreductase (Hao), nitrite reductase (NiR), ammonia monooxygenase (Amo), and nitrate reductase (NaR), were identified. Genome annotation revealed that strain S2 contained a total of nine genes related to nitrogen metabolism, including *NarB* (ferredoxin–nitrate reductase), *NarG*, *NxrA*, *NirBD*, *NRT* (the major facilitator superfamily transporter), *ncd2* (nitronate monooxygenase), *GDH2* (glutamate dehydrogenase), and *gltD* (glutamate synthase (NADPH) small chain) (Figure 5).

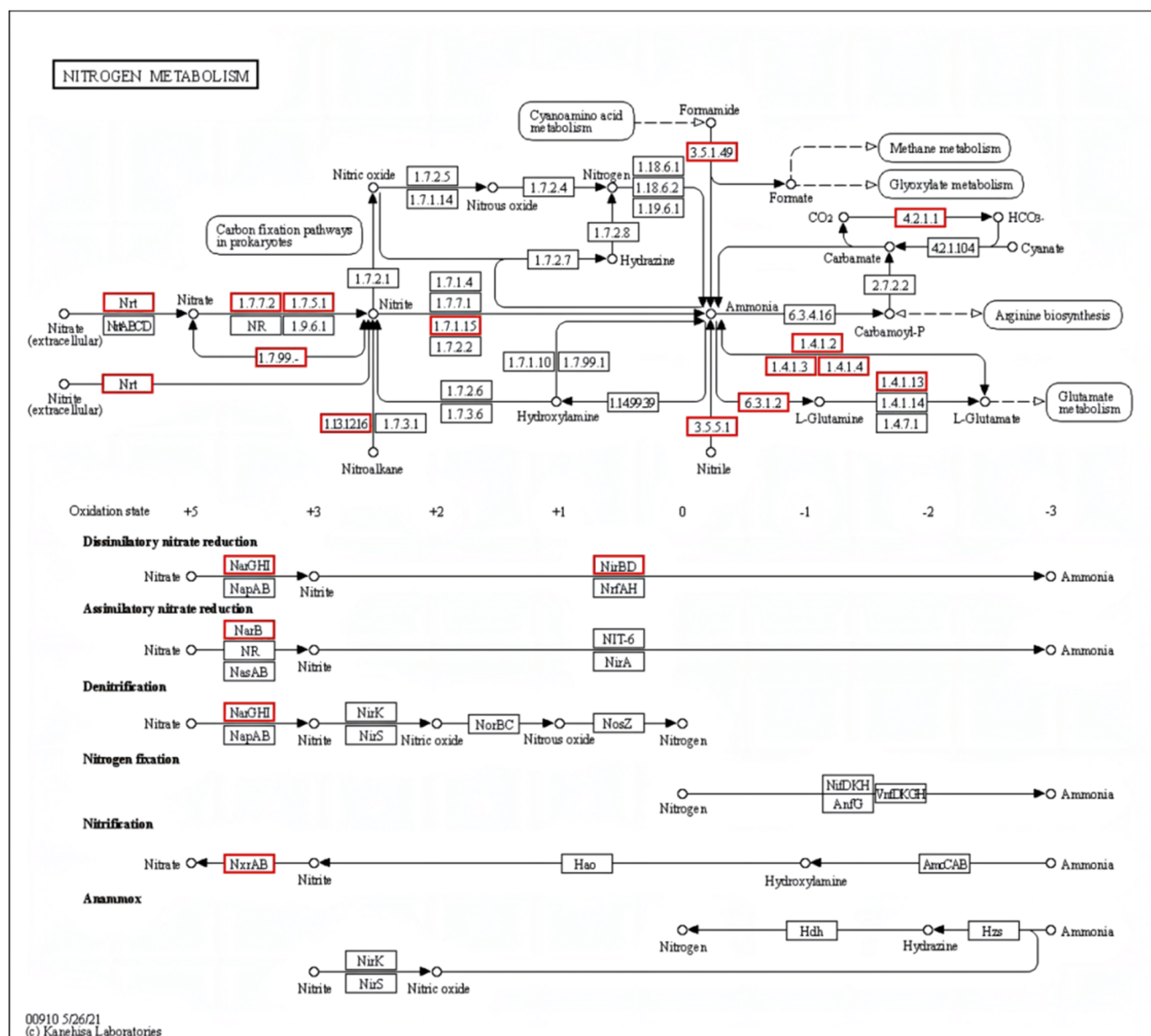


Figure 5. Key genes of S2 in the nitrogen metabolism pathway annotated in the KEGG database. The red boxes indicate the existence of genes in the nitrogen metabolism pathway.

The major facilitator superfamily transporter, responsible for the transport of nitrate and nitrite in the biofilm, is encoded by the *NRT* gene [42]. The genes *ncd2* and *NarB* control the enzymes nitronate monooxygenase and ferredoxin–nitrate reductase, respectively. Gene *gltD* encodes glutamine synthetase (GS), which converts ammonia into glutamine for assimilation into the cell biomass [43]. Additionally, GDH2 encodes Glutamate dehydrogenase (GDH), which plays a crucial role in ammonium detoxification [44]. Nitrate reductases, nitrate oxidoreductase, and nitrite reductase are encoded by *NarG*, *NxrA*, and *NirBD* genes, respectively, and they are essential for the HN–AD nitrogen removal process.

However, genome sequencing did not annotate the *Amo* and *Hao* genes related to the HN–AD process or the key genes (*NirK* and *NirS*) involved in nitrite reductase synthesis. This result is consistent with other HN–AD bacteria, such as *Pseudomonas taiwanensis* J488 (with only the *NirB* gene mapped) [45] and *Klebsiella pneumoniae* EGD-HP19-C (with only *NR* genes annotated) [46]. The inability to map some key genes could be attributed to two factors. First, the new genes associated with the HN–AD process in strain S2 may differ from those found in traditional HN–AD bacteria. The second factor may be the incomplete

genome draft. Our study showed that strain S2 exhibited efficient ammonium, nitrite, and nitrate removal capacity, producing N_2 when using $(NH_4)_2SO_4$ as the sole nitrogen source, thus confirming its classification as an HN-AD bacterium (results shown in Figure 2 and Figure S1).

3.5. Nitrogen Removal Capacity of Strain S2 in Raw Wastewater

The ability of strain S2 to treat wastewater was evaluated. The NH_4^+-N content in raw wastewater decreased from 13.51 ± 0.13 to 6.08 ± 0.41 mg L^{-1} within 12 h when strain S2 and sodium acetate were added, resulting in NH_4^+-N removal efficiency of $55.29 \pm 0.43\%$ (Figure 6a).

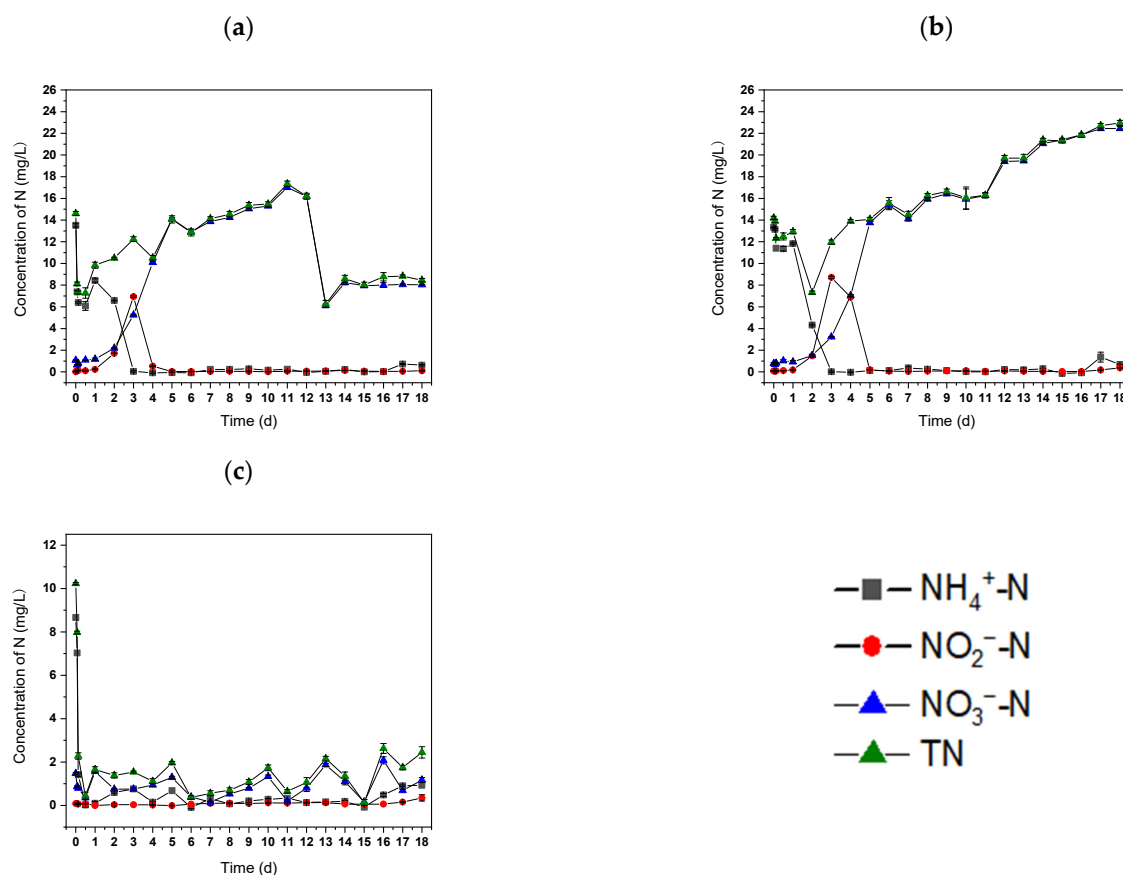


Figure 6. The N concentrations in (a) raw wastewater with *Rhodococcus* sp. S2, (b) raw wastewater, and (c) sterilized wastewater with *Rhodococcus* sp. S2.

However, only $14.83 \pm 0.20\%$ of NH_4^+-N was eliminated in the control group (raw wastewater) (Figure 6b), indicating that the addition of strain S2 and sodium acetate would enhance NH_4^+-N removal from the wastewater. These results validate the substantial NH_4^+-N removal capability of strain S2 in raw wastewater. In comparison, sterilized wastewater, with the inclusion of strain S2 and sodium acetate, exhibited $99.79 \pm 0.45\%$ NH_4^+-N removal efficiency within 12 h (Figure 6c). A relatively lower NH_4^+-N removal efficiency from wastewater as compared to sterilized wastewater may be attributed to the complex composition of raw wastewater and the presence of indigenous microbial groups, which could interfere with the metabolic activity of strain S2. Afterward, the NH_4^+-N concentration decreased to 0.05 ± 0.06 mg L^{-1} on day 3 (Figure 6a). During this period, NH_4^+-N was oxidized to NO_2^--N and finally converted to NO_3^--N . This process was mainly caused by the nitrification of autotrophic bacteria in the raw wastewater. The same phenomenon was observed in the control group (Figure 6b) from day 0 to day 5. This further suggests that the nitrification process is caused by indigenous autotrophic nitrifiers

in raw wastewater. Furthermore, the introduction of additional sodium acetate on day 12 led to a decrease in NO_3^- -N content down to $6.12 \pm 0.14 \text{ mg L}^{-1}$ in the experimental group (Figure 6a). This result further confirms the aerobic denitrification capacity of S2. In the control group, the NO_3^- -N concentration increased gradually afterward, which may have been caused by the transformation of other forms of nitrogen in the culture process, indicating the significant contribution of indigenous microbial populations to nutrient transformation. Slight increases in NH_4^+ -N were observed at the end of the experiment, which was probably mainly due to the release of NH_4^+ -N from the dead bacteria after the exhaustion of organic matter [47]. Another explanation could possibly be the inhibition of the free ammonia produced at elevated pH caused by denitrification [40]. The maximum TN removal efficiency reached $57.16 \pm 0.52\%$ in the experimental group (Figure 6a). Overall, these findings suggest that strain S2 may possess significant nitrogen removal capacity when a carbon source is present in wastewater. The successful isolation and characterization of strain S2 from landfill leachate could offer a promising foundation for future in situ biotechnological treatment of landfill leachate. This strain may be readily adapted to the contaminated environment. The enhanced in situ indigenous HN-AD offers potential advantages for the bioremediation of contaminated sites and wastewater. To reduce carbon source demand and further improve nitrogen removal capabilities in practical wastewater treatment, biochar or other carrier-immobilized bacteria [48] or bacterial consortiums with HN-AD capability are therefore worth future exploration.

4. Conclusions

In this study, *Rhodococcus* sp. S2, isolated from landfill leachate, demonstrated efficient HN-AD capacity. The maximum removal efficiency for NH_4^+ -N, NO_2^- -N, and NO_3^- -N was $99.97 \pm 0.3\%$, $99.79 \pm 0.14\%$, and $83.53 \pm 0.31\%$, respectively. Additionally, the preferential removal of NH_4^+ -N in the mixed nitrogen source and the capacity for simultaneous nitrification and denitrification were observed. The critical genes of *Rhodococcus* sp. S2, namely *NarG*, *NxrA*, and *NirBD*, were ascertained through draft genome sequencing, further confirming its HN-AD capability. Under the experimental conditions, *Rhodococcus* sp. S2 displayed high nitrogen removal performance when used in the treatment of raw wastewater with the addition of a carbon source. These results indicate the potential candidacy of *Rhodococcus* sp. S2 for wastewater nitrogen removal. Subsequent studies focusing on the application of S2 in real raw wastewater without a carbon source, with large-scale experiments, or the progressive complexity of the reaction medium could be pursued.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w16030431/s1>, Figure S1. The composition of gas in gas-tight vials cultivated S2 at 0 h and 24 h. Values are means $\pm$ se, $n = 3$. Table S1. The nitrogen balance of strain S2 during the NH_4^+ -N removal process.

Author Contributions: X.C., investigation, methodology, formal analysis, and writing—original draft; S.L. (Shuangfei Li), conceptualization, project administration, and supervision; W.Z., investigation and methodology; S.L. (Shaofeng Li), funding acquisition and writing—review and editing; Y.G., writing—review and editing; L.O., conceptualization, funding acquisition, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Scientific Research Startup Fund for Shenzhen High-Caliber Personnel of SZPT (No. 6022310040K), the Post-doctoral Later-stage Foundation Project of Shenzhen Polytechnic (6021271015K1), the Shenzhen Science and Technology Program (KCXFZ20201221173203010), the Shenzhen Science and Technology Program (KCXST20221021111206015 and KCXFZ20201221173404012), the National Natural Science Foundation of China (42107396), and the Shenzhen Science and Technology Program (JCYJ20220531094416037).

Data Availability Statement: Some or all data, models, or codes that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to have influenced the work reported in this paper.

References

- Wang, Y.; Zou, Y.L.; Chen, H.; Lv, Y.K. Nitrate removal performances of a new aerobic denitrifier, *Acinetobacter haemolyticus* ZYL, isolated from domestic wastewater. *Bioprocess. Biosyst. Eng.* **2021**, *44*, 391–401. [\[CrossRef\]](#)
- Huang, F.; Pan, L.; Lv, N.; Tang, X. Characterization of novel *Bacillus* strain N31 from mariculture water capable of halophilic heterotrophic nitrification-aerobic denitrification. *J. Biosci. Bioeng.* **2017**, *124*, 564–571. [\[CrossRef\]](#)
- Ke, X.; Liu, C.; Tang, S.Q.; Guo, T.T.; Pan, L.; Xue, Y.P.; Zheng, Y.G. Characterization of *Acinetobacter indicus* ZJB20129 for heterotrophic nitrification and aerobic denitrification isolated from an urban sewage treatment plant. *Bioresour. Technol.* **2022**, *347*, 126423. [\[CrossRef\]](#)
- Xia, L.; Li, X.; Fan, W.; Wang, J. Heterotrophic nitrification and aerobic denitrification by a novel *Acinetobacter* sp. ND7 isolated from municipal activated sludge. *Bioresour. Technol.* **2020**, *301*, 122749. [\[CrossRef\]](#)
- Chen, J.; Gu, S.; Hao, H.; Chen, J. Characteristics and metabolic pathway of *Alcaligenes* sp. TB for simultaneous heterotrophic nitrification-aerobic denitrification. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9787–9794. [\[CrossRef\]](#)
- Li, D.; Liang, X.; Jin, Y.; Wu, C.; Zhou, R. Isolation and Nitrogen Removal Characteristics of an Aerobic Heterotrophic Nitrifying-Denitrifying Bacterium, *Klebsiella* sp. TN-10. *Appl. Biochem. Biotechnol.* **2019**, *188*, 540–554. [\[CrossRef\]](#)
- Feng, L.; Yang, J.; Ma, F.; Pi, S.; Xing, L.; Li, A. Characterisation of *Pseudomonas stutzeri* T13 for aerobic denitrification: Stoichiometry and reaction kinetics. *Sci. Total Environ.* **2020**, *717*, 135181. [\[CrossRef\]](#)
- Lang, X.; Li, Q.; Ji, M.; Yan, G.; Guo, S. Isolation and niche characteristics in simultaneous nitrification and denitrification application of an aerobic denitrifier, *Acinetobacter* sp. YS2. *Bioresour. Technol.* **2020**, *302*, 122799. [\[CrossRef\]](#)
- Kundu, P.; Pramanik, A.; Dasgupta, A.; Mukherjee, S.; Mukherjee, J. Simultaneous heterotrophic nitrification and aerobic denitrification by *Chryseobacterium* sp. R31 isolated from abattoir wastewater. *Biomed. Res. Int.* **2014**, *2014*, 436056. [\[CrossRef\]](#)
- Wang, Q.; He, J. Complete nitrogen removal via simultaneous nitrification and denitrification by a novel phosphate accumulating *Thauera* sp. strain SND5. *Water Res.* **2020**, *185*, 116300. [\[CrossRef\]](#)
- Zou, S.; Yao, S.; Ni, J. High-efficient nitrogen removal by coupling enriched autotrophic-nitrification and aerobic-denitrification consortiums at cold temperature. *Bioresour. Technol.* **2014**, *161*, 288–296. [\[CrossRef\]](#)
- Lei, X.; Jia, Y.; Chen, Y.; Hu, Y. Simultaneous nitrification and denitrification without nitrite accumulation by a novel isolated *Ochrobactrum anthropic* LJ81. *Bioresour. Technol.* **2019**, *272*, 442–450. [\[CrossRef\]](#)
- He, T.; Xie, D.; Li, Z.; Ni, J.; Sun, Q. Ammonium stimulates nitrate reduction during simultaneous nitrification and denitrification process by *Arthrobacter arilaitensis* Y-10. *Bioresour. Technol.* **2017**, *239*, 66–73. [\[CrossRef\]](#)
- Zhao, B.; He, Y.L.; Zhang, X.F. Nitrogen removal capability through simultaneous heterotrophic nitrification and aerobic denitrification by *Bacillus* sp. LY. *Environ. Technol.* **2010**, *31*, 409–416. [\[CrossRef\]](#)
- Sun, Y.; Li, A.; Zhang, X.; Ma, F. Regulation of dissolved oxygen from accumulated nitrite during the heterotrophic nitrification and aerobic denitrification of *Pseudomonas stutzeri* T13. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 3243–3248. [\[CrossRef\]](#)
- Soto, O.; Aspé, E.; Roedel, M. Kinetics of cross-inhibited denitrification of a high load wastewater. *Enzym. Microb. Technol.* **2007**, *40*, 1627–1634. [\[CrossRef\]](#)
- Song, T.; Zhang, X.; Li, J.; Wu, X.; Feng, H.; Dong, W. A review of research progress of heterotrophic nitrification and aerobic denitrification microorganisms (HNADMs). *Sci. Total Environ.* **2021**, *801*, 149319. [\[CrossRef\]](#)
- Wei, R.; Hui, C.; Zhang, Y.; Jiang, H.; Zhao, Y.; Du, L. Nitrogen removal characteristics and predicted conversion pathways of a heterotrophic nitrification-aerobic denitrification bacterium, *Pseudomonas aeruginosa* P-1. *Environ. Sci. Pollut. Res. Int.* **2021**, *28*, 7503–7514. [\[CrossRef\]](#)
- APHA. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washington, DC, USA, 2005. [\[CrossRef\]](#)
- Rout, P.R.; Bhunia, P.; Dash, R.R. Simultaneous removal of nitrogen and phosphorous from domestic wastewater using *Bacillus cereus* GS-5 strain exhibiting heterotrophic nitrification, aerobic denitrification and denitrifying phosphorous removal. *Bioresour. Technol.* **2017**, *244*, 484–495. [\[CrossRef\]](#)
- Yao, N.H.; Du, Y.N.; Xiong, J.X.; Xiao, Y.; He, H.H.; Xie, Z.F.; Huang, D.; Song, Q.; Chen, J.; Yan, D.; et al. Microbial detoxification of 3,5-xyleneol via a novel process with sequential methyl oxidation by *Rhodococcus* sp. CHJ602. *Environ. Res.* **2023**, *220*, 115258. [\[CrossRef\]](#)
- Ye, X.; Peng, T.; Feng, J.; Yang, Q.; Pratush, A.; Xiong, G.; Huang, T.; Hu, Z. A novel dehydrogenase 17beta-HSDx from *Rhodococcus* sp. P14 with potential application in bioremediation of steroids contaminated environment. *J. Hazard. Mater.* **2019**, *362*, 170–177. [\[CrossRef\]](#)
- Mirzakhani, E.; Nejad, F.M. Grasses and *Rhodococcus erythropolis* Bacteria for Bioremediation of Naturally Polluted Soils with Hydrocarbons. *Chem. Eng. Technol.* **2016**, *39*, 1731–1737. [\[CrossRef\]](#)
- Zhang, Y.; Zhang, Y.; Xiong, J.; Zhao, Z.; Chai, T. The enhancement of pyridine degradation by *Rhodococcus* KDPy1 in coking wastewater. *FEMS Microbiol. Lett.* **2019**, *366*, fny271. [\[CrossRef\]](#)
- Qu, C.; Wang, W.; Dong, J.; Wang, X.; Gao, X.; Zhang, H.; Zheng, Z.; Yin, H.; Miao, J. Complete genome sequence of *Rhodococcus* sp. NJ-530, a DMSP-degrading actinobacterium isolated from Antarctic sea ice. *3 Biotech.* **2019**, *9*, 363. [\[CrossRef\]](#)
- Kis, A.E.; Laczi, K.; Zsiros, S.; Kos, P.; Tengolics, R.; Bounedjoum, N.; Kovacs, T.; Rakhely, G.; Perei, K. Characterization of the *Rhodococcus* sp. MK1 strain and its pilot application for bioremediation of diesel oil-contaminated soil. *Acta Microbiol. Immunol. Hung.* **2017**, *64*, 463–482. [\[CrossRef\]](#)

27. Ma, S.; Huang, S.; Tian, Y.; Lu, X. Heterotrophic ammonium assimilation: An important driving force for aerobic denitrification of *Rhodococcus erythropolis* strain Y10. *Chemosphere* **2022**, *291*, 132910. [\[CrossRef\]](#)
28. Chen, P.; Li, J.; Li, Q.X.; Wang, Y.; Li, S.; Ren, T.; Wang, L. Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium *Rhodococcus* sp CPZ24. *Bioresour. Technol.* **2012**, *116*, 266–270. [\[CrossRef\]](#)
29. Jin, R.; Liu, T.; Liu, G.; Zhou, J.; Huang, J.; Wang, A. Simultaneous heterotrophic nitrification and aerobic denitrification by the marine origin bacterium *Pseudomonas* sp. ADN-42. *Appl. Biochem. Biotechnol.* **2015**, *175*, 2000–2011. [\[CrossRef\]](#)
30. He, T.; Li, Z.; Sun, Q.; Xu, Y.; Ye, Q. Heterotrophic nitrification and aerobic denitrification by *Pseudomonas tolaasii* Y-11 without nitrite accumulation during nitrogen conversion. *Bioresour. Technol.* **2016**, *200*, 493–499. [\[CrossRef\]](#)
31. Ren, Y.X.; Yang, L.; Liang, X. The characteristics of a novel heterotrophic nitrifying and aerobic denitrifying bacterium, *Acinetobacter junii* YB. *Bioresour. Technol.* **2014**, *171*, 1–9. [\[CrossRef\]](#)
32. Zhu, L.; Ding, W.; Feng, L.J.; Kong, Y.; Xu, J.; Xu, X.Y. Isolation of aerobic denitrifiers and characterization for their potential application in the bioremediation of oligotrophic ecosystem. *Bioresour. Technol.* **2012**, *108*, 1–7. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Thomas, K.L.; Lloyd, D.; Boddy, L. Effects of oxygen, pH and nitrate concentration on denitrification by *Pseudomonas* species. *FEMS Microbiol. Lett.* **1994**, *118*, 181–186. [\[CrossRef\]](#)
34. Idi, A.; Ibrahim, Z.; Mohamad, S.E.; Majid, Z.A. Biokinetics of nitrogen removal at high concentrations by *Rhodobacter sphaeroides* ADZ101. *Int. Biodeterior. Biodegrad.* **2015**, *105*, 245–251. [\[CrossRef\]](#)
35. Xu, Y.; He, T.; Li, Z.; Ye, Q.; Chen, Y.; Xie, E.; Zhang, X. Nitrogen Removal Characteristics of *Pseudomonas putida* Y-9 Capable of Heterotrophic Nitrification and Aerobic Denitrification at Low Temperature. *Biomed. Res. Int.* **2017**, *2017*, 1429018. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Gu, X.; Leng, J.; Zhu, J.; Zhang, K.; Zhao, J.; Wu, P.; Xing, Q.; Tang, K.; Li, X.; Hu, B. Influence mechanism of C/N ratio on heterotrophic nitrification- aerobic denitrification process. *Bioresour. Technol.* **2022**, *343*, 126116. [\[CrossRef\]](#)
37. Liu, Y.; Ai, G.M.; Miao, L.L.; Liu, Z.P. Marinobacter strain NNA5, a newly isolated and highly efficient aerobic denitrifier with zero N₂O emission. *Bioresour. Technol.* **2016**, *206*, 9–15. [\[CrossRef\]](#)
38. Ouyang, L.; Wang, K.; Liu, X.; Wong, M.H.; Hu, Z.; Chen, H.; Yang, X.; Li, S. A study on the nitrogen removal efficacy of bacterium *Acinetobacter tandoii* MZ-5 from a contaminated river of Shenzhen, Guangdong Province, China. *Bioresour. Technol.* **2020**, *315*, 123888. [\[CrossRef\]](#)
39. Yang, L.; Wang, X.H.; Cui, S.; Ren, Y.X.; Yu, J.; Chen, N.; Xiao, Q.; Guo, L.K.; Wang, R.H. Simultaneous removal of nitrogen and phosphorous by heterotrophic nitrification-aerobic denitrification of a metal resistant bacterium *Pseudomonas putida* strain NP5. *Bioresour. Technol.* **2019**, *285*, 121360. [\[CrossRef\]](#)
40. Chen, L.F.; Chen, L.X.; Pan, D.; Ren, Y.L.; Zhang, J.; Zhou, B.; Lin, J.Q. Ammonium removal characteristics of *Delftia tsuruhatensis* SDU2 with potential application in ammonium-rich wastewater treatment. *Int. J. Environ. Sci. Technol.* **2023**, *20*, 3911–3926. [\[CrossRef\]](#)
41. Kouba, V.; Catrysse, M.; Stryjova, H.; Jonatova, I.; Volcke, E.I.P.; Svehla, P.; Bartacek, J. The impact of influent total ammonium nitrogen concentration on nitrite-oxidizing bacteria inhibition in moving bed biofilm reactor. *Water Sci. Technol.* **2014**, *69*, 1227–1233. [\[CrossRef\]](#)
42. Alvarez, L.; Sanchez-Hevia, D.; Sanchez, M.; Berenguer, J. A new family of nitrate/nitrite transporters involved in denitrification. *Int. Microbiol.* **2019**, *22*, 19–28. [\[CrossRef\]](#)
43. Zhao, Y.; Gao, J.; Su, S.; Shan, X.; Li, S.; Liu, H.; Yuan, Y.; Li, H. Regulation of the activity of maize glutamate dehydrogenase by ammonium and potassium. *Biosci. Biotechnol. Biochem.* **2021**, *85*, 262–271. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Xian, L.; Zhang, Y.; Cao, Y.; Wan, T.; Gong, Y.; Dai, C.; Ochieng, W.A.; Nasimiyyu, A.T.; Li, W.; Liu, F. Glutamate dehydrogenase plays an important role in ammonium detoxification by submerged macrophytes. *Sci. Total Environ.* **2020**, *722*, 137859. [\[CrossRef\]](#) [\[PubMed\]](#)
45. He, T.; Chen, M.; Ding, C.; Wu, Q.; Zhang, M. Hypothermia *Pseudomonas taiwanensis* J488 exhibited strong tolerance capacity to high dosages of divalent metal ions during nitrogen removal process. *Bioresour. Technol.* **2021**, *341*, 125785. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Pal, R.R.; Khardenavis, A.A.; Purohit, H.J. Identification and monitoring of nitrification and denitrification genes in *Klebsiella pneumoniae* EGD-HP19-C for its ability to perform heterotrophic nitrification and aerobic denitrification. *Funct. Integr. Genom.* **2015**, *15*, 63–76. [\[CrossRef\]](#)
47. Wang, X.; Wang, W.; Zhang, Y.; Sun, Z.; Zhang, J.; Chen, G.; Li, J. Simultaneous nitrification and denitrification by a novel isolated *Pseudomonas* sp. JQ-H3 using polycaprolactone as carbon source. *Bioresour. Technol.* **2019**, *288*, 121506. [\[CrossRef\]](#)
48. Liu, T.F.; Wang, B.J.; Liu, M.; Jiang, K.Y.; Wang, L. *Stutzerimonas frequens* strain TF18 with superior heterotrophic nitrification-aerobic denitrification ability for the treatment of aquaculture effluent. *Process Biochem.* **2023**, *130*, 156–165. [\[CrossRef\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.