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Heterotrophic Nitrification–Aerobic Denitrification by *Bacillus* sp. L2: Mechanism of Denitrification and Strain Immobilization

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Abstract: The biological denitrification of low-C/N wastewater is a great challenge in treatment plants due to the lack of microorganisms with heterotrophic nitrification–aerobic denitrification (HN-AD) abilities. In this study, *Bacillus* sp. L2 was isolated from aeration tank water samples using a nitrification medium and screened for its ability to perform HN-AD in low-C/N wastewater. The strain showed a maximum NH_4^+-N removal rate of 98.37% under low-C/N conditions. In the presence of a mixed N source, strain L2 was capable of completely removing NH_4^+-N within 24 h. Furthermore, optimal nitrogen removal conditions for strain L2 were found to be C/N = 9, pH = 9, and sodium acetate as the C source. Under optimal conditions, the strain was able to maintain a high NH_4^+-N removal rate under 0–3% salinity and an NH_4^+-N concentration of 200 mg/L or less. The denitrification pathways of strain L2 were $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- (\leftrightarrow NO_3^-) \rightarrow NO \rightarrow N_2O \rightarrow N_2$ and $NH_4^+ \rightarrow NH_2OH \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Furthermore, semi-continuous wastewater treatment was conducted using immobilized technology, which resulted in more than 82% NH_4^+-N removal after three cycles of reuse. This study demonstrates the great potential of *Bacillus* sp. L2 in wastewater treatment applications.

Keywords: nitrogen removal; wastewater; heterotrophic nitrification–aerobic denitrification; immobilization; denitrification pathways

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1. Introduction

Rapid urbanization, industrialization, and agricultural advancements have led to an increasing discharge of wastewater into water bodies. Domestic sewage, industrial wastewater, and waste leachate are the main sources of wastewater [1]. These wastewaters contain substantial concentrations of nitrogenous organic compounds, and the excessive discharge of nitrogenous wastewater not only leads to the eutrophication of water bodies but also poses a serious threat to the ecological balance [2]. Therefore, the treatment of nitrogenous wastewater is important for the protection of the environment and human health.

In recent years, bioremediation has been widely used for nitrogen removal by microorganisms as this method is highly efficient, low-cost, simple, and does not induce secondary pollution [3]. Nitrification and denitrification are the traditional processes of biological nitrogen removal and are conducted by autotrophic nitrifying microorganisms and heterotrophic denitrifying microorganisms, respectively [4]. In the nitrification process, ammonia nitrogen (NH_4^+-N) is converted to nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) by autotrophic nitrifying microorganisms under aerobic conditions.

Subsequently, NO_3^--N is converted to nitrogen gas by heterotrophic denitrifying microorganisms under anaerobic conditions [5]. However, the conventional nitrification and denitrification processes require strict aerobic and anaerobic conditions and must be carried out in two steps [6]. This leads to the disadvantages of low nitrogen removal efficiency, complex process flow, high operating costs, and long operating cycles [7–9]. Therefore, the conventional biological nitrogen removal method has limitations in practical applications.

The identification of heterotrophic nitrification–aerobic denitrification (HN-AD) microorganisms has provided a new viable pathway for nitrogen removal from wastewater [2]. These microorganisms have the capability to perform both nitrification and denitrification simultaneously (SND) [6]. Such microorganisms have significant advantages over conventional biological nitrogen removal due to the following reasons: (i) these microorganisms demonstrate the capacity to remove nitrogen and chemical oxygen demand (COD) [10]; (ii) the consumption of the acid-base during the nitrification reaction can be auto-compensated through concurrent denitrification [11]; and (iii) HN-AD microbes can save costs, simplify the conversion process, and reduce energy consumption and carbon emissions [10,12]. *Thiosphaera pantotropha* was the first reported HN-AD strain [13]. In recent years, a quantity of HN-AD microorganisms have been discovered, such as *Priestia aryabhattai* KX-3 [14], *Pseudomonas mendocina* SCZ-2 [15], *Stenotrophomonas maltophilia* strain IAM 12423 [16], *Pseudomonas stutzeri* Y2 [17], *Alcaligenes faecalis* strain WT14 [4], *Thauera* sp. SND5 [6], etc. However, most previous studies have focused on high C/N for more favorable nitrogen removal, and less research has been performed on efficient denitrification at lower C/N, which usually results in poor nitrogen removal and denitrification [18]. In addition, the emergence of microbial immobilization techniques has improved the nitrification efficiency of strains, and these techniques have been widely used [19]. The immobilization of HN-AD bacteria is known to involve a variety of carriers, such as porous ceramic carriers [20], polymer polyvinyl alcohol carriers [21], and graphene oxide carriers [22]. Immobilized carriers are essential for practical engineering applications and the scaling-up of wastewater treatment. Nevertheless, the application of HN-AD bacterial immobilized carriers for the treatment of pesticide wastewater is still in its preliminary stages. Meanwhile, HN-AD microorganisms undergo a variety of biochemical processes during denitrification, which lead to unclear denitrification mechanisms [23]. Therefore, more detailed studies need to be conducted to explore the denitrification mechanisms of HN-AD microbes.

Most of the recent reports on HN-AD bacteria have focused on aquaculture wastewater [24], waste leachate, and high-salt wastewater [25,26]. As of our knowledge, there is no report on the biological treatment of pesticide wastewater.

In this study, a novel HN-AD bacterial strain, L2, was isolated from wastewater samples collected from an aeration tank. The strain was highly efficient in removing NH_4^+-N under high-salt and low-C/N conditions. Its denitrification ability was evaluated under the influence of different factors and in the presence of different N sources. The study delved into the influence of diverse environmental factors on the removal of NH_4^+-N and COD, revealing the pathways and mechanisms of nitrogen removal. Immobilized vectors were prepared to explore the performance and feasibility of HN-AD strain L2 for the treatment of pesticide wastewater. The present research provides a novel strain and new ideas for wastewater treatment.

2. Materials and Methods

2.1. Materials

2.1.1. Samples

The strains were isolated from aeration tank water samples provided by Zhonglan Lianhai Design and Research Institute (Lianyungang, China).

2.1.2. Instruments and Reagents

Kaolin powder (95% purity), peanut shell powder (9.6 μm particle size), $(NH_4)_2SO_4$, CH_3COONa, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, NaCl, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}4H_2O$, ammonia (25~28%), and other reagents were purchased from Aladdin Reagent Company, Shanghai, China. Ammonia nitrogen (LH-N2N3), nitrate (LH-NO3-100), and nitrite (LH-NO2-100) consumables were purchased from Lianhua Yongxing Technology Development Co., Beijing, China.

The water quality was assessed using the multi-parameter water quality analyzer (LH-3BA, China), manufactured by Lianhua Yongxing Science and Technology Development Co., Shanghai, China.

2.1.3. Medium Preparation

Media preparation was referred to in previous studies [27,28].

Enrichment medium (LB) composition (g/L): NaCl, 10.0 g; tryptone, 10.0 g; and yeast powder, 5.0 g.

Heterotrophic nitrification solid medium (HNSM) (g/L): CH_3COONa, 6.8 g; $(NH_4)_2SO_4$, 0.5 g; Vickers salt solution, 50 mL; and agar, 15 g.

Vickers salt solution (g/L): K_2HPO_4, 5 g; $MgSO_4{\cdot}7H_2O$, 2.5 g; NaCl, 2 g; $MnSO_4{\cdot}4H_2O$, 0.05 g; and $FeSO_4{\cdot}7H_2O$, 0.05 g.

Heterotrophic nitrification medium (HNM) (g/L): $FeSO_4{\cdot}7H_2O$, 0.03 g; $MgSO_4{\cdot}7H_2O$, 0.3 g; NaCl, 20 g; K_2HPO_4, 1.0 g; CH_3COONa, 0.2 g; and $(NH_4)_2SO_4$, 0.27 g.

Denitrification medium (DM) composition was similar to HNM, with KNO_3 (0.49 g) and $NaNO_2$ (0.34 g) as the sole N sources. Based on the HNM and DM components, the C and N sources were changed according to the experimental needs. The pH of all media mentioned above was adjusted to 7.0. Media were sterilized at 121 °C for 20 min.

2.2. Methods

2.2.1. Screening of Strain L2

The strains isolated from wastewater samples were inoculated in 100 mL of LB medium using an inoculum volume of 2% (V/V) and incubated at 30 °C and 160 rpm for 24 h. The obtained bacterial culture was serially diluted to gradients of 10^{-1}–10^{-8}. Diluted cultures were added to HNSM media and incubated at 30 °C for 72 h. The single colonies of bacteria were isolated and purified by three sequential sub-culturing steps [29]. Total of 13 isolated strains were tested for denitrification performance using HNM and DM media containing different N sources, and finally, strain L2, which showed best HN-AD abilities, was selected for further analysis.

2.2.2. Morphological Analysis and Identification of Strain L2

The strains were inoculated into solid HNM medium and incubated for 12 h. After incubation, morphological characteristics of colonies were observed. Morphology of each individual strain was observed by scanning electron microscopy (SEM, JFC-1600, Tokyo, Japan). DNA of strain L2 was extracted using a DNA extraction kit. DNA was amplified by polymerase chain reaction (PCR) using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGCTT-3′). The PCR products were purified and sequenced by Shanghai San gong Biotechnology Co., China. The obtained sequences were analyzed using NCBI Blast [30]. MEGA 7.0 and neighbor-joining software were used to create the phylogenetic tree.

2.2.3. HN-AD Characteristics of Strain L2

For seed solution, the conserved strain L2 was inoculated in 100 mL of LB medium at 1% (V/V) inoculum to medium ratio [27] and incubated for 12 h. The seed solution was then inoculated into HNM and DM media containing different N sources at 10% inoculum ratio. The initial nitrogen concentration of each N source in media was 50 mg/L. Before inoculation, seed liquid was centrifuged at 12,900× g for 5 min, and the supernatant was discarded. The bacterial cell pellet was washed with PBS and centrifuged again to discard

the supernatant. This washing procedure was repeated three times. Strain L2 was incubated at 30 °C, 160 rpm for 60 h. Samples (5 mL) were taken every 12 h, centrifuged at 12,900× *g* for 5 min, and the concentrations of NH_4^+-N, NO_3^--N, NO_2^--N, OD_{600}, and COD in the samples were determined.

2.2.4. Study of NH4$^+$-N Removal Performance of Strain L2

NH_4^+-N removal efficiency of strain L2 was examined in presence of different C sources, C/N ratios, pHs, NaCl concentrations, and NH_4^+-N concentrations. For C source experiments, sodium propionate, sodium acetate, sodium citrate, maltose, and glucose were used as the only C sources (C/N = 7). For the C/N ratio experiment, C/N was adjusted to 3, 5, 7, 9, and 11 considering the variation in C/N at different stages of wastewater. In pH experiment, NH_4^+-N removal efficiency of strain was studied at different pHs (6, 7, 8, 9, and 10), with C/N of 9. Salinity was set to 0, 1, 2, 3, and 4% to examine the nitrogen removal potential of strain L2 for different wastewater treatment scenarios (C/N = 9). To study the tolerance of the strain to NH_4^+-N, the strain was subjected to media with different NH_4^+-N concentrations of 50, 100, 150, 200, and 250 mg/L (C/N = 9) [31]. The seed solution was added to 100 mL of HNM media at 10% inoculum ratio and incubated at 30 °C, 160 rpm for 60 h. Samples (5 mL) were collected every 12 h interval and centrifuged at 12,900× *g* for 5 min to determine the concentrations of OD_{600}, NH_4^+-N, and COD.

2.2.5. Study of Denitrification Pathways in Strain L2

DNA of strain L2 was used as template to amplify the nitrification-related genes (*amoA* and *hao)* and the denitrification-related genes (*napA*, *nirK*, *norB*, and *nosZ*). Information on primers has been provided in the Supplementary Material. Obtained amplicons were visualized using 1% agarose gel electrophoresis. Selection of primers and amplification conditions was based on previous studies [22–36].

2.2.6. Immobilization of Strain L2

Peanut shell-kaolin (PSK) carriers were prepared according to method described by [20] with some modifications. An amount of 7 g of kaolin powder and 1 g of peanut shell powder were mixed thoroughly, and then water was introduced into the mixture to attain optimal plasticity. Next, PSK particles (diameter: 5–7 mm) were fabricated and air-dried for 12 h until reaching a moisture content of 30–40%. These particles were heated at 1000 °C, allowing them to expand and form a large number of bubbles. Then, the PSK particles were cooled rapidly to stabilize their porous structure. These porous PSK particles were used as carriers for strain L2. Strain L2 was inoculated in 100 mL of LB medium at an inoculum ratio of 1% (*V*/*V*) and incubated at 30 °C and 160 rpm for 12 h. To immobilize the bacterial strain on the PSK carriers, the carriers were submerged in a bacterial mixture consistent with the conditions described above and incubated for 12 h. The density of the bacterial cells adhering to the PSK carrier was consistent with the method described by [20]. A total of 10 mL of LB solution pre-cultured with strain L2 was centrifuged (12,900× *g*, 5 min) and washed three times with PBS.

Ningxia pesticide wastewater (NH_4^+-N = 120 mg/L; C/N = 1:5; salinity 2%) was collected by the institute to explore the nitrogen removal potential of strain L2. Ningxia pesticide wastewater was used after filtration (0.22 µm) for subsequent experiments. The settings of the three cyclic batch experiments are shown in Table 1. In the L2 + C and PSK + C experimental groups, carbon sources were added, the C/N ratio was adjusted to 9, and the flasks were incubated at 30 °C and 160 rpm. All flasks were subjected to 96 h operation cycles. At the end of each cycle, the PSK carriers underwent triple washing with sterile water, and the contents of the flasks were entirely replenished with fresh wastewater. The initial and final concentrations of NH4$^+$-N were determined in each cycle.

Table 1. Batch circulation experiment of pesticide wastewater treatment by immobilized strain L2.

	Blank	L2	L2 + C	PSK + C
Real pesticide wastewater (mL)	100	100	100	100
Added carbon source	-	-	+	+
Inoculated amount of strain L2 (%)	0	10	10	0
Cycle number	3	3	3	3

Notes: PSK, kaolin peanut shell carrier; C, organic carbon; +, carbon source added; -, no carbon source added; for the L2 and L2 + C groups, pre-culture centrifuge solution was added to each cycle.

2.2.7. Analytical Methods

NH_4^+-N, NO_2^--N, NO_3^--N, and COD contents were measured by spectrophotometry using nano reagent, phenol disulfonic acid, naphthalene ethylenediamine hydrochloride, and potassium permanganate, respectively. Optical density was measured at 600 nm by a UV spectrophotometer. pH was measured by a pH meter. Graphs were prepared using Origin 2021. The data analysis was performed using SPSS 26. Three parallel measurements were performed in each experiment.

3. Results and Discussion

3.1. Morphological Characterization of Strain L2

The colonies grown on an enrichment medium were purified to obtain pure strains. The strain L2 on the HNM media plate is shown in Figure 1a. The colony was milky white with a regular shape and lobed edges, and the diameter of the colonies shown was 2–3 mm, as shown in Figure 1b. The SEM images in Figure 1c show that the cells were in the form of short rods with a size of 1.5–1.6 μm × 0.5–0.6 μm.

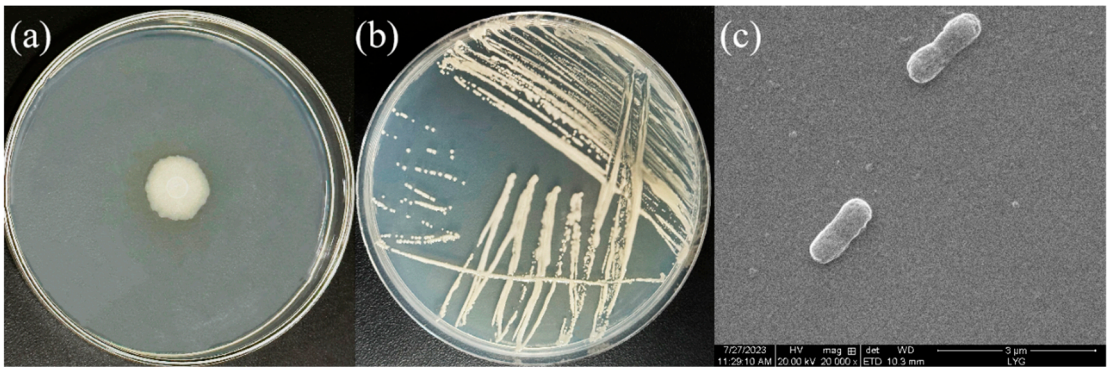


Figure 1. (**a**) Strain L2 spot plate; (**b**) colony morphology of strain L2; (**c**) SEM of strain L2 at 20,000× magnification.

3.2. Identification of Strain L2

The DNA of strain L2 was extracted, and the strain was identified by amplifying 16S rDNA with universal primers. As shown in Figure 2a, the fragment size of the DNA amplicon was about 1.5 kb in 1% agarose gel electrophoresis. After sequencing, the obtained L2 gene sequence was compared to other genomes by BLAST. A comparison of similarity indexes revealed that L2 belonged to *Bacillus*. A phylogenetic tree was constructed by MEGA 7.0. The results showed that L2 was closely related to *Bacillus tropicalis* (Figure 2b). In recent years, many reports have been published on HN-AD *Bacillus*. Yang et al. (2021) found that *Bacillus* sp. H-B could perform HN-AD at low temperatures. Similarly, HN-AD *Bacillus* sp. WXN-23 was reported to be able to perform denitrification in low-nutrient environments [37], indicating that *Bacillus* exhibits potential for widespread application within the domain of biological treatment for wastewater.

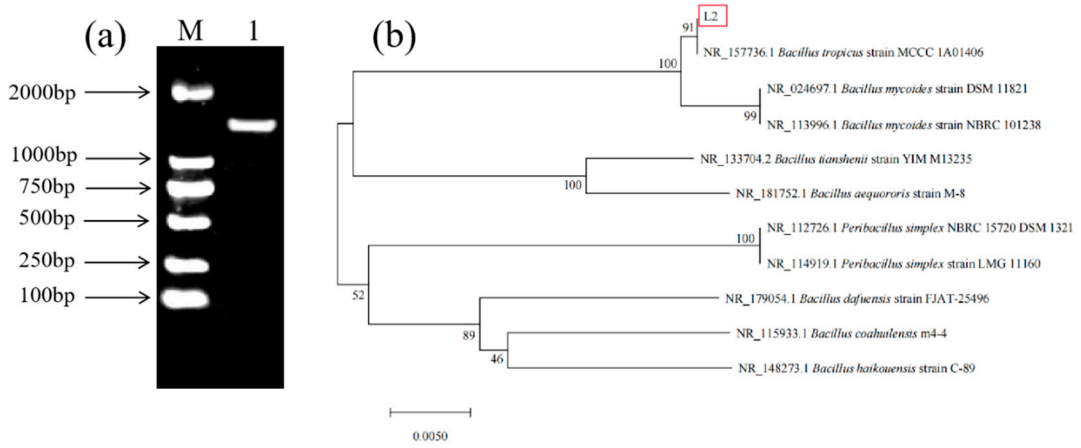


Figure 2. (**a**) Agarose gel electrophoresis of 16S rDNA PCR products; M: DNA marker; 1: 16S rDNA amplified by strain L2; (**b**) phylogenetic tree based on 16S rDNA gene sequences.

3.3. HN-AD Characteristics of Strain L2

3.3.1. Heterotrophic Nitrification

Ammonium sulfate was the sole N source in HNM. As shown in Figure 3a, the cells grew rapidly in the logarithmic phase after 12 h and then entered the stable growth phase after 24 h, followed by the decay phase. At the same time, 95.08% of NH_4^+-N was removed within 24 h, and a maximum NH_4^+-N removal of 98.37% was observed at 36 h. The COD declined rapidly, achieving 70.34% at 36 h, and then increased slightly. Since strain L2 is a heterotrophic bacterium, the decrease in the concentration of bacterium (reflected by COD decline) may be related to the continuous reduction in the C source. After 48 h, NH_4^+-N has a slight increase, which may be caused by the ammonium released from the lysis of dead cells caused by the C source depletion [38]. A small amount of NO_3^--N also accumulated during NH_4^+-N degradation. Unlike previously reported results for *Acinetobacter* sp. JR1 [39], *Cupriavidus* sp. S1 [40], and *Acinetobacter junii* YB [41] HN-AD bacteria, no NO_3^--N accumulation was observed during the NH_4^+-N degradation process.

3.3.2. Aerobic Denitrification

As shown in Figure 3b, when nitrate was the only source of N, strain L2 showed a maximum nitrogen removal of 95.68% at 36 h, with a low nitrite accumulation. Within 12 h, NO_3^--N content in the medium decreased from 51.34 mg/L to 18.06 mg/L, and the average removal rate reached 2.77 mg/(L·h), which was higher than the removal rates reported for many HN-AD strains, such as strain ZJB20129 (1.77 mg/(L·h)) [1], strain Y-11 (1.99 mg/(L·h)) [11], and strain CF-S9 (2.2 mg/(L·h)) [34]. Similar results were reported for *Exiguobacterium mexicanum* strain SND-01 [26].

As shown in Figure 3c, under aerobic conditions with sodium nitrite as the only N source, strain L2 entered a rapid growth phase within 12 h. The strain L2 had a high concentration of sodium nitrite. During this process, the bacteria grew rapidly and then entered a stable growth period. The NO_2^--N removal rate remained high in the first 36 h, with a maximum removal of 65.06%, and then the strain entered a relatively stable state. Meanwhile, COD removal reached 63.94%. Recent studies have shown that NO_2^--N can be toxic to microorganisms when its concentration exceeds 30 mg/L [42]. However, some HN-AD strains, such as *Alcaligenes faecalis* WY-01 and TF-1 strains, lacked the capacity to perform independent aerobic denitrification [4,43] and therefore could not effectively remove nitrite nitrogen. Strain L2 was able to remove NH_4^+-N, NO_3^--N, and NO_2^--N and showed good HN-AD function, showing great potential for the biological treatment of wastewater.

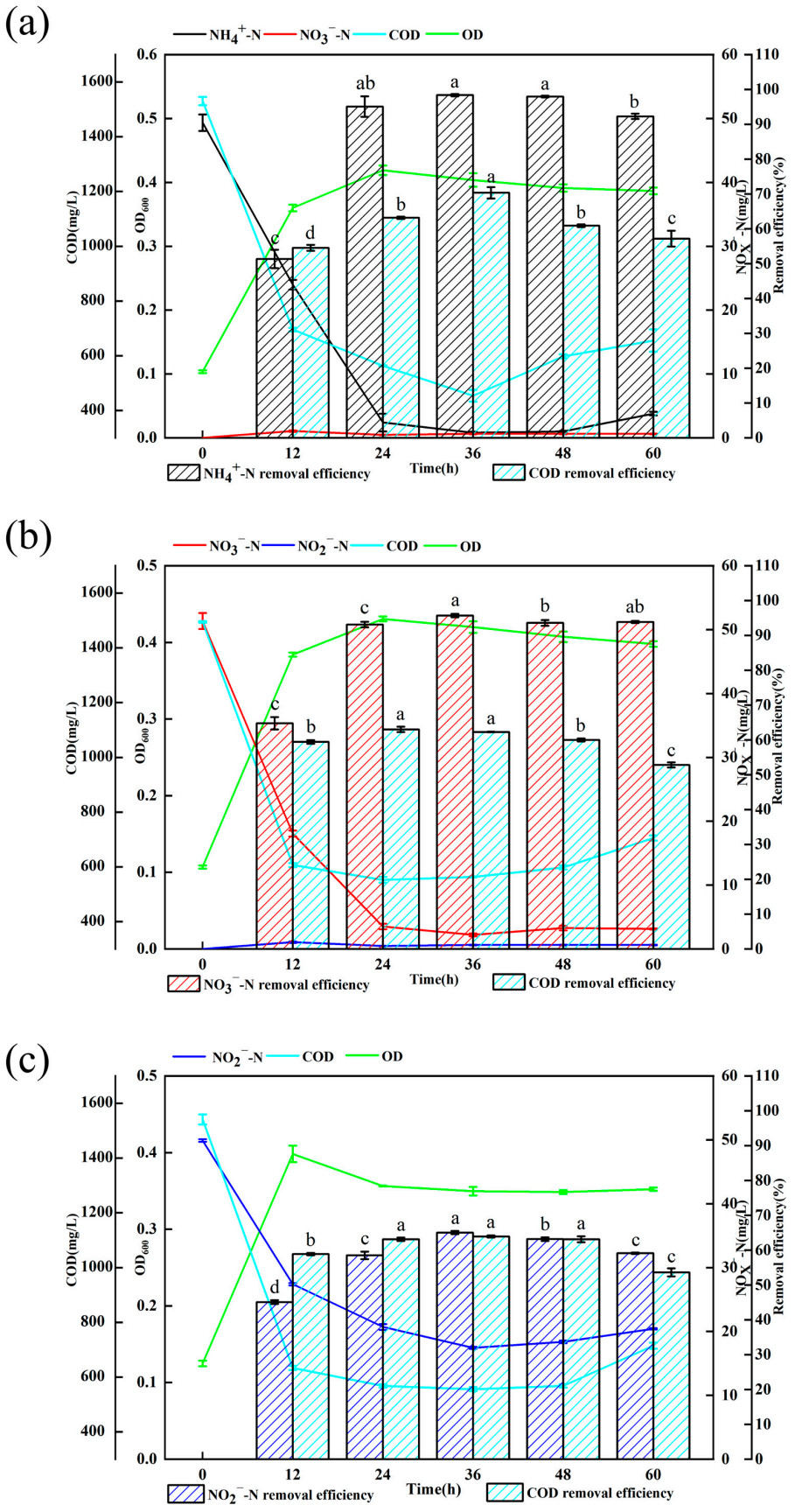


Figure 3. Nitrification–denitrification characteristics of strain L2 under different nitrogen source conditions: (**a**) ammonium sulfate; (**b**) potassium nitrate; (**c**) sodium nitrite.

3.4. Effect of Mixed Nitrogen Sources on the Nitrification Function of Strain L2

Different types of N sources could affect the nitrification performance of heterotrophic nitrifying bacteria. Meanwhile, the accumulation of NO_3^--N and NO_2^--N might inhibit the expression of nitrite reductase and nitrate reductase during nitrification [43]. Therefore, experiments were conducted using a mixture of different N sources in a ratio of 1:1 to investigate the denitrification characteristics of strain L2 and to determine its denitrification pathway. As shown in Figure 4a, when NH_4^+-N and NO_3^--N coexisted in the system, strain L2 rapidly degraded NH_4^+-N and NO_3^--N to 0 mg/L and 1.74 mg/L, respectively, with the highest NO_2^--N accumulation of 10.77 mg/L in 24 h. There was no negative impact of NO_3^--N on the NH_4^+-N degradation rate in the coexisting system. As shown in Figure 4b, strain L2 was still able to rapidly degrade NH_4^+-N to 0 mg/L within 24 h when NH_4^+-N and NO_2^--N coexisted in the system. The changes in NO_2^--N were similar to those in the system of coexistence of NH_4^+-N and NO_3^--N, and a small amount of NO_3^--N also accumulated. NO_2^--N did not affect the nitrification reaction. The performance of the mixed system with three sources of N: NH_4^+-N, NO_3^--N, and NO_2^--N is shown in Figure 4c. In the presence of three N sources, strain L2 exhibited degradation effects on all three N sources, and the concentration of NO_2^--N first increased and then decreased, which indicated that the NO_2^--N accumulation occurred during the degradation of NH_4^+-N and NO_3^--N. However, no significant accumulation of NO_3^--N was observed. This indicated that the nitrification reaction was not affected by the presence of NO_3^--N and NO_2^--N.

In the above three model systems, COD decreased with the depletion of various N sources up to 48 h. After 48 h, a small increase in COD was noticed, which was probably caused by the lysis of dead cells and the release of organic matter. NH_4^+-N removal increased compared to HNM medium with ammonium sulfate as an N source alone, which may be due to the fact that NO_2^--N and NO_3^--N act as electron acceptors and NH_4^+-N acts as an electron donor during the nitrification process to achieve rapid removal of NH_4^+-N [2,44].

Figure 3a–c show that strain L2 can carry out the HN-AD process, with an accumulation of NO_3^--N and NO_2^--N. These findings are consistent with the experimental results shown in Figure 4a,b. However, the highest NO_3^--N and NO_2^--N accumulations were only 0.71 mg/L and 0.63 mg/L, respectively. The findings suggested that strain L2 could partially convert ammonia nitrogen into intracellular nitrogen, and the rest of the ammonia nitrogen was converted into other forms of nitrogen by other means. Therefore, the denitrification pathway of strain L2 was hypothesized to be $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- (\leftrightarrow NO_3^-) \rightarrow NO \rightarrow N_2O \rightarrow N_2$ and $NH_4^+ \rightarrow NH_2OH \rightarrow NO \rightarrow N_2O \rightarrow N_2$. This pathway was further validated by detecting the key genes in strain L2.

3.5. Characterization of NH4$^+$-N Removal by Strain L2

3.5.1. Effect of Carbon Source

As shown in Figure 5a, when maltose, sodium acetate, sodium propionate, sodium citrate, and glucose were used as the only C source (C/N = 7), the best strain growth was achieved in the presence of a glucose carbon source, followed by sodium acetate. According to recent studies, most HN-AD bacteria exhibit a complete inability to utilize glucose as a carbon source [45], and organic acids prove to be more conducive to nitrogen removal [1,46]. Apparently, strain L2 was able to utilize both glucose and organic acids. When glucose and sodium acetate were carbon sources, COD decreased from 1307 and 1339 mg/L to 484 and 543 mg/L, with 62.9% and 59.4% COD removal, respectively, in 60 h. The initial NH_4^+-N concentrations were 47.88 and 47.92 mg/L, respectively, which decreased to 4.58 and 7.50 mg/L, and the final NH_4^+-N removal reached 90.45% and 84.33%, respectively. In HNM media with sodium citrate and sodium propionate as C sources, the growth delay period of the strains was long, and the removal of NH_4^+-N and COD was lower than that in the presence of a maltose carbon source. The difference in the degree of C source utilization by nitrifying bacteria results in different nitrification rates and metabolites. Therefore,

selecting an additional carbon source will affect wastewater management, including nitrification efficiency, operating costs, and environmental conditions [47]. Our results showed that sodium acetate serves as the most effective C source for NH_4^+-N removal by strain L2, followed by glucose and maltose.

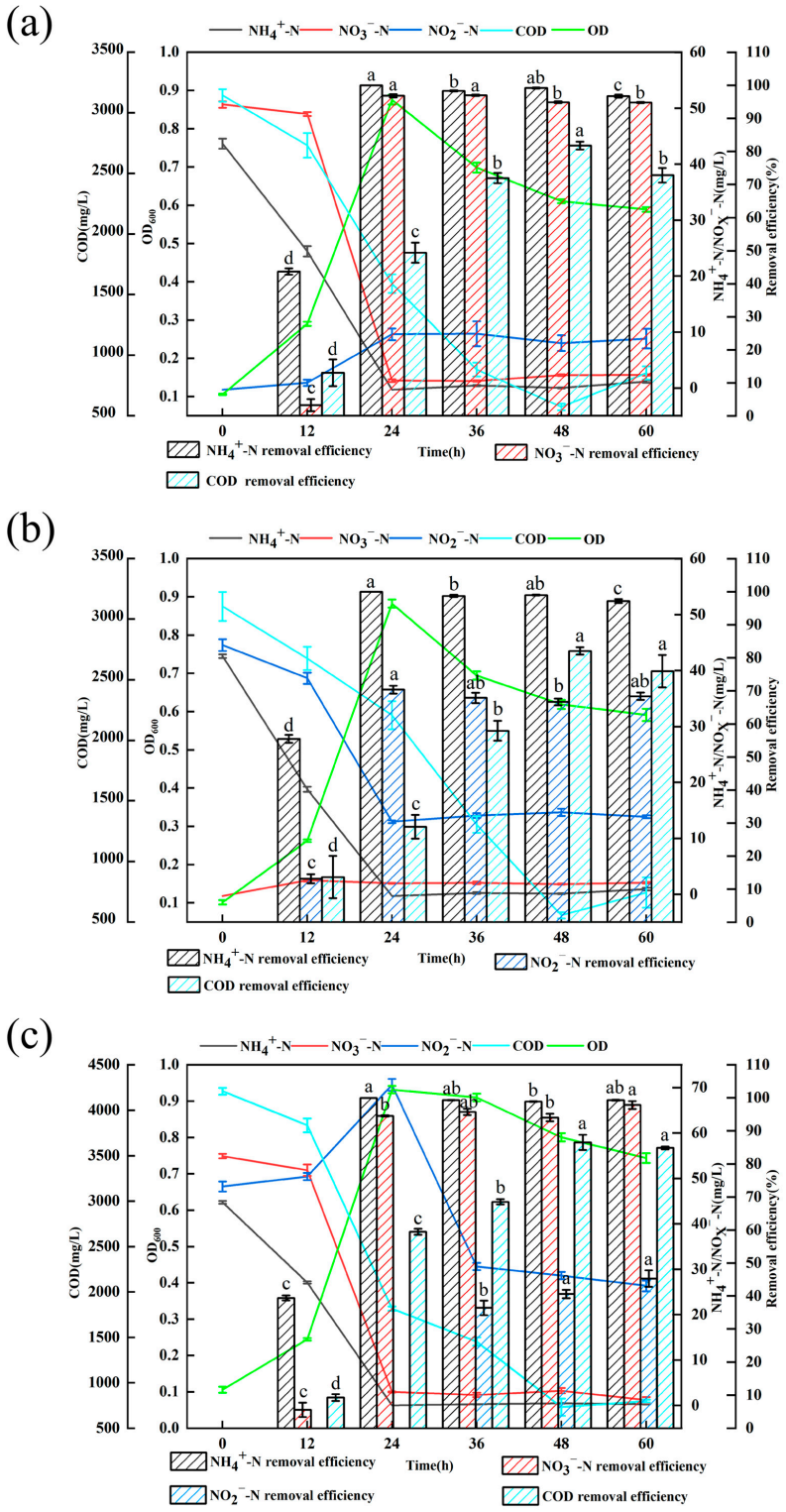


Figure 4. Effect of different mixed nitrogen sources on heterotrophic nitrification of strain L2: (**a**) ammonium sulfate–potassium nitrate (**b**) ammonium sulfate–sodium nitrite (**c**) ammonium sulfate–potassium nitrate–sodium nitrite.

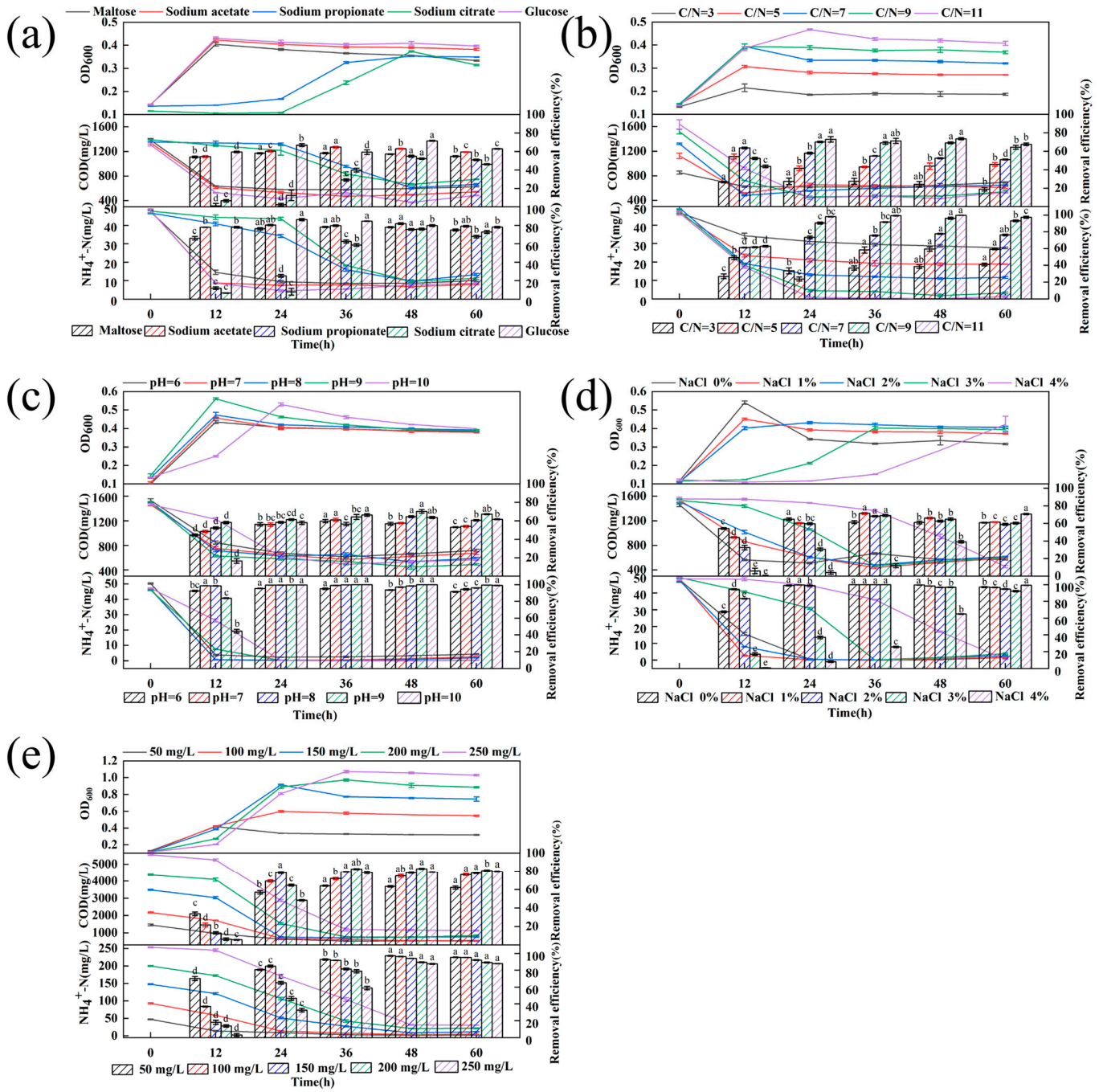


Figure 5. NH_4^+-N removal rates under different conditions: (**a**) carbon source; (**b**) carbon to nitrogen ratio; (**c**) pH; (**d**) salinity; (**e**) initial NH_4^+-N concentration.

3.5.2. Effect of C/N Ratio

The C/N ratio significantly affects the denitrification performance of HN-AD bacteria and plays a key regulatory role in biological denitrification [29,48]. As shown in Figure 5b, the growth condition and denitrification capacity of strain L2 were positively correlated with the C/N ratio. When the C/N was 3 and 5, COD slightly increased after 12 h, and NH_4^+-N degradation was not significant. However, when the C/N ratio was higher than 7, almost all NH_4^+-N was removed. The best NH_4^+-N removal efficiency was achieved at a C/N of 11, with a maximum removal efficiency of 99.75%, which was 3% higher than that at a C/N of 9. Considering the NH_4^+-N removal rate and economic factors, a C/N ratio of 9 was chosen as the best C/N ratio for nitrification by strain L2. Most HN-AD

bacteria require higher C/N to achieve high nitrogen removal efficiency, e.g., HN-AD mode (C/N = 25) and *Acinetobacter* sp. JR1 (C/N > 16) in SBR systems [39,49]. On the other hand, a low C/N resulted in poor HN-AD performance [18]. In contrast to the former studies, strain L2 exhibited efficient nitrogen removal efficiency at a low C/N, which is favorable for practical applications [50].

3.5.3. Effect of pH

Figure 5c showed that strain L2 maintained good growth and NH_4^+-N removal efficiency in the pH range of 6.0–9.0. At pH 6, 7, 8, and 9, the maximum NH_4^+-N removal efficiencies were 95.37%, 99.31%, 99.67%, and 100%, respectively. At pH 10, the growth of the strain was slower in the early stage in the HNM medium, which indicated that the strong alkaline environment had an inhibitory effect on the growth of the strain [51], but the maximum NH_4^+-N removal could still reach 100%. The COD removal rate of the strain at pH = 9 was significantly higher than other pH groups, and the final COD removal was 67.25%.

In general, biological denitrification systems are pH-sensitive, with a low pH inhibiting nitrification and weakly alkaline conditions promoting nitrification. This may be due to the fact that pH affects free ammonia and ammonia nitrogen oxygenase in the medium [2,52]. Therefore, neutral (pH = 7) and alkaline (pH = 8–10) conditions resulted in higher NH_4^+-N and COD removal than acidic (pH = 6) conditions. In summary, strain L2 was found to be efficient in removing NH_4^+-N at pH values ranging from 6–10. Considering strain growth and COD removal performances, the optimal pH for nitrogen removal by strain L2 was determined to be 9. This indicates that the strain has a wide adaptability to complex acidic and alkaline conditions, which proves its potential for wastewater treatment applications.

3.5.4. Effect of Salinity

High-salt wastewater can have an inhibitory effect on microbial activity, which restricts the intracellular substance transport process, inducing cytoplasmic disintegration and even leading to cell death [53,54]. As shown in Figure 5d, strain L2 grew well in the HNM medium with a salinity of 0–2%, showing logarithmic growth from 0 to 12 h, and complete degradation of NH_4^+-N within 36 h. Inhibition of the growth of strain L2 was inhibited by higher salinity in the HNM medium. When the salinity increased to 30 mg/L, the delayed period of strain L2 was 12 h. When the salinity was further elevated to 40 mg/L, the delayed period extended to 24 h. After a delayed period, strain L2 entered the logarithmic growth period, degrading a large amount of NH_4^+-N and COD, with final NH_4^+-N and COD removals of 99.19% and 70.71%, respectively. According to previous studies, the suitable salinity for the growth and metabolism of most HN-AD strains is less than 3%, and at salinity higher than 3%, the nitrogen removal efficiency decreases greatly [55]. For example, the NH_4^+-N removal efficiency of strain ADP-19 at 4% salinity was only 75%. In this study, strain L2 was able to adapt quickly to different salinity conditions. Previously, some nitrifying strains isolated from marine sources could remove N only under high-salinity conditions, such as *Vibrio* sp. SF16 and *Bacillus* sp. N31 [29,56], which cannot remove nitrogen in freshwater. Whereas strain L2 isolated in this study will be able to remove nitrogen efficiently from freshwater or saline water. Based on the results, 0–3% salinity was found to be the optimal salinity range for nitrogen removal by strain L2.

3.5.5. Effect of Initial NH4$^+$-N Concentration

The effects of different initial NH_4^+-N concentrations on the nitrogen removal efficiency of strain L2 are shown in Figure 5e. The maximum NH_4^+-N removal efficiency was 97.13% at an initial NH_4^+-N concentration of 50 mg/L. However, the concentration of L2 cells was low, and the maximum OD_{600} was only 0.42. The initial concentrations of NH_4^+-N in media were 100, 150, 200, and 250 mg/L. After 48 h, NH_4^+-N concentrations decreased to 3.47, 8.77, 21.19, and 31.2 mg/L, respectively. OD_{600} increased with the increase in the initial concentration of NH_4^+-N. NH_4^+-N and COD removal efficiencies of strain L2 were 87.98%

and 79.79% at the initial NH_4^+-N concentration of 250 mg/L (2% salinity). The results showed that the high initial concentration of NH_4^+-N had an inhibitory effect on strain L2, which reduced the NH_4^+-N removal ability of the strain. In a previous study, strain HNDS-1-8 showed 65% to 80% NH_4^+-N removal in 72 h at an initial nitrogen concentration of 256.9 mg/L in wastewater [57]. Compared to these strains, strain L2 was more efficient in NH_4^+-N removal. Considering the NH_4^+-N and COD removal efficiencies of the strain, the optimal initial NH_4^+-N concentration for nitrogen removal by strain L2 was determined to be $\leq$200 mg/L.

3.6. Detection of Genes Related to Nitrogen Removal in HN-AD Strain L2

To investigate the nitrogen removal mechanism of strain L2, *hao* (992 bp), *amoA* (491 bp), *napA* (876 bp), *nirK* (473 bp), *norB* (750 bp), and *nosZ* (700 bp) genes were amplified by PCR. Figure 6a confirms the presence of target gene fragments in strain L2. The nitrification gene *amoA* (encoding ammonia nitrogen oxygenase, NH_4^+-N to NH_2OH) contributes to the conversion of ammonia nitrogen to a high-oxidation form [58]. The *hao* gene encodes hydroxylamine dehydrogenase that oxidizes NH_2OH to NO_2^-. Conversely, *napA* encodes the NAR enzyme, which is crucial for the conversion of NO_3^--N to NO_2^--N in the environment and is a key participant in aerobic denitrification. *nirK* encodes the NIR enzyme that reduces NO_2^- to NO, and the successful amplification of *nirK* suggests that NIR produced by strain L2 is a Cu-type reductase rather than a cytochrome cd1-type reductase [59]. *norB* encodes a nitric oxide reductase that reduces NO to N_2O. The nitrous oxide reductase encoded by *nosZ* participates in the final step of denitrification to reduce N_2O to N_2. Therefore, based on the experimental results in Section 3.3 and 3.4 and the successful amplification of the abovementioned genes, it can be concluded that the denitrification pathway of strain L2 is $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- (\leftrightarrow NO_3^-) \rightarrow NO \rightarrow N_2O \rightarrow N_2$ and $NH_4^+ \rightarrow NH_2OH \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Figure 6b). This is similar to the findings reported by [60].

3.7. Application of Immobilized Strain L2 for Wastewater Treatment

Microbial immobilized PSK carriers were used to investigate the persistence and performance of strain L2 in the pesticide wastewater environment in Ningxia. As shown in Figure 7e, ammonia nitrogen (initial concentration of 120 mg/L) was the main nitrogen pollutant in the pesticide wastewater, and the NH_4^+-N removal by immobilized strain L2 was greater than 78% and 82% in the L2 + C and PSK + C experimental groups after three reuse cycles.

It is worth noting that the final NH_4^+-N removal rate in the PSK + C group during cycle 2 reached 86%, which was significantly higher than that in the other groups. This can be attributed to the abrasion of the carrier, resulting in more pores on its surface, as shown in Figure 7c. An increase in pores enables the carrier to attach more bacterial cells to its surface, thus enhancing its biological activity. On the contrary, compared to the blank experimental group, the NH_4^+-N removal rate was very low (<6%), which might be due to the low concentration of organic matter in the pesticide wastewater (COD only around 20 mg/L), leading to a deficiency in effective organic carbon and electron donors for strain L2. The results suggest that a biological treatment using organic carbon sources and immobilized carrier technology improves the efficiency of strain L2 in treating pesticide wastewater.

During the treatment of pesticide wastewater, the nitrogen removal efficiencies of L2 in the L2+C and PSK+C experimental groups did not reach the optimal level because the pesticide wastewater contained biotoxic substances, such as methyl chloroformate, carbendazim, chloroformate, o-phenylenediamine, and benzotriazole, which inhibited the activity of bacteria. However, the efficiency of NH_4^+-N removal remained high. Overall, strain L2 showed strong activity and stability in real wastewater and has a promising potential for the treatment of different environmental wastewater.

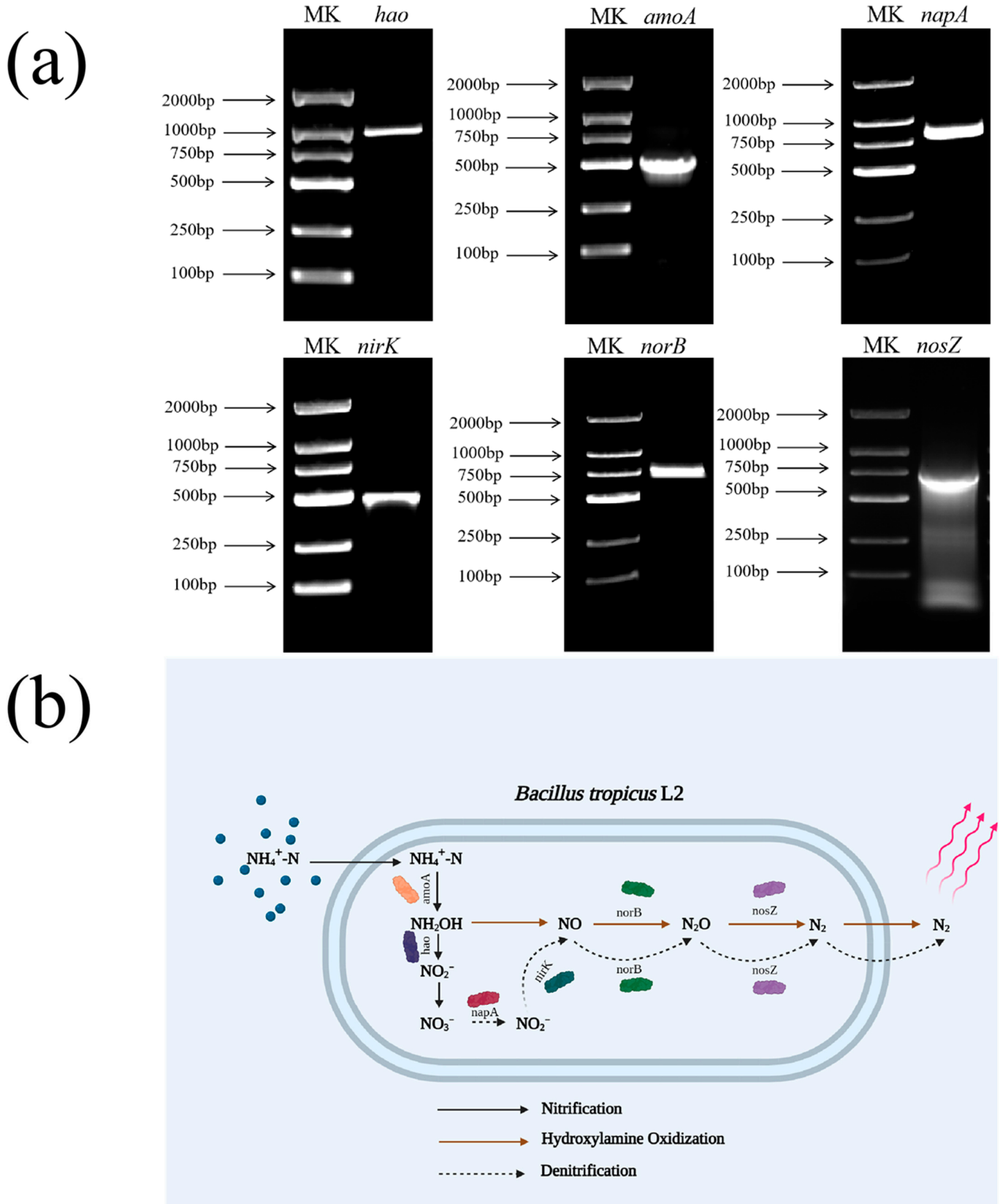


Figure 6. (**a**) Agarose gel electrophoresis results after amplification of denitrification-related genes of strain L2; M: DNA marker; (**b**) N metabolic pathways.

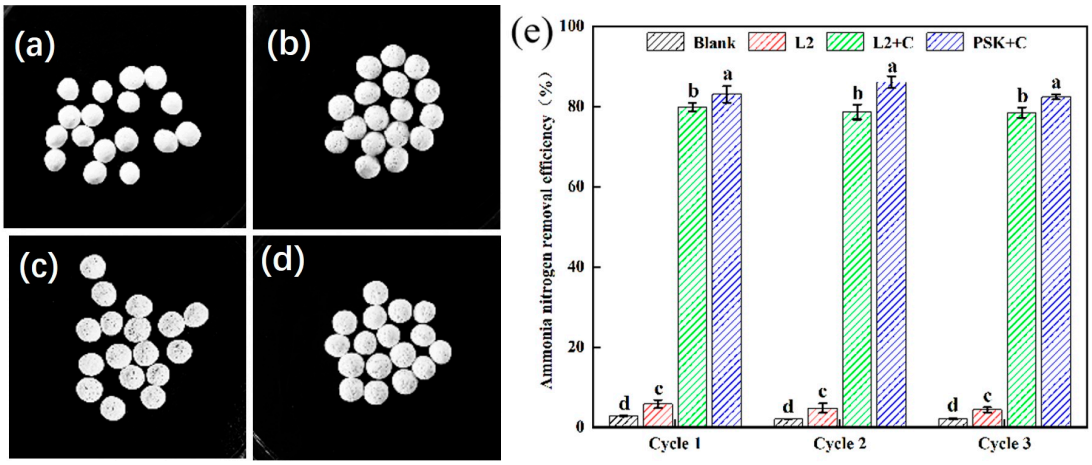


Figure 7. (**a**) Uncycled PSK carrier; (**b**) PSK carrier after cycle 1; (**c**) PSK carrier after cycle 2; (**d**) PSK carrier after cycle 3; (**e**) NH_4^+-N removal in pesticide wastewater by strain L2 and semi-continuous treatment with PSK carrier.

4. Conclusions

An HN-AD bacterial strain L2 was isolated from the aeration tank water samples of Zhonglan Lianhai Design and Research Institute, which was identified as *Bacillus* sp. L2 by morphological characterization and 16S rDNA amplification. In the presence of a mixed N source, strain L2 could completely remove NH_4^+-N within 24 h. The optimal denitrification conditions for strain L2 were as follows: sodium acetate as the C source, C/N = 9, pH = 9, salinity $\leq$ 3%, and an initial NH_4^+-N concentration $\leq$ 200 mg/L. At an NH_4^+-N concentration of 250 mg/L (salinity 2%), the NH_4^+-N and COD removal efficiencies of strain L2 could reach 87.98% and 79.79%. The denitrification pathway was determined and validated using different N sources and PCR amplification of genes related to denitrification. Results showed that the denitrification pathways of strain L2 were $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- (\leftrightarrow NO_3^-) \rightarrow NO \rightarrow N_2O \rightarrow N_2$ and $NH_4^+ \rightarrow NH_2OH \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Both strain L2 and immobilized carriers are suitable for the treatment of pesticide wastewater. This study provided a novel HN–AD strain L2 for the treatment of wastewater.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16030416/s1, Table S1: List of PCR primers used for target genes amplification.

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