

Article

Characteristics of Nitrogen Removal and Functional Gene Transcription of Heterotrophic Nitrification-Aerobic Denitrification Strain, *Acinetobacter* sp. JQ1004

Liangang Hou ^{1,*}, Feng Huang ¹, Zhengwei Pan ¹, Wei Chen ^{1,3} and Xiujie Wang ^{2,*}¹ Water & Environmental Protection Department, China Construction First Group Construction & Development Co., Ltd., Beijing 100102, China² The College of Environmental and Chemical Engineering, Jiangsu University of Science and Technology, Zhenjiang 212100, China³ China Construction First Group Corporation Limited, Beijing 100161, China

* Correspondence: houliangang@chinaonebuild.com (L.H.); wangxiujie@just.edu.cn (X.W.)

Abstract: In this study, the heterotrophic nitrification–aerobic denitrification strain JQ1004 was investigated in terms of its nitrogen removal mechanism and kinetic properties, laying the foundation for its application in the field of wastewater treatment. Nitrogen balance analysis revealed that the final metabolic product was N₂, and approximately 54.61% of N was converted into cellular structure through assimilation. According to the fitting of the Compertz model, the maximum degradation rates of ammonia and nitrate were 7.93 mg/(L·h) and 4.08 mg/(L·h), respectively. A weakly alkaline environment was conducive to N removal, and the sensitivity of functional genes to acidic environments was *amoA* > *nirS* > *narG*. An appropriate increase in dissolved oxygen significantly enhanced heterotrophic nitrification activity, and notably, the denitrification-related functional gene *narG* exhibited greater tolerance to dissolved oxygen compared to *nirS*. The transcription level of *amoA* was significantly higher than that of *narG* or *nirS*, confirming that there might have been direct ammonia oxidation metabolic pathways (NH₄⁺ → NH₂OH → N₂) besides the complete nitrification and denitrification pathway. The annotation of nitrogen assimilation-related functional genes (including *gltB*, *gltD*, *glnA*, *nasA*, *nirB*, *narK*, *nrtP*, *cynT*, and *gdhA* genes) in the whole-genome sequencing analysis further confirmed the high assimilation nitrogen activity of the HN-AD strain.

Keywords: wastewater; nitrogen balance; kinetics; *amoA*; whole genome

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1. Introduction

With the rapid growth of the global population and the development of the social economy, a large amount of nitrogen-containing wastewater is being released, seriously damaging the aquatic environment ecosystem [1]. Excessive nitrogen discharge not only leads to the eutrophication of water bodies and hypoxia in animals or plants, but also poses a threat to human health [2]. Microbial biotechnology is one of the most common methods for nitrogen removal in wastewater treatment. However, conventional nitrifiers or denitrifiers require separate aerobic or anaerobic environments, respectively, for bacterial growth and metabolism [3,4]. This has led to limitations of traditional biotechnology for nitrogen removal, further causing low treatment efficiency and high infrastructure investment [5].

Recent research has focused on heterotrophic nitrification and aerobic denitrification (HNAD), which can effectively overcome the shortcomings of traditional biological nitrogen removal technologies. The HNAD process is considered to convert NH₄⁺ into NO₃[−] through heterotrophic nitrification reactions under aerobic conditions while further denitrifying NO₃[−] or NO₂[−] into gaseous nitrogen, achieving a complete nitrogen cycle in a single reactor [6]. At present, an increasing number of HNAD bacteria have been isolated

and purified from different natural environments, such as *Vibrio* sp. [7], *Zobellella* sp. [8], and *Pseudomonas* sp. [9]. Compared with traditional nitrifying bacteria, HNAD bacteria have several advantages, such as a fast growth rate, high nitrogen removal efficiency, strong environmental tolerance, and the ability to nitrify and denitrify simultaneously in the same aerobic environments [10]. The HNAD process has broad application prospects in the field of wastewater treatment due to the simplification of nitrogen conversion processes and the effective improvement of nitrogen removal efficiency [3,11].

Currently, the study of HNAD bacteria mainly focuses on bacterial isolation and identification, nitrogen removal efficiency, nitrogenous gas composition, and nitrogen metabolic mechanisms [12,13]. There are fewer studies on the kinetics and molecular mechanism of HNAD, so it is significant to search for a universal HNAD bacterium. The aim of this study is to elucidate the characteristics of the growth dynamics of HNAD bacteria, including the rules of nitrogen transformation and the differences in gene transcription under various environmental conditions. In the present study, the biomass proliferation pattern of HNAD strain JQ1004, isolated in previous work [14], was simulated using the Logistic model. Nitrogen balance analysis was performed to determine the nitrogen conversion mechanism. The effects of environmental conditions on the strain JQ1004 were investigated based on the performance of nitrogen removal and the expression levels of functional genes. The whole genome was further analyzed to study the characteristics of cellular molecules and the potential degrading ability of pollutants in wastewater.

2. Materials and Methods

2.1. Media and Strains

The HNAD strain *Acinetobacter* sp. JQ1004 was reported in the published research with the GenBank ID: MF033517.1 [14]. The strain was obtained using the methods of gradient dilution and plate scratching. The components of the heterotrophic nitrification medium (HM) and denitrification medium (DM) referred to the description in the reported work, with some modifications [15]. 1 mol/L HCl or NaOH solution was used to adjust the pH. All culture media were required to be sterilized at 121 °C for 15 min using high-pressure steam before use.

2.2. Nitrogen Balance Analysis

The analysis of nitrogen balance by strain JQ1004 was based on the published work [15]. Firstly, 200 mL of liquid HM containing 50 mg/L ammonia was put into a 500 mL serum bottle, and an O₂/He gas mixture (2 L/min, for 10 min) was injected to ensure complete air displacement. The bottle was sealed and sterilized at 121 °C for 30 min. Bacterial suspensions were inoculated into the serum vials at a 2% (*v/v*) inoculation ratio. The inoculation process ensured that the vials remained sealed to avoid mixing with external nitrogen sources. The bacterial mixture was cultured at 140 rpm and 30 °C. Gas samples were taken with a 1000 µL gas-tight needle, and the sampling volume was 200 µL. N₂O and N₂ were determined by gas chromatography. Cells were centrifuged and freeze-dried to determine the intracellular nitrogen using an element analyzer, and the supernatant was taken to detect NH₄⁺, NO₂[−], NO₃[−], and total nitrogen (TN).

2.3. Kinetics of Nitrogen Degradation and Cell Growth

The bacterial suspension was inoculated into the liquid HM or DM to conduct experiments on the degradation of different nitrogen compounds by the strain JQ1004. The mixed solution was incubated continuously at 140 rpm and 30 °C for 48 h. N concentration changes were monitored through timed sampling. The degradation kinetics model for different nitrogen compounds could be fitted using a modified Compertz model [16]:

$$S = S_0 \left\{ 1 - \exp \left[- \exp \left(\frac{eR_m}{S_0} (t_0 - t) + 1 \right) \right] \right\} \quad (1)$$

where R_m is the maximum degrading rate (mg/(L·h)), S is the N concentration (mg/L) at a certain time, S_0 is the initial N concentration (mg/L), t_0 is the lag time (h), t is the culture time (h), and e is a mathematical constant (Euler's number, approximately equal to 2.71828). The Logistic model [17] was employed to fit the cell growth curves when using ammonia as the nitrogen source. The model equations were:

$$y(t) = \frac{a}{1 + be^{-kt}} \quad (2)$$

where a , b , and k are the constants of the growth equation and e is the mathematical constant.

2.4. The Effect of Environmental Conditions on Nitrogen Removal

To investigate the characteristics of nitrogen removal by the strain JQ1004 under different environmental conditions, sodium succinate and ammonium sulfate were used as carbon and nitrogen sources, respectively, with the initial ammonia concentration set at 100 mg/L and a C/N ratio of 7.5. The control culture conditions were set as pH 7.0, 30 °C, and 140 rpm. For different pH experiments, the initial pH was set to 5.5, 6.5, 7.0, 7.5, or 8.5, respectively. For different rotational speed experiments, the speed was set to 120, 140, 160, 180, or 200 rpm, respectively. For different temperature experiments, the temperature was set to 20 °C, 25 °C, 30 °C, or 35 °C. Except for the variables being tested, all other cultivation conditions were the same as those of the control group. Samples were taken at regular intervals for the determination of NH_4^+ and OD_{600} , and the cultured cells were collected for the determination of the relative expression of functional genes.

2.5. Functional Genes Analysis

Functional genes involved in the nitrogen removal process were amplified using polymerase chain reaction (PCR), and their transcription levels were tested by quantitative PCR (Q-PCR). The primer pairs for each functional gene used in PCR are shown in Table 1. The lengths of the PCR-amplified fragments were detected via 2% agarose gel electrophoresis. The sequences were submitted to the BLAST program at the National Center for Biotechnology Information (NCBI) for online comparison, and representative sequences with high homology were downloaded for analysis based on the comparison results. Total RNA was extracted using an RNAPrep Pure Cell/Bacteria Kit (Tiangen Biochemistry, Beijing, China) for Q-PCR. The 16S rDNA gene was used as an internal reference gene to correct for differences in the cDNA content between different samples [18]. Q-PCR was also performed on the target and internal reference genes of each sample using SYBR Green I. The data were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method [19].

Table 1. The PCR amplification primers list.

Functional Genes	Primer Sequence (5'-3')	Product Length (bp)
<i>amoA</i>	AAGGATTGGCCATTGCTCTG GTGGACCTAAAATCCAAGCATT	606
<i>nirS</i>	TAAAAGTTCACACACAAAAGCAACGC ACAAGTACTGCACCCAGTAATTGG	517
<i>narG</i>	AATCGCAGATCAATTCCAAGCG TCAGCTTCAGTCTGACTAGATTCTAGT	537

2.6. Whole Genome Sequencing and Analysis

The DNA sample was extracted using the magnetic bead method for subsequent gene library construction. The NEB Next[®] Ultra[™] DNA Library Prep Kit (Shanghai, China, Sangon) was used for the construction of sequencing libraries, and 2% agarose gel was used for electrophoresis to check the size of the library. Samples were subjected to paired-end sequencing using the Illumina HiSeq XTen. The data obtained from sequencing were trimmed and filtered using Trimmomatic, and genome assembly was performed

on the clean data after quality control. The assembled genome was subjected to Denovo prediction of repetitive sequences. The predicted open reading frame (ORF) sequences were converted into protein sequences and compared to the Gene Ontology (GO), Clusters of Orthologous Groups of proteins (COG), or Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to obtain the GO, COG, and KEGG pathway annotations of the genes.

2.7. Analytical Methods

The contents of NH_4^+ , NO_3^- , NO_2^- , and TN were detected according to standard methods [20]. The pH and temperature were measured using a portable tester (Munich, German, Water Testing Worldwide). OD_{600} was expressed as the absorbance value of the bacterial suspension at 600 nm.

3. Results and Discussion

3.1. Nitrogen Balance Analysis

From the results of the nitrogen balance analysis in Table 2, it could be seen that the strain JQ1004 converted ammonia into N_2O and N_2 through the HNAD process, in which 12.28% and 22.26% of nitrogen were transformed into N_2O and N_2 , respectively, and emitted into the atmosphere. In addition, up to 54.61% of nitrogen was assimilated into the intracellular nitrogen of the biomass. Xu et al. [21] stated that a novel fungal strain, *Fusarium keratoplasticum* FSP1, with the HNAD function, assimilated 64.28% of nitrogen into cell substances and dissimilated 20.41% into gaseous nitrogen. During the nitrogen metabolism process, only a very small amount (<0.1 mg) of nitrate or nitrite was detected, which was negligible within the detection error range, indicating that JQ1004 was able to secrete nitrate reductase and nitrite reductase with high activity [22]. There was a 2.02% ammonia deficiency in the nitrogen removal process, which might have been due to an experimental error.

Table 2. The nitrogen balance of strain JQ1004 during the heterotrophic nitrogen removal process.

Initial TN(mg/L)	Nitrogen Content (mg/L)						Error
	NH_4^+	NO_3^-	NO_2^-	Intracellular N	N_2O	N_2	
50.58 ± 0.74	4.37 ± 0.08	0.04 ± 0.01	0.06 ± 0.02	27.62 ± 0.57	6.21 ± 0.14	11.26 ± 0.94	2.02%

Note: Error% = $100 \times (\text{Initial TN content} - \text{Final TN content}) / \text{Initial TN content}$.

3.2. Kinetic Analysis of Nitrogen Degradation and Cell Growth

As shown in Figure 1a, the modified Compertz model aptly described the degradation patterns of different nitrogen sources by strain JQ1004. According to Table 3, the correlation coefficients (R^2) of the fitted curves for the degradation of ammonia and nitrate were 0.997 and 0.985, respectively. From the fitting results, it could be seen that strain JQ1004 had an obvious delay period in degrading nitrogen pollutants, which was 5.91 h or 12.74 h, respectively, for metabolizing ammonia or nitrate. These results indicated that the strain JQ1004 showed a preference for degrading ammonia, which was in accordance with previously published work [23]. When using different nitrogen sources, the average degradation rate of ammonia was significantly higher than that of nitrate based on the kinetic parameters. Ammonia was rapidly degraded within 6–24 h, and the ammonia removal efficiency reached 97.3% at $t = 24$ h, with the highest degradation rate of $R_m = 7.93 \text{ mg}/(\text{L}\cdot\text{h})$. Ren et al. [24] studied the ammonia removal efficiency of *Acinetobacter junii* YB under different C/N conditions and found that the maximum ammonia removal rate ranged from 4.04 to 10.09 $\text{mg}/(\text{L}\cdot\text{h})$. Similarly, nitrate entered a quick consumption period at 13–35 h, with a nitrate removal rate of 82.3% at $t = 36$ h and a maximum nitrate degradation rate of $R_m = 4.08 \text{ mg}/(\text{L}\cdot\text{h})$. The strain JQ1004 had higher degradation activity in metabolizing ammonia than nitrate, possibly due to higher enzyme activity during ammonia oxidation compared to denitrification [25].

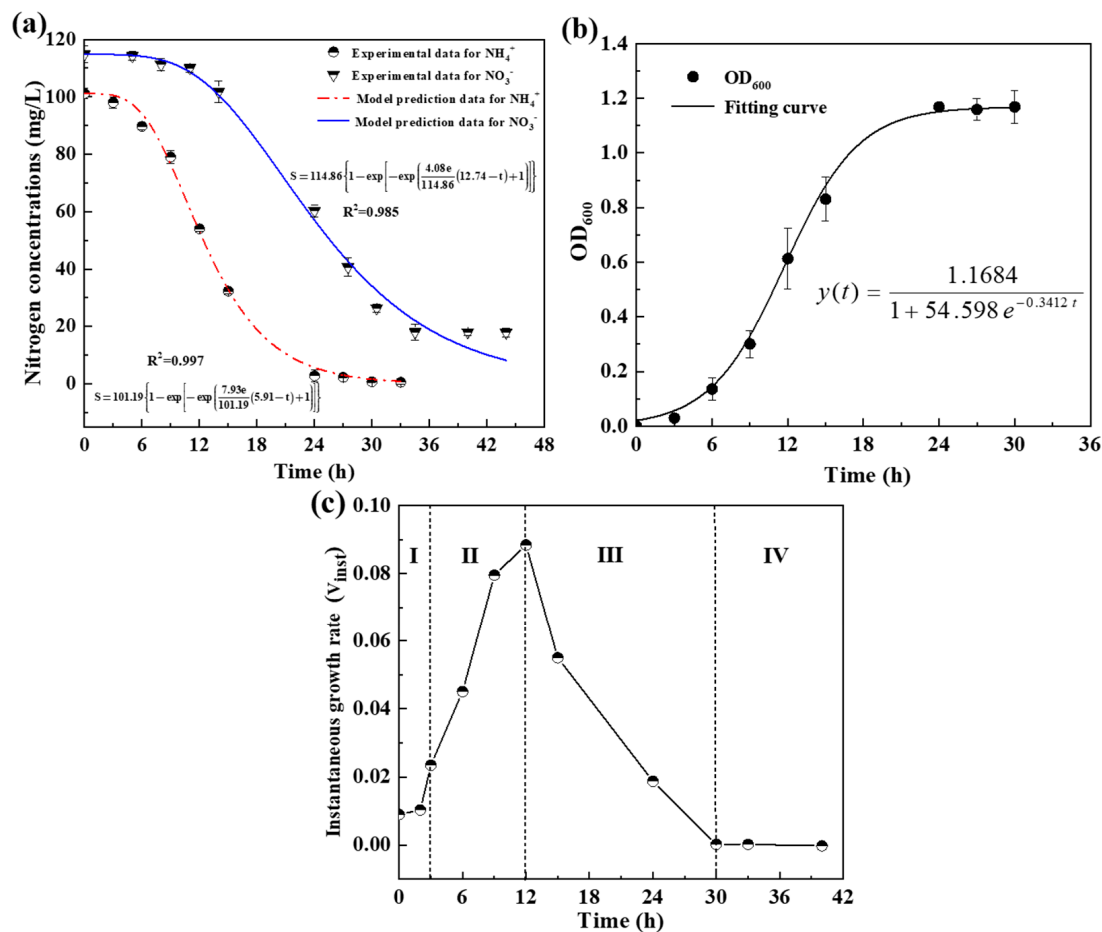


Figure 1. The nitrogen degradation curve (a), growth curve (b), and its first derivative (c) of heterotrophic nitrifying strain JQ1004.

Table 3. The kinetic parameters of degradation of different nitrogen by strain JQ1004.

Kinetic Parameters	Ammonia	Nitrate
S_0 (mg/L)	101.19	114.86
t_0 (h)	5.91	12.74
R_m [mg/(L·h)]	7.93	4.08
R^2	0.997	0.985

The Logistic equation, modeled as a typical S-curve, can be used to describe the effect of increasing cell concentration on the microbial self-metabolism rate during growth and proliferation [26]. As shown in Figure 1b, the equation was able to effectively fit the actual experimental data of cell growth with an R^2 of 0.99. From the results of the regression analysis, it can be seen that the maximum specific growth rate of strain JQ1004 using ammonia as a nitrogen source was 0.34 h^{-1} . Furthermore, the biomass of strain JQ1004 reached the maximum $\text{OD}_{600} = 1.16$ A at 24 h, according to the fitting curve. The growth curves were first-order derived using the Origin 2018 software to obtain the instantaneous growth rate, V_{inst} . The instantaneous growth rate was calculated as:

$$V_{\text{inst}} = \frac{dy}{dt} = \lim_{\Delta t \rightarrow 0} \frac{\Delta y}{\Delta t} = \lim_{\Delta t \rightarrow 0} \frac{f(t + \Delta t) - f(t)}{\Delta t} \quad (3)$$

where the proliferation rate was taken into account when $\Delta t \rightarrow 0$, which could more effectively describe cell proliferation than the average growth rate. As shown in Figure 1c, the proliferation process of strain JQ1004 was obviously divided into four stages. Stage I was

a slow-growth hysteresis period which lasted for about 0–3 h. In this stage, microorganisms mainly adapted to the environment by adjusting their own metabolic patterns and produced functional enzymes that could be used for subsequent metabolism. Subsequently, the strain entered the logarithmic growth period (stage II), during which substrate was consumed and utilized in large quantities to synthesize microbial cellular matter. Bacterial biomass grew rapidly, and the duration of this stage was 3–12 h. After the logarithmic period, the bacterial growth gradually entered a period of slow growth due to the large amount of substrate consumption, and the duration of this stage (Stage III) was 12–30 h. Finally, the strain entered the stable period (Stage IV), in which the growth rate was close to zero, and the biomass no longer increased or even showed a slight decline due to cellular demise and lysis.

3.3. Amplification and Expression of Functional Genes

3.3.1. PCR Amplification of Functional Genes

To further investigate the nitrogen removal mechanism, functional genes related to nitrogen metabolism, including *amoA*, *narG*, *nirS*, and *nirK*, were amplified by PCR. Ammonia monooxygenase (AMO), encoded by the *amoA* gene, catalyzed the conversion of ammonia into hydroxylamine, which was the beginning of the nitrification reaction [27]. Generally, the *amoA* gene is used as a marker functional gene for identifying nitrifying bacteria, as it is widely found among autotrophic or heterotrophic nitrifying bacteria [28]. As shown in Figure 2a, an *amoA* gene fragment with a length of 606 bp was amplified from the genome of strain JQ1004. The sequences were submitted to NCBI for BLAST online comparison, and the results showed that the gene was homologous to the strain *Acinetobacter pittii* AB17H194 (CP040911.1), with up to 99% homology. Nitrate reduction ($\text{NO}_3^- \rightarrow \text{NO}_2^-$) in the aerobic denitrification process was mainly completed by membrane-bound nitrate reductase (*narG*) or periplasmic nitrate reductase (*napA*), which were located in the cell membrane or periplasmic space [29]. According to the characteristics of aerobic denitrification exhibited by JQ1004, the *napA* gene, as the signature gene, was theoretically present in its genome. However, as shown in Figure 2b, only the *narG* gene was detected, with a fragment length of 537 bp, due to the limitations of PCR technology. Furthermore, the *nirS* gene, coding for the nitrite reductase containing cytochrome cd1, was obtained with a fragment length of 381 bp. The results of the homology comparison showed that the *nirS* gene had the highest similarity (97.64%) with the strain *Acinetobacter haemolyticus* XH900 (CP018260.1). The successful acquisition of the *nirS* gene further verified the denitrification ability of strain JQ1004 at the molecular level [30].

3.3.2. The Patterns of Functional Genes Expression

The transcriptional patterns of three genes—*amoA*, *narG*, and *nirS*—during nitrogen removal by strain JQ1004 are shown in Figure 2d. The findings indicated that the *amoA* gene was expressed first, and AMO was synthesized and secreted with high activity, leading to ammonia being degraded rapidly. The transcription level of the *amoA* gene reached a maximum value of 1.98×10^4 copies at 12 h, and then decreased with ammonia consumption. There was an obvious delay in the expression of the *narG* and *nirS* genes, and their expression reached a maximum value of about 1.81×10^4 copies and 1.69×10^4 copies, respectively, at 16 h. It has been shown that nitrite reductase is an inducible enzyme requiring the presence of nitrate or nitrite to induce its expression [31]. In addition, the transcription level of the *amoA* gene was significantly higher than that of the *narG* and *nirS* genes, yet strain JQ1004 did not produce accumulation of intermediates such as nitrate or nitrite, suggesting that the strain had other metabolic pathways in addition to the complete nitrification and denitrification pathways.

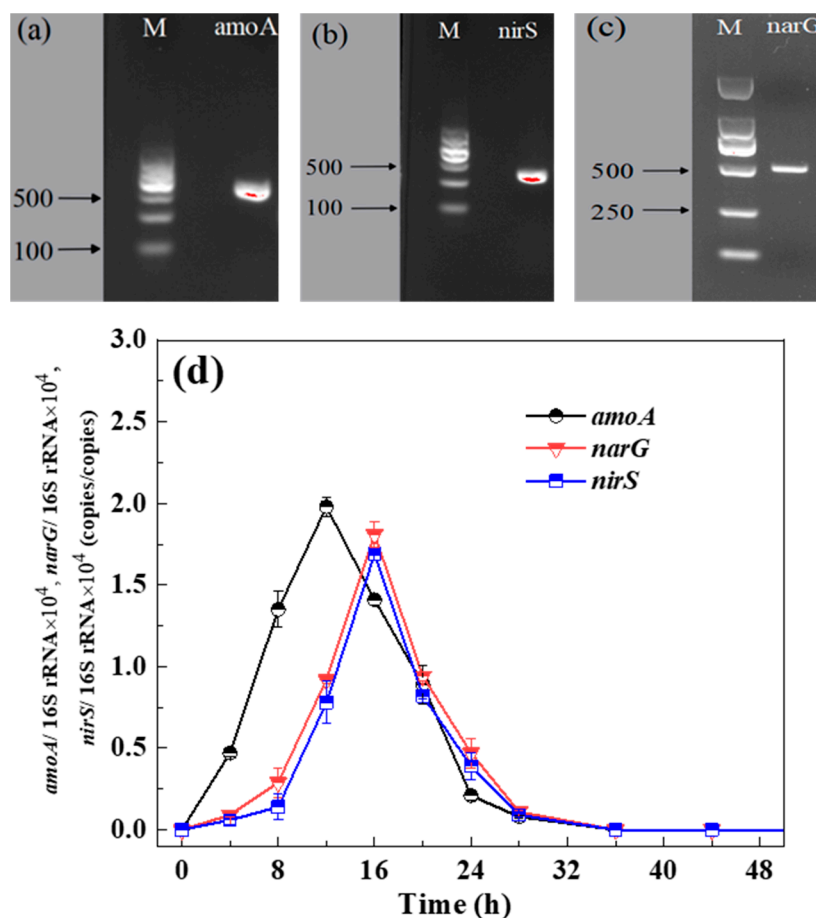


Figure 2. The PCR amplification results (a–c) and expression levels (d) of different functional genes (*amoA*, *narG*, and *nirS*) for nitrogen removal.

3.4. Effect of Environmental Conditions

3.4.1. Different pH

Figure 3a shows the nitrogen removal ability and growth characteristics of strain JQ1004 under different pH conditions, with sodium succinate as the carbon source. After 24 h of continuous culture, the average TN degrading efficiencies were up to 71.44%, 83.28%, 90.22%, 91.87%, and 88.12% at pH values of 5.5, 6.5, 7.0, 7.5, and 8.5, respectively, and the maximum specific growth rates were 0.24 h^{-1} , 0.28 h^{-1} , 0.30 h^{-1} , 0.34 h^{-1} , and 0.30 h^{-1} , respectively. It was concluded that a weakly alkaline environment (such as $\text{pH} = 7.5$) promoted ammonia degradation and the growth rate of the strain, while a too-high or too-low pH environment inhibited nitrogen removal and bacterial growth. There are two main reasons for the increase in nitrogen removal ability when the pH is weakly alkaline. On the one hand, according to Loh et al. [32], aerobic bacteria tend to change their metabolic mechanisms to adapt to environmental variations. When the pH increases due to the alkalinity consumption of the nitrifying reaction, microorganisms will accelerate their nitrification activity in order to produce more acid to lower the pH, thus keeping the cells in an optimal pH environment. On the other hand, the metabolic substrate of AMO is NH_3 rather than NH_4^+ , and an alkaline environment will accelerate NH_3 production, which will improve the nitrification activity and further promote TN removal efficiency [33]. Figure 3b shows the changes in the relative expression of *amoA*, *nirS*, and *narG* in the strain JQ1004 under different pH conditions. The results indicated that the alkaline environment was beneficial for the expression of the *amoA*, *nirS*, and *narG* genes. Compared with the control, *amoA*, *nirS*, and *narG* expression increased by 34%, 23%, and 24%, respectively, at pH 7.5, while *amoA*, *nirS*, and *narG* expression increased by 36%, 21%, and 19%, respectively, at pH 8.5. As mentioned above, an alkaline environment promoted the activity of AMO

and induced more NH_4^+ to be converted to NO_2^- or NO_3^- , which further enhanced the expression of the *narG* and *nirS* genes. In addition, all acidic environments inhibited the expression of functional genes. Compared to the neutral environment, the expression of *amoA*, *nirS*, and *narG* decreased by 37%, 26%, and 18%, respectively, at pH 5.5. These results revealed that the sensitivities of these three genes to an acidic environment were in the order of *amoA* > *nirS* > *narG*.

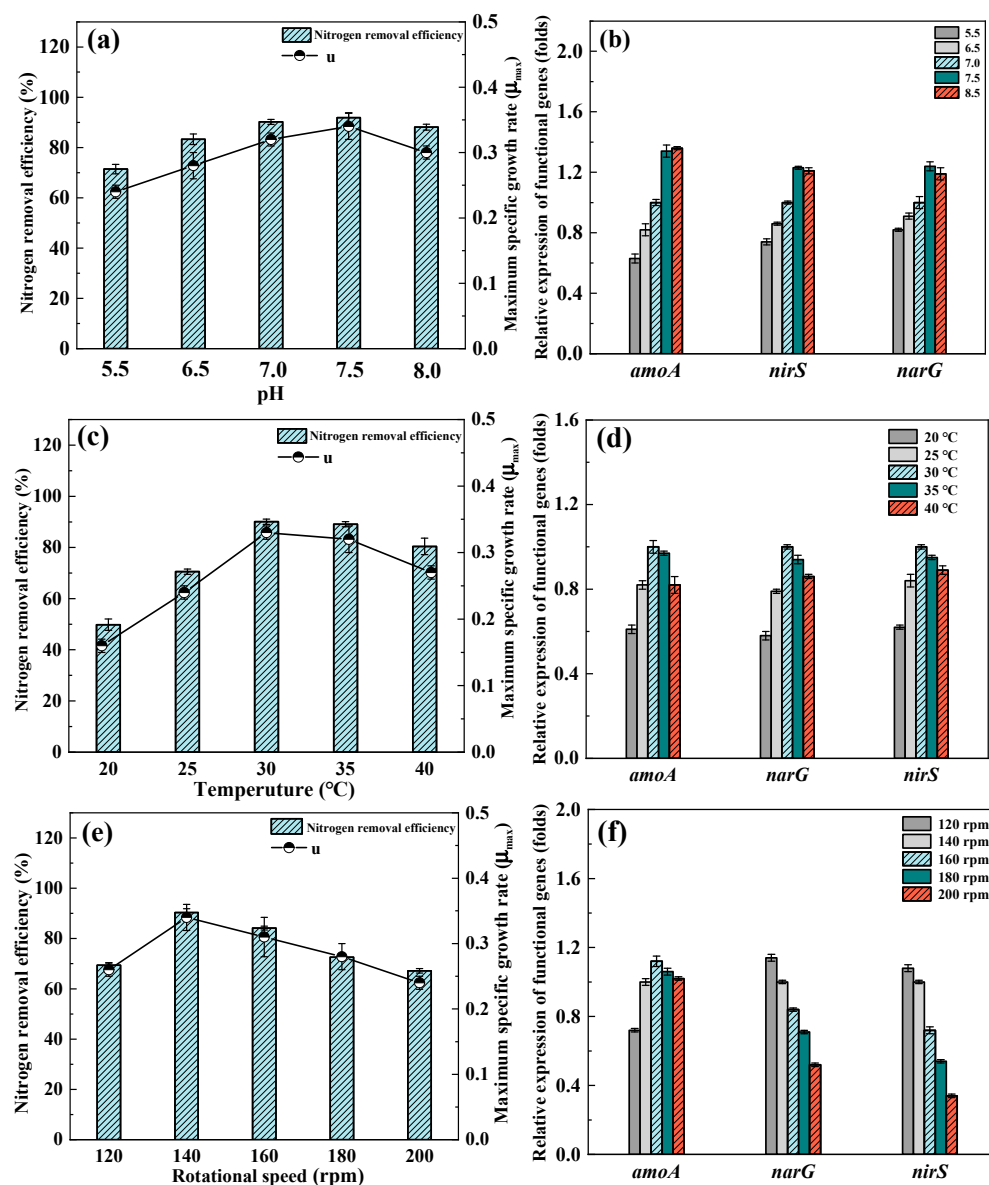


Figure 3. The effect of environmental conditions on nitrogen removal and gene expression of strain JQ1004: (a,b) different pH; (c,d) different temperatures; (e,f) different rotational speeds.

3.4.2. Different Temperatures

As shown in Figure 3c, the temperature significantly affected nitrogen removal and the corresponding gene expression of strain JQ1004. The highest growth and nitrogen removal efficiency were observed at 30 °C, with a maximum specific growth rate of 0.33 h^{-1} and an average TN degrading efficiency of 90.08% at 24 h, respectively. When the temperature increased to 35 °C, the growth and nitrogen degradation were not overly affected. The maximum specific growth rate and average TN removal efficiency were 0.32 h^{-1} and 89.11% at 24 h, respectively. It could be seen that the optimal temperature range for strain JQ1004 was 30–35 °C, which was consistent with the results of previous studies, i.e., the

optimal temperature for the metabolic growth of heterotrophic nitrifying bacteria was 30–37 °C [33,34]. Furthermore, the maximum specific growth rates were 0.16 h⁻¹, 0.24 h⁻¹, and 0.27 h⁻¹, with average TN removal efficiencies of 49.82%, 70.53%, and 80.41%, when the temperature was 20 °C, 25 °C, and 40 °C, respectively. The findings suggested that low temperatures resulted in a slow microbial growth rate and weak metabolic vitality, further leading to a decrease in the rate of nitrogen removal. When the temperature was too high, it easily led to the denaturation of nucleic acids or proteins, reducing the activity of related metabolic enzymes and affecting bacterial growth and reproduction [25]. Figure 3d demonstrates that temperatures which were either too high or too low inhibited the expression of *amoA*, *nirS*, and *narG*. Compared with 30 °C, the transcriptional levels of the *amoA* gene were reduced by 39%, 18%, 3%, and 18% at 20 °C, 25 °C, 35 °C, and 40 °C, respectively. The transcriptional levels of the *nirS* gene were decreased by 38%, 16%, 5%, and 11%, while those of the *narG* gene were decreased by 42%, 21%, 6%, and 14%, respectively. It can be concluded that the different functional enzymes involved in the nitrogen cycle of strain JQ1004 were sensitive to temperature changes, and the enzyme activities were decreased by both high and low temperatures.

3.4.3. Different Rotational Speeds

Figure 3e shows that the maximum specific growth rate of strain JQ1004 was 0.26 h⁻¹ at 120 rpm, and the average TN removal efficiency was only 69.41% after 24 h. When the shaking speed increased to 140 rpm, the bacterial growth and metabolism rate were accelerated with the rising DO content. The maximum specific growth rate was 0.34 h⁻¹, and the average TN removal efficiency was 90.36% after 24 h. When the speed successively increased to 160 rpm, 180 rpm, and 200 rpm, the specific growth rates of the strain in 24 h decreased to 0.31 h⁻¹, 0.28 h⁻¹, and 0.24 h⁻¹, respectively, and the corresponding TN removal efficiencies reduced to 84.19%, 72.58%, and 67.16%. The reasons for this phenomenon might lie in the increase in dissolved oxygen (DO) caused by the ascending rotational speed, promoting the nitrification activity within a certain concentration range and further enhancing the nitrogen removal performance. However, an excessively high DO concentration could inhibit the denitrification process, thereby reducing the TN removal efficiency. According to the coupling mechanism proposed by Wehrfritz et al. [35], O₂, as one of the essential substrates for HNAD bacteria, was involved in nitrogen conversion under the catalysis of AMO and synergistic respiration with nitrate or nitrite. Although HNAD bacteria mostly grow under aerobic conditions, nitrite reductase in the denitrification process is sensitive to oxygen, and too high a DO concentration will inhibit its activity [36]. On the contrary, a shortage of DO will also limit the nitrification reaction, so controlling the DO at the optimal concentration is especially important for microbial growth and the nitrogen degradation process. As shown in Figure 3f, compared with the control group, the expression of the *amoA* gene decreased by 28% in the 120 rpm group and increased by 12%, 6%, and 2% in the 160, 180, and 200 rpm groups, respectively. The results demonstrated that the reduction in DO concentration inhibited the expression of the *amoA* gene and thus reduced the heterotrophic nitrification activity of the strain. Shoda and Ishikawa [37] studied the effect of DO on the HNAD strain *Alcaligenes faecalis* strain No. 4, stating that the microorganism still retained a high heterotrophic nitrification capacity when the DO concentration was 2 mg/L. The nitrogen removal ability of the HNAD strain decreased significantly once the DO concentration fell below 1 mg/L. In addition, the expressions of functional genes related to the denitrification process were inhibited to different degrees due to the increase in rotational speed. Compared with the control group, the expression of the *narG* gene decreased by 16%, 29%, and 48% at 160 rpm, 180 rpm, and 200 rpm, respectively, while the expression of the *nirS* gene decreased by 28%, 46%, and 64%. The findings confirm that denitrification genes have different degrees of sensitivity to DO, and that the *narG* gene is more tolerant than the *nirS* gene to DO.

In summary, *Acinetobacter* sp. JQ1004 is capable of nitrogen transformation through the heterotrophic nitrification–aerobic denitrification process. The overexpression of *amoA*

genes compared to denitrification-related genes suggests the potential existence of a direct ammonia oxidation pathway ($\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{N}_2$). This direct ammonia oxidation reaction utilizes ammonia as an electron donor and O_2 as an electron acceptor, transforming ammonia directly to N_2 via NH_2OH as an intermediate [38]. Unlike the common complete nitrification–denitrification process, the direct ammonia oxidation process significantly reduces the consumption of electrons, demonstrating immense application potential in the field of biological nitrogen removal [39]. Kinetic parameters related to the growth and metabolism of HN-AD bacteria, such as the growth rate and substrate consumption rate, can be obtained through the Logistic model and the modified Compertz model, providing a theoretical basis for practical applications. These kinetic models can predict the metabolic patterns of the strain towards pollutants under different conditions, enabling the optimization of reactor design and operational parameters and ultimately enhancing treatment efficiency.

3.5. Analysis of the Whole Genome

3.5.1. Molecular Characterization of the Genome

The genome of strain JQ1004 was sequenced using the Illumina HiSeq™ sequencing platform, and the total number of reads obtained after quality control of the raw data was 9.19×10^6 . The average sequence length was 146.2 bp, of which the high-quality data standardized by Q20 were 1.33×10^9 bp, accounting for 99.1% of the total number of bases. The genome was assembled to obtain a genome size of 1.34×10^9 bp and divided into 35 contigs with a GC content of 40.1%. The assembled results were subjected to gene element prediction and preliminary annotation, while Denovo prediction of repetitive sequences was carried out at the same time. A total of 3291 genes were predicted and involved in encoding proteins. Among them, the numbers of genes containing bases ≥ 500 bp and ≥ 1000 bp were 2408 and 1171, respectively, and the numbers of tRNAs and rRNAs were 62 and 8, respectively.

3.5.2. Functional Genes Annotation

As shown in Figure 4a, the bacterial genome was subjected to functional annotation using the COG database. There were 3201 functional genes predicted by ORF, of which 2351 genes could be annotated in the COG database, accounting for 73.5% of the total number of genes. Genes could be annotated in the COG database under the categories of “Energy production and conversion”, “Amino acid transport and metabolism”, “Translation, ribosomal structure and biogenesis”, “Transcription”, “Cell wall/membrane/envelope biogenesis”, and “Inorganic ion transport and metabolism”, which accounted for 6.2%, 7.2%, 7.3%, 7.9%, 6.1%, and 6.2%, respectively. In addition, there were 256 genes (approximately 10.9%) in strain JQ1004 with uncertain protein functions, which need to be further investigated. The GO database was utilized to enrich the genes of strain JQ1004 from three aspects: biological process, cellular component, and molecular function. A total of 2057 genes were annotated in the GO database, accounting for about 64.3% of the total functional genes. As shown in Figure 4b, most of the functional genes were enriched in the “metabolic process”, “cellular process”, or “single-organism process” of the biological pathway; “cell”, “cell part”, “membrane”, or “membrane part” of the cellular components; and “catalytic activity”, “transporter activity”, or “binding” of the molecular functions related to catalytic binding. The genome was enriched and analyzed in terms of pathways using the KEGG database. A total of 1672 genes, accounting for about 52.2%, were annotated in the KEGG database. As shown in Figure 4c, the strain JQ1004 had a large number of genes enriched in the “Amino acid metabolism”, “Carbohydrate metabolism”, and “Energy metabolism” pathways, and the most enriched pathway was the “Amino acid metabolism” pathway, with a total of 242 enriched genes.

Analysis of the metabolic pathways showed that the strain JQ1004 contained not only general essential metabolic pathways, such as carbon metabolism (ko01200), the tricarboxylic acid cycle (TCA cycle, ko00020), glycolysis/gluconeogenesis metabolic pathways (ko00010), and pyru-

vate metabolism (ko00620), but also nitrogen metabolism (ko00910), sulfur metabolism (ko00920), fatty acid metabolism (ko01212), methane metabolism (ko00680), selenium metabolism (ko00450), propionic acid metabolism (ko00640), butyric acid metabolism (ko00650), glyoxylate and dibasic acid metabolism (ko00630), fructose and mannose metabolism (ko00051), starch and sucrose metabolism (ko00500), and other basic metabolic pathways. Among them, the genes that could be annotated to the nitrogen metabolic pathway were mainly the *gltB*, *gltD*, *glnA*, *nasA*, *nirB*, *narK*, *nrtP*, *cynT*, and *gdhA* genes. In addition to the ability to metabolize ammonia, nitrate, and other nitrogen compounds, the results of functional annotation showed that the strain JQ1004 was able to degrade aromatic compounds, limonene, pinene, aminobenzoic acid, 2-carboxysalicylic acid, benzoic acid, geraniol, caprolactam, bisphenol, chloroalkanes, styrene, ethylbenzene, nitrotoluene, xylenes, atrazine, dioxin, naphthalene, etc. This indicates that the bacterium has great potential for application in wastewater treatment.

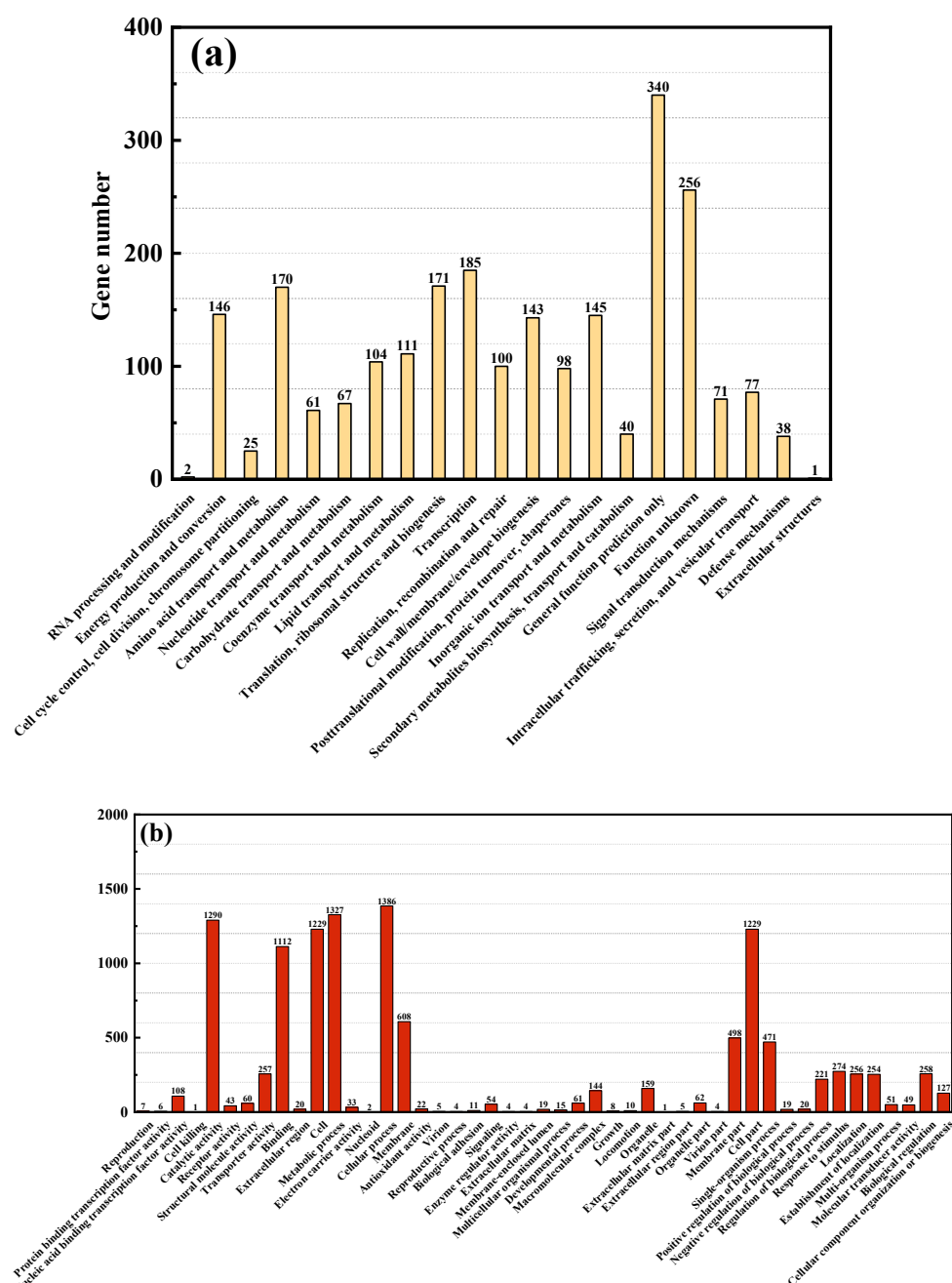


Figure 4. Cont.

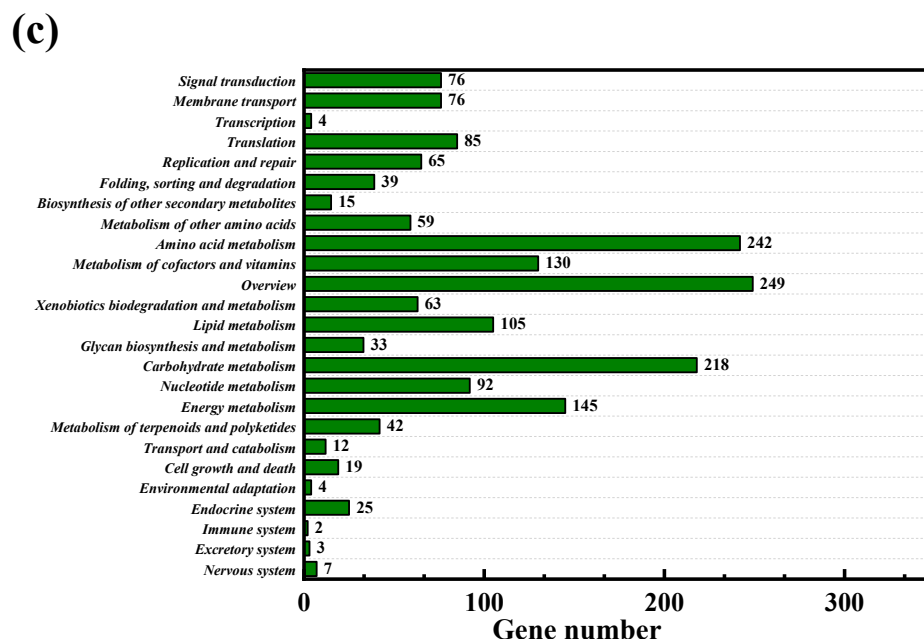


Figure 4. The functional annotation classifications of strain JQ1004 in the COG (a), GO (b), and KEGG (c) databases.

4. Conclusions

The present study mainly investigated the nitrogen removal characteristics of the strain JQ1004 under different environmental conditions, as well as its whole genome. Nitrogen balance analysis showed that about 12.3% and 22.3% of nitrogen was converted to N_2O and N_2 , respectively. Up to 54.6% of nitrogen was converted to intracellular nitrogen by assimilation. The *amoA*, *narG*, and *nirS* genes were successfully expressed, and the transcription of denitrification-related genes lagged obviously behind *amoA* genes. A weakly alkaline environment promoted the expression of the *amoA*, *narG*, and *nirS* genes, while environments with too-high or too-low pH values inhibited nitrogen removal and cell growth. The optimal temperature was 30–35 °C. Increasing the rotational speed was able to improve the heterotrophic nitrification ability, but suppressed the expression of genes related to the denitrification process. The total number of functional genes predicted by the ORF was 3201, among which 2351 genes could be annotated to the COG database, accounting for 73.45%. About 2057 genes were annotated to the GO database, accounting for about 64.26%, while 1672 genes (52.23%) were annotated to the KEGG database. Among these, the most enriched pathway was “Amino acid metabolism”, collecting approximately 242 genes.

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the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Shu, H.; Sun, H.; Huang, W.; Zhao, Y.; Ma, Y.; Chen, W.; Sun, Y.; Chen, X.; Zhong, P.; Yang, H.; et al. Nitrogen removal characteristics and potential application of the heterotrophic nitrifying-aerobic denitrifying bacteria *Pseudomonas mendocina* S16 and *Enterobacter cloacae* DS'5 isolated from aquaculture wastewater ponds. *Bioresour. Technol.* **2022**, *345*, 126541. [\[CrossRef\]](#) [\[PubMed\]](#)
- Joo, H.; Mitsuyo, H.; Makoto, S. Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. *J. Biosci. Bioeng.* **2005**, *100*, 184–191. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wang, X.; Hou, H.; Liu, P.; Hou, L.; Yang, T.; Dai, H.; Li, J. Acceleration of nitrogen removal performance in a biofilm reactor augmented with *Pseudomonas* sp. using polycaprolactone as carbon source for treating low carbon to nitrogen wastewater. *Bioresour. Technol.* **2023**, *386*, 129507. [\[CrossRef\]](#)
- Hou, L.; Li, J.; Liu, Y. Microbial communities variation analysis of denitrifying bacteria immobilized particles. *Process Biochem.* **2019**, *87*, 151–156. [\[CrossRef\]](#)
- 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. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zhao, B.; Tian, M.; An, Q.; Ye, J.; Guo, J. Characteristics of a heterotrophic nitrogen removal bacterium and its potential application on treatment of ammonium-rich wastewater. *Bioresour. Technol.* **2017**, *226*, 46–54. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ren, J.; Di, F. Sensitivity analysis strategy to assess the salting-out problem during CO₂ geological storage process. *Shock Vib.* **2021**, *2021*, 2809467. [\[CrossRef\]](#)
- Fu, G.; Zhao, L.; Huangshen, L.; Wu, J. Isolation and identification of a salt-tolerant aerobic denitrifying bacterial strain and its application to saline wastewater treatment in constructed wetlands. *Bioresour. Technol.* **2019**, *290*, 121725. [\[CrossRef\]](#) [\[PubMed\]](#)
- Xie, F.; Myat, T.; Wang, H. Simultaneous heterotrophic nitrification and aerobic denitrification by a novel isolated *Pseudomonas mendocina* X49. *Bioresour. Technol.* **2021**, *319*, 124198. [\[CrossRef\]](#)
- Yao, S.; Ni, J.; Ma, T.; Li, C. Heterotrophic nitrification and aerobic denitrification at low temperature by a newly isolated bacterium, *Acinetobacter* sp. HA2. *Bioresour. Technol.* **2013**, *139*, 80–86. [\[CrossRef\]](#)
- Xi, H.; Zhou, X.; Arslan, M.; Luo, Z.; Wei, J.; Wu, Z.; El-Din, M. Heterotrophic nitrification and aerobic denitrification process: Promising but a long way to go in the wastewater treatment. *Sci. Total Environ.* **2022**, *805*, 150212. [\[CrossRef\]](#) [\[PubMed\]](#)
- 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. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zhang, Q.; Liu, H.; Liang, S.; Chen, W.; Tan, S.; Yang, C.; Qin, S.; Long, K. Comparison of moving bed biofilm reactor and bio-contact oxidation reactor start-up with heterotrophic nitrification-aerobic denitrification bacteria and activated sludge inoculation under high ammonia nitrogen conditions. *Bioresour. Technol.* **2024**, *395*, 130408. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wang, X.; Wang, W.; Zhang, Y.; Zhang, J.; Li, J.; Wang, S.; Chen, G. Isolation and characterization of *Acinetobacter* sp. JQ1004 and evaluation of its inhibitory kinetics by free ammonia. *Desalin. Water Treat.* **2019**, *147*, 316–325. [\[CrossRef\]](#)
- 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. [\[CrossRef\]](#)
- Li, J.; Gu, J.; Pan, L. Transformation of dimethyl phthalate, dimethyl isophthalate and dimethyl terephthalate by *Rhodococcus ruber* Sa and modeling the processes using the modified Gompertz model. *Int. Biodeter. Biodegr.* **2005**, *55*, 223–232. [\[CrossRef\]](#)
- Liu, H.; Zeng, W.; Fan, Z.; Li, J.; Zhan, M.; Peng, Y. Effect of iron on enhanced nitrogen removal from wastewater by sulfur autotrophic denitrification coupled to heterotrophic denitrification under different substrate ratios. *Chem. Eng. J.* **2021**, *421*, 129828. [\[CrossRef\]](#)
- Chai, Y.; Huang, C.; Sui, M.; Yin, Y.; Sun, N.; Chen, Y.; Liao, Z.; Sun, X.; Shen, W.; Tang, S. Fe-loaded alginate hydrogel beads activating peroxymonosulfate for enhancing anaerobic fermentation of waste activated sludge: Performance and potential mechanism. *J. Environ. Manag.* **2023**, *341*, 118079. [\[CrossRef\]](#)
- Zhou, X.; Wang, Y.; Tan, X.; Sheng, Y.; Li, Y.; Zhang, Q.; Xu, J.; Shi, Z. Genomics and nitrogen metabolic characteristics of a novel heterotrophic nitrifying-aerobic denitrifying bacterium *Acinetobacter oleivorans* AHP123. *Bioresour. Technol.* **2023**, *375*, 128822. [\[CrossRef\]](#)
- APHA; AWWA; WEF. *Standards Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 2005.
- Xu, M.; Cui, Y.; Huang, M.; Sui, Y. Simultaneous inorganic nitrogen and phosphate removal by aerobic-heterotrophic fungus *Fusarium keratoplasticum* FSP1: Performance, pathway and application. *Bioresour. Technol.* **2024**, *393*, 130141. [\[CrossRef\]](#)
- Chen, M.; He, T.; Wu, Q.; Zhang, M.; He, K. Enhanced heterotrophic nitrification and aerobic denitrification performance of *Glutamicibacter arilaitensis* EM-H8 with different carbon sources. *Chemosphere* **2023**, *323*, 138266. [\[CrossRef\]](#)
- 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. [\[CrossRef\]](#)
- Ren, Y.; Yang, L.; Liang, X. The characteristics of a novel heterotrophic nitrifying and aerobic denitrifying bacterium, *Acinetobacter junii* YB. *Bioresour. Technol.* **2014**, *171*, 1–9. [\[CrossRef\]](#)

25. Ke, X.; Liu, C.; Tang, S.; Guo, T.; Pan, L.; Xue, Y.; Zheng, Y. Characterization of *Acinetobacter indicus* ZJB20129 for heterotrophic nitrification and aerobic denitrification isolated from an urban sewage treatment plant. *Bioresour. Technol.* **2022**, *347*, 126423. [[CrossRef](#)] [[PubMed](#)]
26. Gupta, S.; Ansari, F.; Nasr, M.; Rawat, I.; Nayunigari, K.; Bux, F. Cultivation of *Chlorella sorokiniana* and *Scenedesmus obliquus* in wastewater: Fuzzy intelligence for evaluation of growth parameters and metabolites extraction. *J. Clean. Prod.* **2017**, *147*, 419–430. [[CrossRef](#)]
27. Huang, X.; Yi, X.; He, T.; Jia, H.; Feng, M.; Xiang, S.; Wang, S.; Ni, J.; Xie, D.; Li, Z. Ammonium transformed into nitrous oxide via nitric oxide by *Pseudomonas putida* Y-9 under aerobic conditions without hydroxylamine as intermediate. *Bioresour. Technol.* **2019**, *277*, 87–93. [[CrossRef](#)]
28. Chen, P.; Wang, J.; Lv, J.; Wang, Q.; Zhang, C.; Zhao, W.; Li, S. Nitrogen removal by *Rhodococcus* sp. SY24 under linear alkylbenzene sulphonate stress: Carbon source metabolism activity, kinetics, and optimum culture conditions. *Bioresour. Technol.* **2023**, *368*, 128348. [[CrossRef](#)] [[PubMed](#)]
29. Zhao, B.; Yang, C.; Pan, T.; An, Q.; Guo, J. Characterization of an aerobic denitrifier *Pseudomonas stutzeri* strain XL-2 to achieve efficient nitrate removal. *Bioresour. Technol.* **2018**, *250*, 564–573. [[CrossRef](#)] [[PubMed](#)]
30. Braker, G.; Zhou, J.; Wu, L.; Devol, A.; Tiedje, J. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. *Appl. Environ. Microb.* **2000**, *66*, 2096–2104. [[CrossRef](#)]
31. Härtig, E.; Walter, Z. Kinetics of *nirS* expression (cytochromecd1 nitrite reductase) in *Pseudomonas stutzeri* during the transition from aerobic respiration to denitrification: Evidence for a denitrification specific nitrate and nitrite responsive regulatory system. *J. Bacteriol.* **1999**, *181*, 161–166. [[CrossRef](#)]
32. Loh, X. The effect of pH on the hydrolytic degradation of poly(ϵ -caprolactone)-block-poly(ethylene glycol) copolymers. *J. Appl. Polym. Sci.* **2013**, *127*, 2046–2056. [[CrossRef](#)]
33. Chen, Q.; Ni, J. Ammonium removal by *Agrobacterium* sp. LAD9 capable of heterotrophic nitrification-aerobic denitrification. *J. Biosci. Bioeng.* **2012**, *113*, 619–623. [[CrossRef](#)] [[PubMed](#)]
34. Zhang, Q.; Ying, L.; Ai, G.; Miao, L.; Zheng, H.; Liu, Z. The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. *Bioresour. Technol.* **2012**, *108*, 35–44. [[CrossRef](#)] [[PubMed](#)]
35. Wehrfritz, J.; Reilly, A.; Spiro, S.; Richardson, D. Purification of hydroxylamine oxidase from *Thiosphaera pantotropha*. Identification of electron acceptors that couple heterotrophic nitrification to aerobic denitrification. *FEBS Lett.* **1993**, *335*, 246–250. [[CrossRef](#)] [[PubMed](#)]
36. Ka, J.; Urbance, J.; Ye, W.; Ahn, T.; Tiedje, J. Diversity of oxygen and N-oxide regulation of nitrite reductases in denitrifying bacteria. *FEMS Microbiol. Lett.* **1997**, *156*, 55–60. [[CrossRef](#)] [[PubMed](#)]
37. Shoda, M.; Yoichi, I. Heterotrophic nitrification and aerobic denitrification of high-strength ammonium in anaerobically digested sludge by *Alcaligenes faecalis* strain No. 4. *J. Biosci. Bioeng.* **2014**, *117*, 737–741. [[CrossRef](#)] [[PubMed](#)]
38. Pous, N.; Bañeras, L.; Corvini, P.; Liu, S.; Puig, S. Direct ammonium oxidation to nitrogen gas (Dirammox) in *Alcaligenes* strain HO-1: The electrode role. *Environ. Sci. Ecotechnol.* **2023**, *15*, 100253. [[CrossRef](#)]
39. Pan, Y.; Liu, D. Tapping the potential of wastewater treatment with Direct ammonia oxidation (Dirammox). *Environ. Sci. Technol.* **2023**, *57*, 7106–7108. [[CrossRef](#)]

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