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Nitrite Degradation by a Novel Marine Bacterial Strain *Pseudomonas aeruginosa* DM6: Characterization and Metabolic Pathway Analysis

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Abstract: High concentrations of nitrite in marine aquaculture wastewater not only pose a threat to the survival and immune systems of aquatic organisms but also contribute to eutrophication, thereby impacting the balance of coastal ecosystems. Compared to traditional physical and chemical methods, utilizing microorganism-mediated biological denitrification is a cost-effective and efficient solution. However, the osmotic pressure changes and salt-induced enzyme precipitation in high-salinity seawater aquaculture environments may inhibit the growth and metabolism of freshwater bacterial strains, making it more suitable to select salt-tolerant marine microorganisms for treating nitrite in marine aquaculture wastewater. In this study, a salt-tolerant nitrite-degrading bacterium, designated as DM6, was isolated from the seawater (salinity of 25–30‰) of *Portunus trituberculatus* cultivation. The molecular identification of strain DM6 was conducted using 16S rRNA gene sequencing technology. The impacts of various environmental factors on the nitrite degradation performance of strain DM6 were investigated through single-factor and orthogonal experiments, with the selected conditions considered to be the key factors affecting the denitrification efficiency of microorganisms in actual wastewater treatment. PCR amplification of key genes involved in the nitrite metabolism pathway of strain DM6 was conducted, including denitrification pathway-related genes narG, narH, narI, nirS, and norB, as well as assimilation pathway-related genes nasC, nasD, nasE, glnA, gltB, gltD, gdhB, and gdhA. The findings indicated that strain DM6 is classified as *Pseudomonas aeruginosa* and exhibits efficient nitrite degradation even under a salinity of 35‰. The optimal nitrite degradation efficiency of DM6 was observed when using sodium citrate as the carbon source, a C/N ratio of 20, a salinity of 13‰, pH 8.0, and a temperature of 35 °C. Under these conditions, DM6 could completely degrade an initial nitrite concentration of 156.33 ± 1.17 mg/L within 36 h. Additionally, the successful amplification of key genes involved in the nitrite denitrification and assimilation pathways suggests that strain DM6 may possess both denitrification and assimilation pathways for nitrite degradation simultaneously. Compared to freshwater strains, strain DM6 demonstrates higher salt tolerance and exhibits strong nitrite degradation capability even at high concentrations. However, it may be more suitable for application in the treatment of wastewater from marine aquaculture systems during summer, high-temperature, or moderately alkaline conditions.

Keywords: marine bacterial; *Pseudomonas aeruginosa*; nitrite degradation characteristics; nitrite transformation pathways

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1. Introduction

The excessive accumulation of nitrite is a major challenge in marine aquaculture [1]. Intensive marine aquaculture, characterized by high stocking densities, necessitates substantial feeding to meet the nutritional requirements of aquatic animals. However, undigested feed, excreta, and urea released during microbial decomposition can contribute to the release of soluble nitrogen compounds (including nitrite) and phosphorus. The excessive input of these nutrients can lead to coastal eutrophication, causing adverse impacts on marine ecosystems [2]. Nitrite present in effluents from aquaculture facilities is toxic [3], even at low concentrations, posing a threat to the health and immune systems of aquatic organisms. For instance, the harmful concentration for *Litopenaeus vannamei* is reported to be 6.67 mg/L [4]. Moreover, nitrite is more prone to accumulate in water bodies compared to ammonia [4], possibly due to factors such as high dissolved oxygen demand resulting from high stocking densities and overfeeding [5]. Therefore, there is an urgent need to explore a technological approach to address the issue of nitrite accumulation in marine aquaculture.

Currently, there are various techniques available for removing nitrite from seawater in aquaculture, including rotating biological contactors, trickle filters, bead filters, and fluidized sand biofilters [1]. Physical and chemical methods typically require high levels of technical expertise and equipment investment, leading to high operating costs and a risk of secondary pollution. In comparison, microbiologically mediated biological denitrification utilizes the growth metabolism of microorganisms to convert nitrogen compounds in water into nitrogen gas or biomass, making it more efficient and cost-effective [6,7]. Microorganisms are mainly employed for the removal of ammonia nitrogen and nitrate nitrogen in the treatment of aquaculture wastewater [8], but research on nitrite nitrogen removal is relatively limited [9]. This may be attributed to the typically higher concentrations of ammonia nitrogen and nitrate nitrogen in water, which have a broader and more significant environmental impact. However, even low levels of nitrite nitrogen can pose a threat to aquatic organisms [4], and its removal from water is more challenging compared to ammonia nitrogen [4,5]. Therefore, researching the microbial removal of nitrite nitrogen in marine aquaculture effluents is also crucial. In addition, there are differences in the characteristics between marine aquaculture wastewater and freshwater sewage, as the former contains higher concentrations of salts [10], trace elements, and COD. In particular, the osmotic changes caused by high salinity and the salt-induced precipitation of dehydrogenases may hinder the effective performance of most freshwater-derived microorganisms in the treatment of marine aquaculture wastewater. In particular, the osmotic changes caused by high salinity may hinder the effective performance of most freshwater-derived microorganisms in treating marine aquaculture wastewater [8]. Therefore, strains selected from seawater environments, due to their long-term adaptation and reproduction in the marine environment, may be more suitable for the removal of nitrite nitrogen in marine aquaculture wastewater.

Pseudomonas aeruginosa, a widely distributed bacterium in natural environments, comprises multiple species and subspecies. Previous studies have indicated that *Pseudomonas aeruginosa* exhibits strong adaptability and tolerance, enabling it to better adapt to diverse environments in practical applications. It has demonstrated a wide range of application potential in the management of nitrogen pollution in water [11–14]. However, as each species or subspecies may have differences in growth conditions, metabolic pathways, and adaptability to environmental factors, they exhibit varying abilities in denitrification. Therefore, further research is needed on the denitrification characteristics and degradation mechanisms of *Pseudomonas aeruginosa* before its practical application in order to guide actual production.

This study screened a strain of *Pseudomonas aeruginosa* with high efficiency in nitrite degradation capable of completely degrading 100 mg/L of nitrite within 24 h, demonstrating its great potential for nitrite treatment in marine aquaculture wastewater. The paper focuses on studying the growth and nitrite degradation characteristics of this strain under different environmental factors, including carbon source, C/N ratio, temperature,

salinity, pH, and nitrite concentration. The optimal denitrification conditions were optimized through orthogonal experiments to enhance the efficiency of nitrite degradation by the strain. Furthermore, the key genes involved in the nitrite degradation pathway were amplified by PCR to elucidate their roles and regulatory mechanisms in the nitrite degradation process of strain DM6. This study provides theoretical support and experimental evidence for further optimizing treatment conditions, enhancing degradation efficiency, and developing related applications.

2. Materials and Methods

2.1. Experimental Materials

We initiated the strain screening process using the 2216 medium with seawater obtained from *Portunus trituberculatus* aquaculture (salinity 25–30‰). Two types of media were primarily used in this study. The 2216 seawater medium [15] was utilized for strain activation following modifications and contained the following components (g/L): peptone 5.0, yeast extract 1.0, iron citrate 0.1, and sea salt 30.0, with a pH range of 7.0–7.4. Agar plates were prepared by adding 1.5% (*w*/*v*) agar to the 2216 liquid medium. The nitrite degradation medium, derived from the modified M9 medium, was utilized to investigate the denitrification characteristics of the strain and contained the following components (g/L): $NaNO_2$ 0.5, sodium citrate 4.13, Na_2HPO_4 6.76, KH_2PO_4 3.0, NaCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.49, $CaCl_2$ 0.01, with a pH range of 7.0–7.4. All media were sterilized at 121 °C under high pressure for 20 min.

2.2. Strain Identification

The 16S rDNA gene fragment of strain DM6 was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′). The PCR amplification was conducted in a total volume of 25 μL, comprising 12.5 μL of 2× Es Taq MasterMix, 1 μL of DNA template, 1 μL of F-primer (10 μM), 1 μL of R-primer (10 μM), and 9.5 μL of ddH_2O. The PCR reaction was performed with an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 70 °C for 1 min. The reaction was concluded with a final extension step at 72 °C for 2 min. The PCR products were then sent to YOKANG Biotechnology Co., Ltd. (Hangzhou, China), for sequencing. The obtained sequences were subjected to Blast homology analysis, and highly homologous 16S rDNA gene sequences from reference strains were selected. A phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA 11.0 software to determine the taxonomic classification of strain DM6.

2.3. Effects of Environmental Factors

In order to investigate the optimal denitrification conditions for the target strain, this study explored the effects of five factors on strain growth and nitrite degradation ability, including carbon source, C/N ratio, initial pH, salinity, and temperature. For the carbon source experiment, six different carbon sources, including sucrose, sodium citrate, sodium acetate, sodium succinate, glucose, and sodium pyruvate, were tested as the sole carbon source under the condition of an initial NO_2^--N concentration of 100 mg/L, C/N ratio of 10, and initial pH of 7.0. For the C/N ratio experiment, the C/N ratio was adjusted to 0, 2, 5, 10, 15, and 20 by adding sodium citrate, and the cultures were incubated at 30 °C with shaking at 200 rpm. For the initial pH experiment, the initial pH was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 by adding HCl/NaOH (0.1 M). For the salinity experiment, the salinity of the medium was adjusted to 15‰, 25‰, 35‰, 45‰, 55‰, and 65‰ by adding NaCl. For the temperature experiment, temperatures of 15, 20, 25, 30, 35, and 40 °C were tested. All experiments were conducted in triplicate, with an initial nitrite concentration of 100 mg/L and an inoculum size of 1% (*v*/*v*). Each experiment was carried out for a period of 108 h, with samples taken every 12 h to measure OD_{600} and NO_2^--N concentration.

2.4. Orthogonal Experimental Optimization

Based on the results of single-factor experiments, an L_9 (3^4) orthogonal experiment was designed to further optimize the nitrite reduction conditions for strain DM6. The orthogonal experiment was conducted using sodium citrate as the carbon source, taking into account the influence of four major environmental factors on the nitrite degradation efficiency of the strain. The selection of levels was adjusted based on the nitrite degradation efficiency at 24 h, as shown in Table 1. The initial concentration of nitrite was 100 mg/L, and the nitrite reduction efficiency was measured after 24 h of cultivation for each group. Finally, a verification experiment was conducted under the optimal denitrification conditions.

Table 1. Orthogonal experimental factor level.

Level	A	B	C	D
	Temperature (°C)	Salinity (‰)	C/N	pH
1	30	13	10	7.0
2	35	16	20	8.0
3	40	19	30	9.0

2.5. Nitrite Tolerance Test

Under the optimal denitrification conditions, the nitrite tolerance of strain DM6 was investigated at different initial concentrations of nitrite. The initial concentrations of nitrite were adjusted to 100, 150, 200, 250, 300, and 350 mg/L by varying the amount of sodium nitrite added. Simultaneously, the addition of sodium citrate was adjusted to maintain a C/N ratio of 20. NaCl and NaOH solutions were used to adjust the salinity and pH to 13‰ and 8.0, respectively. Each experiment was conducted in triplicate, with an inoculum size of 1% (*v*/*v*), and incubated at 35 °C with agitation at 200 rpm. Samples were collected at 12 h intervals and analyzed for OD_{600} and NO_2^--N concentration.

2.6. Amplification of Denitrification Functional Genes

According to the manufacturer's instructions, total genomic DNA of strain DM6 was extracted using the Bacterial DNA Kit (Omega, Norcross, GA, USA). The genomic DNA served as a template for amplifying the denitrification functional genes of this strain, including narG, narH, narI, nirS, norB, nasC, nasD, nasE, glnA, gltB, gdhB, and gdhA. The PCR reaction system (50 μL) consisted of 2× Es Taq MasterMix 25 μL, DNA template 1 μL, F-primer (10 μM) 2 μL, R-primer (10 μM) 2 μL, and ddH_2O 20 μL. The PCR amplification conditions for the denitrification functional genes were as follows: initial denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 70 °C for 1 min; and final extension at 72 °C for 2 min. The annealing temperature and cycle number for all gene amplifications were 60 °C and 30 cycles, respectively. The extension time was determined by the length of the genes: 1 min for narI, norB, nasE, glnA, gltD, and gdhA; 1.5 min for narH, nirS, and nasD; and 2.5 min for nasC, narG, gltB, and gdhB. The primer sequences are detailed in Table 2. The PCR products were separated by 1% agarose gel electrophoresis and sequenced by YOKANG Biotechnology Co., Ltd.

Table 2. Primer sequences for denitrification-related genes.

Gene	Primer Sequence (5′–3′)	Fragment Length (bp)
narG	narG-F: CGTGTTCATCGCCTACTACCTGAG narG-R: GGCTTGGTCTCGACGTTGTTGA	4097
narH	narH-F: CAAGGACGGCATGGTGATGATGT narH-R: ACGGCAGCAGCGACAGGTAT	2247
narI	narI-F: GGCGTCCACCGAACAACCATT narI-R: TTGACCTTCAGTTGCGGCAGTT	884

Table 2. *Cont.*

Gene	Primer Sequence (5′–3′)	Fragment Length (bp)
nirS	nirS-F: CCACGCAGCCCTTGTTCTTGA nirS-R: CGCACGCTATTCACAGTTGGAAG	2050
norB	norB-F: CCATGCCGCAGTTCCATCTCA norB-R: TCGCCGTTGAACCAGGACAC	1774
nasC	nasC-F: CCTGATCTACGGCAAGCGAGTG nasC-R: CGGACGGACAGTGCTCCAGTAT	3075
nasD	nasD-F: CTGCTTGGGTTCTCCCGACAATC nasD-R: ATATCCAGCCAGTTCATCGGTTCAC	2673
nasE	nasE-F: ACAGCCGCAATTGAAGAAGGAGTT nasE-R: TGTTCGATCAGGACGCCACAAC	546
glnA	glnA-F: CGGAGTGGCAGGTTCTGACGAA glnA-R: TTGGCGTTGGTGATTTGGCTGTT	1579
gltB	gltB-F: TGGCGCAGCAGCTTCTTCTTC gltB-R: GCCTCCCTTTGCCTGTCGAAA	4654
gltD	gltD-F: AAGGGCCGCTATTGTCGCAAAG gltD-R: CGTCGGTTCTGGCTGGTGAAAC	1640
gdhB	gdhB-F: GCTTCTGCTGAACTTCATGGACG gdhB-R: ACCGCAAACACCGCAGGTT	5063
gdhA	gdhA-F: AAGTGGAGAACATCGTCGCCTTC gdhA-R: CCGCCTGGCTTGTTAGAGTCAC	1569

2.7. Data Analysis

NO_2^--N concentration was measured using the NED spectrophotometric method [16], and the cell growth (OD_{600}) of strain DM6 was spectrophotometrically determined at the wavelength of 600 nm [17]. Data in this experiment were analyzed by one-way ANOVA with Tukey's HSD test ($p < 0.05$) using SPSS Statistics 17.0 software and the figures were made by Origin 2018 software. Additionally, the results of the orthogonal experiment were analyzed using Orthogonal Design Assistant II 3.1 software. Each experiment had three repetitions, and the results are presented as means ± SD (standard deviation of means).

The calculation method for nitrite degradation efficiency (E) is as follows:

$$E\ (\%) = (C_0 - C_t)/C_0 \times 100\%$$

where C_0 and Ct represent the initial and corresponding NO_2^--N concentrations (mg/L) at time t, respectively [18]. The concentration units for C_0 and C_t are mg/L.

3. Results

3.1. Strain Identification

This study conducted strain identification of a nitrite-degrading bacterium isolated from seawater using 16S rRNA sequencing, and it was designated as sp.DM6. BLAST analysis of the strain's 16S rRNA sequence revealed 100% similarity with *Pseudomonas aeruginosa* DSM 50071 (NR 117678.1), *Pseudomonas aeruginosa* ATCC 10145 (NR 114471.1), and *Pseudomonas aeruginosa* NBRC 12689 (NR 113599.1). Furthermore, a phylogenetic tree was constructed using the neighbor-joining method in MEGA 11.0 software (Figure 1), which confirmed that strain DM6 clustered with *Pseudomonas aeruginosa*. Therefore, it can be concluded that strain DM6 belongs to *Pseudomonas aeruginosa*.

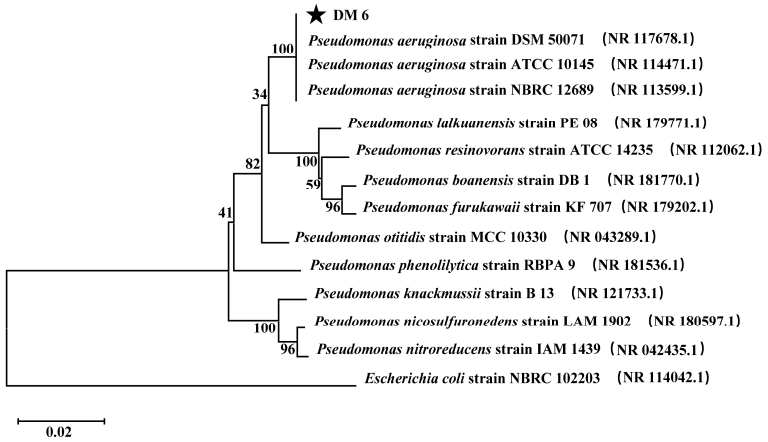

Figure 1. Phylogenetic tree of strain DM6.

3.2. Effects of Environmental Factors

3.2.1. Carbon Sources

Figure 2 shows the effect of different carbon sources on the growth and nitrite-degrading ability of strain DM6. It was found that strain DM6 exhibited optimal growth and denitrification performance when sodium citrate was used as the carbon source, reaching an OD_{600} peak (1.11 ± 0.06) at 36 h, while the nitrite degradation rate was 82.66 ± 1.99%. Although strain DM6 could use sodium succinate, glucose, or sodium pyruvate as the sole carbon source for growth, the growth rate was relatively slow, and the maximum nitrite degradation rate achieved was not as good as that achieved using sodium citrate. It should be noted that strain DM6 could not utilize sucrose or sodium acetate for growth.

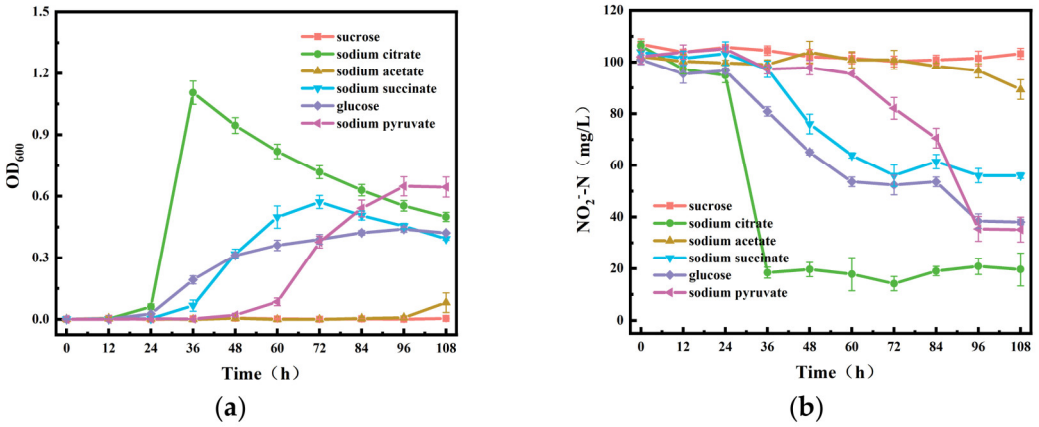

Figure 2. Effects of different carbon sources on the growth and nitrite degradation ability of strain DM6. (**a**) Growth curve of strain DM6. (**b**) Nitrite concentration decrease curve.

3.2.2. C/N

Figure 3 shows the effect of different C/N ratios on the growth and nitrite-degrading ability of strain DM6. The results showed that strain DM6 was a heterotrophic bacterium that could not grow without the addition of external organic carbon sources. With the increase in the C/N ratio, the growth and nitrite-degrading ability of strain DM6 were significantly improved. Especially under the condition of a C/N ratio of 20, strain DM6 completely degraded the initial concentration of 100.41 ± 2.37 mg/L nitrite within 36 h, reaching an OD_{600} peak (1.46 ± 0.01). This indicated that the C/N ratio had an important influence on the growth and nitrite-degrading ability of strain DM6.

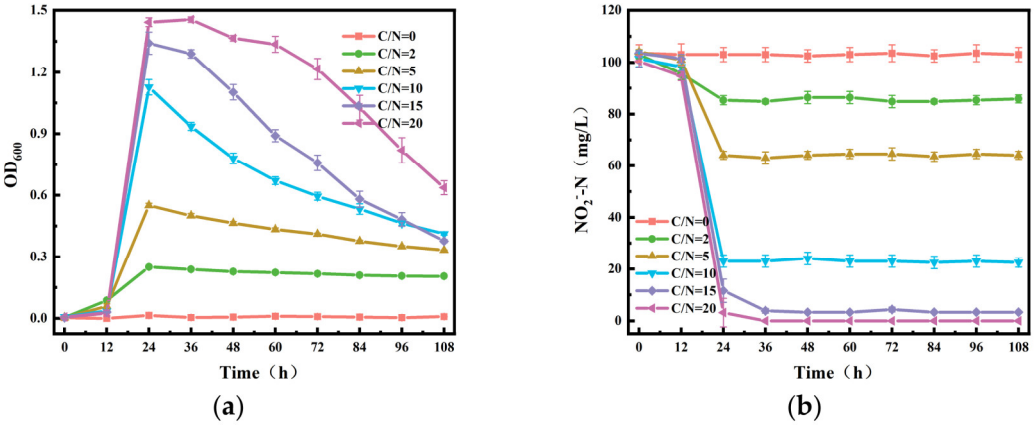

Figure 3. Effects of different C/N ratios on the growth and nitrite degradation ability of strain DM6. (**a**) Growth curve of strain DM6. (**b**) Nitrite concentration decrease curve.

3.2.3. pH

Figure 4 illustrates the effect of different initial pH on the growth and nitrite-degrading ability of strain DM6. The research results indicated that strain DM6 could not grow when the initial pH was 4.0 or 5.0. However, under neutral or alkaline conditions (pH 7.0–10.0), strain DM6 exhibited good growth and nitrite-degrading performance. At 24 h, strain DM6 reached its OD_{600} peak, and the nitrite degradation rate remained above 65.33 ± 3.25%. This suggested that the initial pH had a significant impact on the growth and nitrite-degrading ability of strain DM6, and an appropriate initial pH range (7.0–10.0) was beneficial for the growth and nitrite degradation of strain DM6.

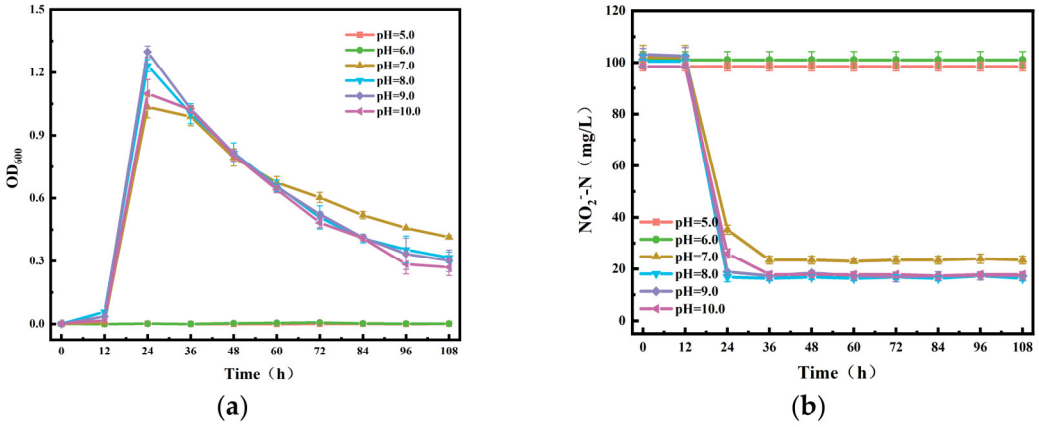

Figure 4. Effects of different initial pH on the growth and nitrite degradation ability of strain DM6. (**a**) Growth curve of strain DM6. (**b**) Nitrite concentration decrease curve.

3.2.4. Salinity

Figure 5 shows the effect of different salinities on the growth and nitrite-degrading ability of strain DM6. The results indicated that strain DM6 exhibited the fastest growth rate at a salinity of 15‰, reaching its OD_{600} peak (0.97 ± 0.03) at 36 h, with a nitrite degradation rate of 73.35 ± 1.16%. As salinity increased, strain DM6 required a longer adaptation period during the initial growth stage. Under high nitrite concentration conditions (100 mg/L), strain DM6 demonstrated good salinity tolerance and could tolerate at least 35‰ salinity. At this salinity, the maximum nitrite degradation rate that strain DM6 could achieve was 67.89 ± 2.07%. This suggested that strain DM6 had good adaptability and tolerance, allowing it to survive and degrade nitrite in high-nitrite and high-salinity environments. This was of great significance for the application of strain DM6 in pollution control in high-salinity environments such as seawater.

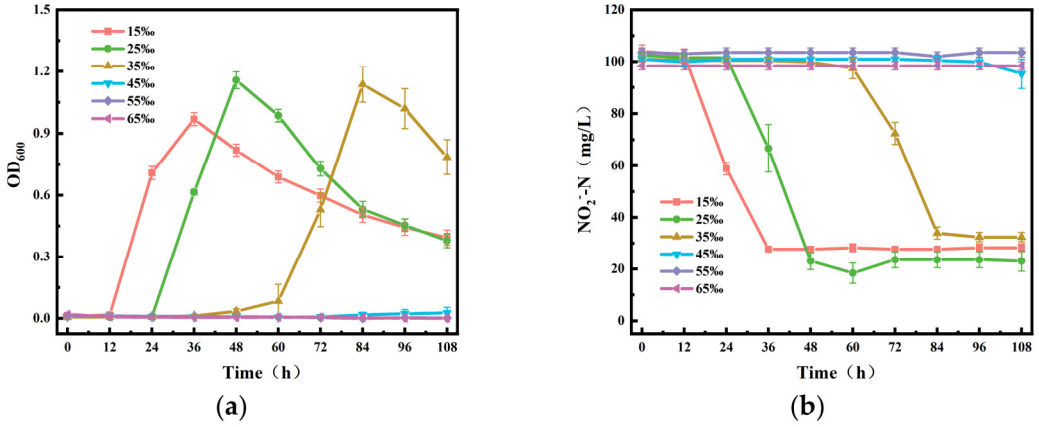

Figure 5. Effects of different salinity on the growth and nitrite degradation ability of strain DM6. (**a**) Growth curve of strain DM6. (**b**) Nitrite concentration decrease curve.

3.2.5. Temperature

Figure 6 illustrates the impact of different temperatures on the growth and nitrite-degrading ability of strain DM6. The results indicated that strain DM6 exhibited optimal growth rate and nitrite degradation ability at temperatures of 30 or 35 °C. When the temperature decreased to 25 or 20 °C, the initial growth rate of strain DM6 slowed down, but it had little effect on the final nitrite degradation rate (both above 73.32 ± 1.59%). Strain DM6 could tolerate high temperatures up to 40 °C, but it rapidly declined after 24 h, with a maximum nitrite degradation rate of only 53.99 ± 2.60%. It is worth noting that strain DM6 did not grow under 15 °C conditions. These results demonstrated that strain DM6 had a wide temperature adaptability range and could grow and degrade nitrite under different temperature conditions. However, the optimal growth and degradation abilities were still observed either during summer or in systems controlled at higher water temperatures.

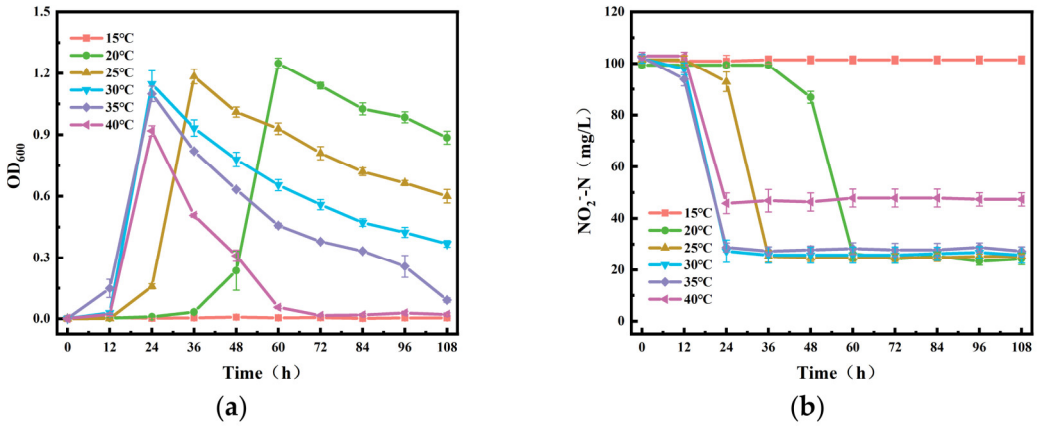

Figure 6. Effects of different temperatures on the growth and nitrite degradation ability of strain DM6. (**a**) Growth curve of strain DM6. (**b**) Nitrite concentration decrease curve.

3.3. Orthogonal Experimental Optimization

Based on the results of single-factor experiments, a four-factor, three-level orthogonal experiment was conducted (as shown in Table 3) with nitrite degradation efficiency as the indicator to further optimize the optimal process conditions for strain DM6 denitrification. According to Table 3, the nitrite degradation efficiency of the strain was highest under the No. 2 culture conditions, reaching 92.38 ± 0.05%, whereas it was lowest under the No. 9 culture conditions, at only 16.72 ± 7.05%. Based on the range (R) values of various factors, the primary and secondary order of factors affecting the nitrite degradation efficiency of strain DM6 were salinity > temperature > pH > C/N. Salinity was the most important environmental factor that affected the denitrification performance of this strain. By comparing

the average nitrite degradation efficiency (K value) of different levels of each factor, the optimal level combination was obtained: temperature of 35 °C, salinity of 13‰, C/N of 20, and pH of 8.0.

Table 3. Optimization results of nitrite degradation conditions for strain DM6.

Experiment Number	A	B	C	D	Nitrite Degradation Efficiency (%)
1	1	1	1	1	78.76 ± 0.51
2	1	2	2	2	92.38 ± 0.05
3	1	3	3	3	23.2 ± 7.37
4	2	1	2	3	91.56 ± 0.07
5	2	2	3	1	85.95 ± 5.25
6	2	3	1	2	74.04 ± 1.69
7	3	1	3	2	86.31 ± 0.89
8	3	2	1	3	47.79 ± 5.17
9	3	3	2	1	16.72 ± 7.05
K1	64.78	85.54	66.86	60.48	
K2	83.85	75.37	66.89	84.24	
K3	50.27	37.99	65.15	54.18	
R	33.58	47.56	1.73	30.06	
Priority of factors			B > A > D > C		
Optimal composition			A_2 B_1 C_2 D_2		

Note: The K value represents the average nitrite degradation efficiency of each factor at different levels; the R value represents the range.

Under the optimal conditions of the orthogonal experiment, the strain DM6 achieved a nitrite degradation efficiency of 100.00% after 24 h of cultivation, which was higher than the maximum nitrite degradation efficiency in Table 3.

3.4. Nitrite Tolerance Test

Figure 7 illustrates the growth and denitrification characteristics of strain DM6 under different nitrite concentrations, using the optimized denitrification conditions. Strain DM6 demonstrated adaptability to various nitrite concentrations, exhibiting robust growth across the range of concentrations. Notably, strain DM6 could completely degrade an initial nitrite concentration of 156.33 ± 1.17 mg/L within 36 h, showcasing its highly efficient denitrification capability (100%). Furthermore, strain DM6 exhibited tolerance to nitrite concentrations as high as 350.96 ± 0.56 mg/L, achieving a degradation rate of 50.54 ± 0.21%. These results indicated the potential of strain DM6 to treat nitrite pollution at different concentrations, particularly for high-concentration nitrite treatment.

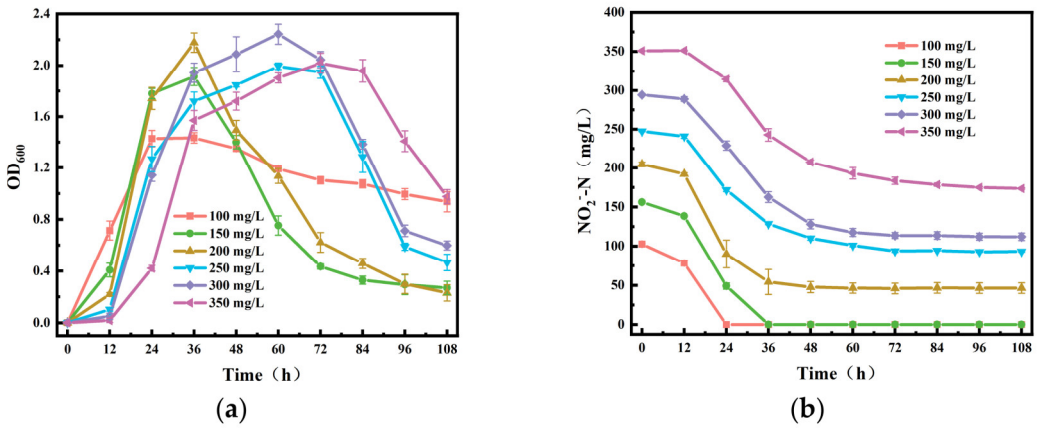

Figure 7. Effects of different nitrite concentrations on the growth and nitrite degradation ability of strain DM6. (**a**) Growth curve of strain DM6. (**b**) Nitrite concentration decrease curve.

3.5. Amplification of Denitrification Functional Genes

To further understand the nitrite metabolism mechanism of strain DM6, a PCR amplification analysis was performed to determine the presence of denitrification functional genes. The gel electrophoresis image of the PCR products is shown in Figure 8a. The denitrification functional genes, including narG, narH, narI, nirS, and norB, as well as assimilation enzyme coding genes, such as nasC, nasD, nasE, glnA, gltB, gltD, gdhB, and gdhA, were successfully amplified from the genomic DNA of strain DM6. Based on the PCR amplification results, the potential nitrite transformation pathway of strain DM6 was predicted, as depicted in Figure 8b.

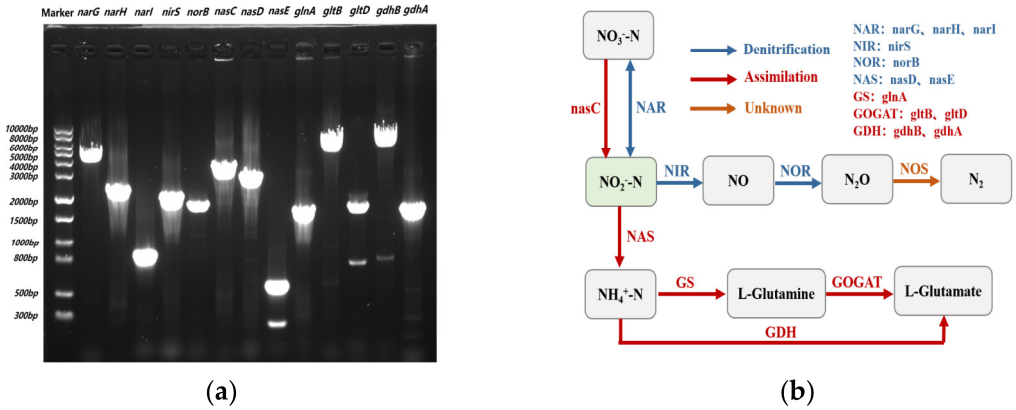

Figure 8. (**a**) Gel electrophoresis image of denitrification functional genes. (**b**) Refined predicted nitrate metabolism pathway of strain DM6.

4. Discussion

In addressing the issue of nitrite accumulation in wastewater from marine aquaculture, studies showed that microbially mediated biological denitrification methods were more efficient and cost-effective compared to traditional physical and chemical approaches [6,14]. However, there are significant differences in salinity effects between wastewater from marine aquaculture and freshwater sewage. Typically, the salinity of marine aquaculture ranges from 10 to 30‰ [10], but in high-salinity environments (salinity $\geq$ 10‰), osmotic changes and salt-induced enzyme precipitation can inhibit microbial growth and metabolism [8]. As a result, many microorganisms isolated from freshwater or soil habitats may not be effective at treating wastewater from marine aquaculture [19]. In addition, studies have shown that with the increase in salinity, the abundance and activity of microorganisms in biofilms gradually decrease [20]. The growth and metabolism of ammonia-oxidizing bacteria (AOB) and other microbial populations are significantly inhibited [21], leading to a reduction in denitrification efficiency. Therefore, salt-tolerant bacteria may have better adaptability and survival ability and may play an important role in the formation of biofilms and nitrogen removal in high-salinity environments. Previous research has confirmed the advantages of denitrifying bacteria sourced from the marine environment, such as *Vibrio diabolicus* SF16 isolated from marine sediment [8], *Rhodococcus* sp. LS-2 isolated from deep-sea sediments [22], and *Enterococcus faecalis* XH1 isolated from seawater [23]. These marine strains were of significant importance in the treatment of nitrogen-containing wastewater under high-salinity conditions. However, it is worth noting that previous studies primarily focused on the removal of ammonia and nitrate. In this study, strain DM6, selected from aquaculture seawater in this study, demonstrated the ability to tolerate a salinity of 35‰ under high nitrite concentration conditions (100 mg/L), indicating its adaptability to most aquaculture seawater salinity stress. It is known that nitrite oxidizers are more sensitive to high salinity concentration than ammonium oxidizers [21], which suggests that nitrite may accumulate more readily in high-salinity aquaculture wastewater. Meanwhile, other marine bacterial strains such as *Vibrio diabolicus* SF16 are currently more focused on ammonia and nitrate removal. This

suggests that stain DM6 may have a certain advantage in treating aquaculture wastewater with high nitrite concentrations. In the future, biofilm reinforcement can be employed to fully leverage the robust nitrite degradation capabilities of DM6 in practical applications, thereby enhancing water quality.

Pseudomonas aeruginosa is a crucial microbial resource in water-quality management and environmental protection. This class of bacteria is capable of utilizing organic compounds in wastewater for metabolism, thereby promoting the degradation and purification of organic matter in water bodies. For instance, *Pseudomonas aeruginosa* SNDPR-01 had the ability to denitrify and remove phosphorus, thereby reducing nitrogen pollutants, COD, and phosphorus levels in water bodies [14]. Additionally, *Pseudomonas aeruginosa* can metabolize certain heavy metal ions, converting them into harmless substances and effectively removing heavy metal contamination from water. For example, *Pseudomonas aeruginosa* P-1 could tolerate high concentrations of heavy metal ions such as Cu^{2+}, Zn^{2+}, Cr^{2+}, Pb^{2+}, and Cd^{2+} [12], while *Pseudomonas aeruginosa* G12 and PCN-2 could simultaneously denitrify (nitrate nitrogen) and remove chromium [11,13]. However, existing studies have primarily focused on the ability of *Pseudomonas aeruginosa* to remove ammonia nitrogen, nitrate nitrogen, phosphorus, and heavy metal ions, with limited research on their removal characteristics concerning nitrite. Furthermore, due to differences in genetic information and habitat sources, different species or subspecies of *Pseudomonas aeruginosa* exhibit variations in denitrification characteristics, mechanisms, and environmental adaptability. The findings of this study demonstrated that the marine strain *Pseudomonas aeruginosa* DM6 possessed highly efficient (100%) nitrite degradation capability and strong environmental adaptability. This further confirms the significant role of *Pseudomonas aeruginosa* in water-quality management and reveals the differences in environmental requirements among different strains. *Pseudomonas aeruginosa* currently identified are mostly isolated from activated sludge or leachate, primarily applied in industrial wastewater treatment. In comparison, strain DM6 was isolated from marine aquaculture water, showcasing superior adaptability to seawater. However, due to the common opportunistic pathogenic nature of *Pseudomonas aeruginosa*, safety testing should be conducted before applying strain DM6 to marine aquaculture effluent.

Currently, nitrite-degrading bacteria mainly include heterotrophic denitrifying bacteria and autotrophic nitrifying bacteria. Generally speaking, heterotrophic denitrifying bacteria have the advantage of faster growth and metabolism speed due to their ability to use organic matter to obtain energy and carbon sources [24]. In addition, autotrophic nitrifying bacteria are more sensitive to environmental factors (such as pH, temperature, and heavy metals) [25,26], leading to their inferior denitrification performance compared to heterotrophic denitrifying bacteria. The results of this study showed that strain DM6 had good tolerance to high concentrations of nitrite (350 mg/L) and could achieve a 100% degradation efficiency when the nitrite concentration was 150 mg/L, which is among the best of the reported heterotrophic nitrite-degrading bacteria (as shown in Table 4). This means that strain DM6 has significant advantages in treating nitrite-contaminated wastewater, especially for the treatment of high-concentration nitrite. In addition, strain DM6 had a wide range of adaptation to environmental conditions (temperature 20–40 °C, pH 7.0–10.0). Studies have shown that the optimum growth temperature for most denitrifying bacteria strains is between 25 and 37 °C, and their metabolism will be inhibited under high- or low-temperature conditions [27]. The optimum temperature for strain DM6 to degrade nitrite was 30–35 °C, and it could still grow and denitrify at high temperatures of 40 °C. This means that this strain is most suitable for growth and denitrification in summer or systems with higher water temperatures. Furthermore, as shown in Table 4, most nitrite-degrading bacteria prefer neutral or weakly alkaline environments and cannot grow in acidic or strongly alkaline conditions. However, strain DM6 not only adapted to a mildly alkaline environment but also tolerated a strongly alkaline environment with pH 10.0, showing good growth and denitrification performance. In addition, due to its tolerance to salt, this strain also has the potential to be applied in salt–alkali land aquaculture fields other than

nearshore aquaculture, but further research is still needed. In summary, compared to other degrading strains, strain DM6 demonstrates greater tolerance to high concentrations of nitrite. Furthermore, DM6 is better suited for use in summer or high-temperature water bodies, and its resilience to strong alkaline conditions also suggests potential applications in saline–alkali aquaculture areas.

Table 4. Comparison of nitrogen removal ability between strain DM6 and different nitrite-degrading bacteria.

Strain Name	Origin of Strains	Optimal Temperature (°C)	Optimal pH	Nitrite Concentration (mg/L)	Degradation Efficiency (%)	References
Pseudomonas aeruginosa DM6	Aquaculture seawater	30–35	7.0–10.0	350 150	50.54 100	
Acinetobacter bereziniae ZQ-A1	Bioreactor	25–35	8.0–9.0	200	87.13	[28]
Pseudomonas qingdaonensis L3	Pond sediment	29.1	6.2	150	85.34	[29]
Pseudomonas denitrificans G1	Aquaculture ecosystem	30	7.0–9.5	140	99.96	[30]
Photobacterium ganghwense NNA4	Aquaculture seawater	30	7.0	139	100	[31]
Klebsiella oxytoca TN-10	Tanyard waste	30	7.0	101	99.87	[25]
Paracoccus pantotrophus QD-19	Activated sludge	30	7.0	100	99	[32]
Acinetobacter junii YB	Activated sludge	37	7.5	100	87.01	[24]
Alcaligenes faecalis WT14	Constructed wetland	20.3	8.4	100	100	[33]
Bacillus megaterium S379	Aquaculture ponds	20–40	7.0–9.0	65	96.82	[1]
Pseudomonas aeruginosa P-1	Sludge	20–30	8.0	60	36.68	[12]
Bacillus cereus GS-5	Bioreactor	35	7.5	50	83.8	[6]
Ochrobactrum anthropic LJ81	Living sludge	30	5.0–9.0	50	99.8	[34]
Rhicobium pusense WS7	Activated sludge	15–30	7.0	50	100	[35]
Acinetobacter calcoaceticus TY1	Activated sludge	8	6.0–8.0	35	97.51	[36]
Bacillus litoralis N31	Shrimp aquatic water	30	7.5–8.5	20	89.3	[37]
Acinetobacter junii ZHG-1	Landfill leachate	30	9.0	50	96.7	[38]

Based on the nitrogen metabolism pathways in the KEGG database (KO00910), it can be concluded that there are two pathways for nitrite degradation, namely denitrification and assimilation. The amplification of denitrification-related genes in strain DM6 confirms its ability to degrade nitrite through both pathways. Specifically, narG, narH, and narI encode the α, β, and γ subunits of membrane-bound nitrate reductase (NAR), which catalyzes the interconversion between NO_3^--N and NO_2^--N but generally plays a dominant role under anaerobic conditions [39]. Thus, NAR may not function effectively in high-oxygen environments. In addition, nirS is a key denitrification gene commonly amplified from aerobic denitrifying bacteria [40]. The nirS gene encodes a homodimeric cytochrome cd1 nitrite reductase (cd1-NIR), which catalyzes the step of nitrite denitrification into nitrogen-containing gases [17]. Furthermore, norB encodes nitric oxide reductase (NOR), which plays a crucial role in converting NO to N_2O [41]. The successful amplification of these denitrification genes in strain DM6 suggests its potential to convert nitrite to nitrogen-containing gases through the denitrification pathway.

In addition, the successful amplification of assimilatory nitrite reductase-coding genes (nasD and nasE) in the genome of strain DM6 indicates the existence of another pathway, where nitrite is first converted to ammonium nitrogen and then enters the ammonium assimilation pathway to ultimately synthesize glutamate for biomass production. Bacteria

typically assimilate ammonium in two ways: When sufficient nitrogen is provided, they use the energy-independent glutamate dehydrogenase (GDH) pathway; however, when nitrogen is limited, they employ the energy-dependent glutamine synthetase–glutamate synthase (GS-GOGAT) pathway [42]. The successful amplification of the GS-coding gene (glnA), GOGAT-coding genes (gltB and gltD), and GDH-coding genes (gdhB and gdhA) in the genome of strain DM6 suggests that it possesses a complete ammonium assimilation pathway and can convert nitrite to biological nitrogen. Previous studies had demonstrated effective nitrogen removal and fixation of nitrogen in *Vibrio* sp. Y1-5, a heterotrophic nitrogen assimilating strain, without nitrogen loss [43]. In the future, it may be possible to utilize the nitrogen assimilation pathway of strain DM6 to recover nitrogen from wastewater in the form of biomass, enabling the conversion and utilization of waste. Subsequently, the converted nitrogen can be used for the production of value-added products and processes [44], thereby achieving the goal of turning waste into treasure. In conclusion, the successful amplification of key genes in the nitrite degradation pathway indicates that strain DM6 may possess both denitrification and assimilation pathways for nitrite degradation, further confirming its ability to degrade nitrite. The successful amplification of key genes in the ammonia assimilation pathway suggests that strain DM6 could potentially convert nitrogen in wastewater into biomass or other usable forms, facilitating resource recovery and waste reduction in wastewater treatment systems. Furthermore, future research combining metabolite profiling and other techniques can provide deeper insights into the metabolic mechanisms of strain DM6. Additionally, gene editing may be employed to enhance the application potential of the strain.

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References

1. Gao, J.; Gao, D.; Liu, H.; Cai, J.; Zhang, J.; Qi, Z. Biopotentiality of High Efficient Aerobic Denitrifier *Bacillus megaterium* S379 for Intensive Aquaculture Water Quality Management. *Environ. Manag.* **2018**, *222*, 104–111. [CrossRef]
2. Zheng, L.; Liu, Q.; Liu, J.; Xiao, J.; Xu, G. Pollution Control of Industrial Mariculture Wastewater: A Mini-Review. *Water* **2022**, *14*, 1390. [CrossRef]
3. Siikavuopio, S.I.; Dale, T.; Christiansen, J.S.; Nevermo, I. Effects of chronic nitrite exposure on gonad growth in green sea urchin Strongylocentrotus droebachiensis. *Aquaculture* **2004**, *242*, 357–363. [CrossRef]
4. Huang, M.; Xie, J.; Yu, Q.; Xu, C.; Zhou, L.; Qin, J.G.; Chen, L.; Li, E. Toxic effect of chronic nitrite exposure on growth and health in Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture* **2020**, *529*, 735664. [CrossRef]
5. Hu, D.; Wang, L.; Zhao, R.; Zeng, J.; Shao, Z. Core microbiome involved in nitrite removal in shrimp culture ponds. *Aquac. Res.* **2021**, *53*, 1663–1675. [CrossRef]
6. 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]
7. Cao, X.; Zhao, B.; Wu, Y.; Huang, J.; Wang, H.; Sun, X.; Li, S. Characterization of *Alcaligenes aquatilis* as a novel member of heterotrophic nitrifier-aerobic denitrifier and its performance in treating piggery wastewater. *Bioresour. Technol.* **2022**, *354*, 127176. [CrossRef] [PubMed]
8. Duan, J.; Fang, H.; Su, B.; Chen, J.; Lin, J. Characterization of a halophilic heterotrophic nitrification-aerobic denitrification bacterium and its application on treatment of saline wastewater. *Bioresour. Technol.* **2015**, *179*, 421–428. [CrossRef]
9. Yang, L.; Ren, Y.X.; Liang, X.; Zhao, S.-Q.; Wang, J.-P.; Xia, Z.-H. Nitrogen removal characteristics of a heterotrophic nitrifier *Acinetobacter junii* YB and its potential application for the treatment of high-strength nitrogenous wastewater. *Bioresour. Technol.* **2015**, *193*, 227–233. [CrossRef] [PubMed]

10. Dong, W.X.; Li, J.F.; Teng, Y.; Cui, Z.G.; Cui, H.W.; Liang, S.K.; Zhao, M.J. Denitrification characteristics of a salt-tolerant and cold-tolerant heterotrophic nitrification-aerobic denitrification bacteria. *Environ. Sci. Technol.* **2023**, *46*, 1–7. [CrossRef]
11. An, Q.; Deng, S.; Xu, J.; Nan, H.; Li, Z.; Song, J.-L. Simultaneous reduction of nitrate and Cr (VI) by *Pseudomonas aeruginosa* strain G12 in wastewater. *Ecotoxicol. Environ. Saf.* **2020**, *191*, 110001. [CrossRef] [PubMed]
12. 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.* **2020**, *28*, 7503–7514. [CrossRef] [PubMed]
13. He, D.; Zheng, M.; Ma, T.; Li, C.; Ni, J. Interaction of Cr (VI) reduction and denitrification by strain *Pseudomonas aeruginosa* PCN-2 under aerobic conditions. *Bioresour. Technol.* **2015**, *185*, 346–352. [CrossRef] [PubMed]
14. Huang, M.Q.; Cui, Y.W.; Huang, J.L.; Sun, F.-L.; Chen, S. A novel *Pseudomonas aeruginosa* strain performs simultaneous heterotrophic nitrification-aerobic denitrification and aerobic phosphate removal. *Water Res.* **2022**, *221*, 118823. [CrossRef]
15. Chen, Z.H.; Zhang, Y.H.; Wang, J.Y.; Zhang, Y.L.; Zhu, X.Y.; Sun, C.; Zhang, X.H. Exploring the diversity of cultivable bacteria in the Western Pacific Ocean using modified culture media. *Acta Microbiol. Sin.* **2021**, *61*, 845–861. [CrossRef]
16. Greenberg, A.E.; Trussell, R.R.; Clesceri, L.S. Standard methods for the examination of water and wastewater: Supplement to the sixteenth edition. *Am. J. Public Health Nations Health* **2005**, *56*, 387. [CrossRef]
17. Yang, J.R.; Wang, Y.; Chen, H.; Lyu, Y.-K. Ammonium removal characteristics of an acid-resistant bacterium *Acinetobacter* sp. JR1 from pharmaceutical wastewater capable of heterotrophic nitrification-aerobic denitrification. *Bioresour. Technol.* **2019**, *274*, 56–64. [CrossRef]
18. 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]
19. Zhou, P.; Liu, Y.; Mu, X.T.; Su, X.; Wu, Y.H.; Han, R. Research progress of heterotrophic nitrification-aerobic denitrification bacteria in nitrogen removal of mariculture wastewater. *J. Water Ecol.* **2023**, *44*, 148–157. [CrossRef]
20. Xia, Z.; Wang, Q.; She, Z.; Gao, M.; Zhao, Y.; Guo, L.; Jin, C. Nitrogen removal pathway and dynamics of microbial community with the increase of salinity in simultaneous nitrification and denitrification process. *Sci. Total Environ.* **2019**, *697*, 134047. [CrossRef]
21. García-Ruiz, M.J.; Castellano-Hinojosa, A.; González-López, J.; Osorio, F. Effects of salinity on the nitrogen removal efficiency and bacterial community structure in fixed-bed biofilm CANON bioreactors. *Chem. Eng. J.* **2018**, *347*, 156–164. [CrossRef]
22. Hou, D.M.; Zhang, L.; Li, C.C.; Chen, L.T.; Zou, J.P. Isolation, identification and nitrogen removal performance of a salt-tolerant heterotrophic nitrification-aerobic denitrification bacterium *Rhodococcus* sp. LS-2. *J. Nanchang Hangkong Univ.* **2023**, *37*, 50–58. [CrossRef]
23. Yin, L.L.; Lv, J.; Wang, J.H.; Wu, J.; Zhang, C. Isolation, identification and performance study of a sucrose-preferred marine heterotrophic nitrification-aerobic denitrification bacteria. *Mar. Environ. Sci.* **2023**, *42*, 425–431.
24. 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]
25. 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] [PubMed]
26. Chen, M.; Wang, W.; Feng, Y.; Zhu, X.; Zhou, H.; Tan, Z.; Li, X. Impact resistance of different factors on ammonia removal by heterotrophic nitrification-aerobic denitrification bacterium *Aeromonas* sp. HN-02. *Bioresour. Technol.* **2014**, *167*, 456–461. [CrossRef]
27. He, T.; Ye, Q.; Sun, Q.; Cai, X.; Ni, J.; Li, Z.; Xie, D. Removal of Nitrate in Simulated Water at Low Temperature by a Novel Psychrotrophic and Aerobic Bacterium, *Pseudomonas taiwanensis* Strain J. *BioMed Res. Int.* **2018**, *2018*, 1–9. [CrossRef]
28. Cao, Z.; Huang, F.; Zhang, R.; Zhao, X.; Wang, Y.; Wu, Y.; Liao, X.; Feng, Y.; Ma, J.; Lan, T. Nitrogen removal characteristics of heterotrophic nitrification-aerobic denitrification bacterium *Acinetobacter* ZQ-A1 and community characteristics analysis of its application in pig farm wastewater. *Environ. Sci. Pollut. Res.* **2023**, *30*, 104029–104042. [CrossRef]
29. Liu, Y.Q.; Zhang, Y.Y.; Zhan, X.Q.; Li, Y.Y.; Chen, Y.Z.; Chen, Q.H. Screening and identification of a heterotrophic nitrification-aerobic denitrification strain L3 and its denitrification characteristics. *Water Treat. Technol.* **2023**, *49*, 94–100. [CrossRef]
30. Chen, Z.; Jiang, Y.; Chang, Z.; Wang, J.; Song, X.; Huang, Z.; Chen, S.; Li, J. Denitrification characteristics and pathways of a facultative anaerobic denitrifying strain, *Pseudomonas denitrificans* G1. *J. Biosci. Bioeng.* **2020**, *129*, 715–722. [CrossRef]
31. Liu, Y.; Ai, G.M.; Wu, M.R.; Li, S.-S.; Miao, L.-L.; Liu, Z.-P. *Photobacterium* sp. NNA4, an efficient hydroxylamine-transforming heterotrophic nitrifier/aerobic denitrifier. *J. Biosci. Bioeng.* **2019**, *128*, 64–71. [CrossRef]
32. Zhang, Y.H.; Dong, X.B.; Liu, X.Y.; Xu, J.Q.; Xu, Z.L. Isolation and denitrification characteristics of a novel heterotrophic nitrification-aerobic denitrification bacteria *Paracoccus* sp. QD-19. *Biotechnol. Bull.* **2023**, *39*, 301–310. [CrossRef]
33. Chen, J.; Xu, J.; Zhang, S.; Liu, F.; Peng, J.; Peng, Y.; Wu, J. Nitrogen removal characteristics of a novel heterotrophic nitrification and aerobic denitrification bacteria, *Alcaligenes faecalis* strain WT14. *J. Environ. Manag.* **2021**, *282*, 111961. [CrossRef]
34. 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]
35. Wei, B.; Luo, X.; Ma, W.; Lv, P. Biological nitrogen removal and metabolic characteristics of a novel cold-resistant heterotrophic nitrification and aerobic denitrification *Rhizobium* sp. WS7. *Bioresour. Technol.* **2022**, *362*, 127756. [CrossRef]

36. Wu, L.; Ding, X.; Lin, Y.; Lu, X.; Lv, H.; Zhao, M.; Yu, R. Nitrogen removal by a novel heterotrophic nitrification and aerobic denitrification bacterium *Acinetobacter calcoaceticus* TY1 under low temperatures. *Bioresour. Technol.* **2022**, *353*, 127148. [CrossRef]
37. 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]
38. Gu, X.; Leng, J.; Zhu, 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]
39. Yang, T.; Xin, Y.; Zhang, L.; Gu, Z.; Li, Y.; Ding, Z.; Shi, G. Characterization on the aerobic denitrification process of *Bacillus* strains. *Biomass Bioenergy* **2020**, *140*, 105677. [CrossRef]
40. 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]
41. Yang, Q.; Yang, T.; Shi, Y.; Xin, Y.; Zhang, L.; Gu, Z.; Li, Y.; Ding, Z.; Shi, G. The nitrogen removal characterization of a cold-adapted bacterium: *Bacillus simplex* H-b. *Bioresour. Technol.* **2021**, *323*, 124554. [CrossRef] [PubMed]
42. Tang, Q.; Zeng, M.; Zou, W.; Jiang, W.; Kahaer, A.; Liu, S.; Hong, C.; Ye, Y.; Jiang, W.; Kang, J.; et al. A new strategy to simultaneous removal and recovery of nitrogen from wastewater without N_2O emission by heterotrophic nitrogen-assimilating bacterium. *Sci. Total Environ.* **2023**, *872*, 162211. [CrossRef] [PubMed]
43. Li, Y.; Wang, Y.; Fu, L.; Gao, Y.; Zhao, H.; Zhou, W. Aerobic-heterotrophic nitrogen removal through nitrate reduction and ammonium assimilation by marine bacterium *Vibrio* sp. Y1-5. *Bioresour. Technol.* **2017**, *230*, 103–111. [CrossRef] [PubMed]
44. Falcone, P.M.; Imbert, E. Tackling Uncertainty in the Bio-Based Economy. *Int. J. Stand. Res.* **2019**, *17*, 74–84. [CrossRef]