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Isolation and Characterization of an Aerobic Denitrifier *Bacillus* sp. SC16 from an Intensive Aquaculture Pond

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Abstract: Overloading of ammonia and nitrite nitrogen in aquaculture can result in toxicity to aquatic animals. In order to eliminate the hazardous substances, a highly efficient denitrifying bacterium, *Bacillus* sp. SC16, was identified in a fishery pond and isolated subsequently. The strain SC16 could remove nitrate up to 97%, ammonia up to 36.6%, and nitrite up to 99.99% when incubated with nitrate at an initial concentration of 306.9 mg·L^{-1} for 72 h, ammonia at 165.49 mg·L^{-1} for 48 h, and nitrite at 200 mg·L^{-1} for 24 h under aerobic conditions. The nitrite reductase gene was identified as the *nirK* gene. The transcriptional levels of the *nirK* gene in strain SC16 incubated with ammonia, nitrate, and nitrite showed similar expression patterns. When the strain SC16 was used to treat the aquaculture water, the concentration of ammonia decreased significantly, from 8.35 mg·L^{-1} to 4.56 mg·L^{-1}, and there was almost no accumulation of nitrite by the end of experiment. Therefore, the results indicated that *Bacillus* sp. SC16 could be a promising candidate for aquaculture water treatment.

Keywords: *Bacillus* sp.; denitrification; *nirK* gene; transcriptional expression; aquaculture

1. Introduction

During the past two decades, the total production of farmed fish in China increased three-fold, with the greatest contribution coming from fresh water aquaculture (25.4 million metric tons in 2018) [1,2]. To achieve such a high yield, an intensive feed-driven culture model was implemented by fishery farmers. However, this intensive culture model has resulted in the accumulation of nitrogenous pollutants due to the aquafeed residues and feces, thereby causing environmental concerns, such as eutrophication, algae blooms, and water contamination [3,4]. The nitrogen-enriched aquaculture water discharged into adjacent rivers might result in eutrophication and thus affect the aquatic ecosystems. Therefore, there is a need to improve aquaculture water through decreasing the accumulation of nitrogenous pollutants.

Many conventional techniques, e.g., rotating biological contactors, trickling filters, bead filters, and fluidized sand biofilters, have been applied to remove the nitrogen from water [5]. In addition, biological treatments, including the bio-flocs technology and microbiological method, have also attracted wide attention due to their simple operation, lower maintenance costs, and high pollutant-removal efficiency [5,6]. Aerobic denitrifiers, also named as heterotrophic nitrification and aerobic denitrification (HN-AD) bacteria [7], combine the processes of both nitrification and denitrification. The bacteria are

potential candidates in bioremediation due to their high removal rate of nitrogen [8]. Aerobic denitrifiers belonging to different species have been screened and characterized, for example, *Pseudomonas putida* [9], *Alcaligenes faecalis* [10], *Thiosphaera pantotropha* [11], *Rhodococcus* sp. [6], and *Bacillus subtilis* [12]. Nevertheless, there are limited aerobic denitrifiers used to treat intensive aquaculture water [13]. Thus, it is still necessary to find bacterial organism with high nitrogen-removal efficiency to eliminate nitrogen contamination in aquaculture.

Aerobic denitrification is considered as the assemblage of NO_3^- respiration, NO_2^- respiration, NO reduction, and N_2O respiration: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ [14]. The four consecutive steps are catalyzed by nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS), respectively [15]. Nitrite respiration, the key process in aerobic denitrification, is catalyzed by NIR, the enzyme that determines the denitrification properties of bacteria [16]. A high activity of nitrite reductase results in a high efficacy of nitrite respiration, as the enzyme makes aerobic denitrifying strains use nitrite preferentially. The inefficiency, impaired activity, and lack of nitrite reductase in some denitrifiers would cause the strains to lose the capability of complete denitrification and result in the accumulation of nitrite [17]. There are commonly two types of NIR in denitrifiers, copper nitrite reductase and cytochrome cd1 nitrite reductase encoded by *nirK* and *nirS* genes, respectively. It has been reported that *nirS* and *nirK* don't usually coexist in denitrifiers [18].

The present study aimed to investigate the performance of *Bacillus* sp. SC16 isolated from a high-density culture pond in eliminating nitrogen under aerobic conditions. Our objectives were to clone the nitrite reductase gene and assess the expression levels of nitrite reductase genes using real-time PCR when strain SC16 was incubated with nitrate, ammonium, or nitrite.

2. Materials and Methods

2.1. Media

The basal medium of denitrifying medium (DM) contained the following components per liter [19]: CH_3COONa 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, $CaCl_2 \cdot 2H_2O$ 0.07 g, KH_2PO_4 0.4 g, trace elements 1 mL, and Tris buffer 12 mL. The trace elements per liter was composed of $ZnSO_4$ 2.2 g, $FeSO_4 \cdot 7H_2O$ 3.0 g, $CaCl_2$ 5.5g, $MnCl_2 \cdot 4H_2O$ 5.0 g, $CuSO_4 \cdot 5H_2O$ 1.6 g, $CoCl_2 \cdot 6H_2O$ 1.6 g, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 1.1 g. The Tris buffer (per liter) contained 121.1 g $C_4H_{11}NO_3$. KNO_3 0.5 g, NH_4Cl 0.5 g, and $NaNO_2$ 0.3 g were added into the DM to form the media DM-1, DM-2 and DM-3, respectively. The ingredients of BTB per liter were as follows: L-Asparagine 10.0 g, KNO_3 1.0 g, KH_2PO_4 1.0 g, $FeCl_2 \cdot 6H_2O$ 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, and BTB regent (1% (*w*/*v*)) in alcohol 1 mL. Luria-Bertani (LB) medium used for seed preparation and culture preservation contained 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract, and 10 g L^{-1} of NaCl. The pH of all media was adjusted to 7.0. The agar at 2% (*w*/*v*) was added in liquid media to prepare the solid media. All media were autoclaved for 20 min at 121 °C, followed by adding BTB regent.

2.2. Isolation and Identification

The sludge samples were obtained from intensive aquaculture ponds in the Huadu district, Guangzhou, China. First, several 500 mL vitreous flasks containing 5 mL of the sludge sample and 100 mL DM were transferred to a rotary shaker (WSQ; SCIENTZ Co., Ltd., Ningbo, China) with 200 rpm at 28 °C for 3 d. Next, 5 mL of the bacteria solution was inoculated into 100 mL DM and cultured under the same conditions as described above, repeating once to enrich denitrifying bacteria. Then, the bacteria solution was diluted using 10-fold serial dilutions to concentrations of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. After dilution, 0.1 mL bacterial suspension of each concentration was inoculated on solid BTB, followed by incubating at 28 °C for 2 d. Denitrifying bacteria showed blue colonies on the solid BTB. Thus, the blue colonies were selected and purified. Finally, the purified colonies were suspended in 40% sterile glycerol solution (*v*/*v*) and then stored at −80 °C for further analysis.

The morphology of the strain isolated was examined using a scanning electron microscope (SEM) (XL-30ESEM; PHILIPS Co., Ltd., Amsterdam, The Netherlands) at 24 h post-cultivation. Genomic DNA of the strain was extracted using a DNA extraction kit (Rapid Bacterial Genomic DNA Isolation Kit, Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The 16S rRNA gene was amplified using a PCR thermal cycler (Veriti®Thermal Cycler, ThermoFisher Co., Ltd., Waltham, MA, USA) with the general primers 27F and 1492R (in Supplementary, Table S1). Reaction mixtures with a final volume of 25 μL contained each of the primers (10 μmol L^{-1}), 2.5 mM dNTPs, 10 reaction buffer, 2.5 U Taq polymerase (TaKaRa Co., Ltd., Kyoto, Japan), 1 μg DNA template, and double-distilled water (ddH_2O). PCR was conducted with the following conditions: 4 min at 94 °C for pre-denaturation, 35 cycles of 10 s at 98 °C for denaturation, 30 s at 54 °C for annealing, 1 min at 68 °C for extension, and 7 min at 68 °C for final extension. The PCR product was sequenced by BGI Genomics Co., Ltd. (Shenzhen, Guangdong, China). The partial sequence was analyzed and compared with that of other strains obtained from the NCBI database using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed using the maximum-likelihood method implemented in the MEGA 6.0 software (MEGA software development team, Phoenix, AZ, USA), with 1000 bootstrap replicates.

2.3. Nitrogen Removal Performance

The optimum incubation conditions for growth and nitrogen removal performance of strain SC16 was determined with single-factor experiments. The basal conditions were assigned as follows: KNO_3 concentration of 500 mg/L, temperature of 28 °C, rotation of 200 rpm, and 1% inoculation (*v*/*v*). For carbon resources, sodium acetate, citric acid, glucose, ethanol, and methanol were selected. For C/N ratios, 1, 2, 4, 8, 16, and 32 were set. For the pH experiment, the initial pH values of 5, 6, 7, 8, and 9 were adjusted. For the temperature experiment, 15 °C, 20 °C, 28 °C, and 37 °C were designed. All of the tests were conducted with three replications, and non-seeded samples were used as blank controls.

To estimate the nitrogen removal ability of strain SC16, a single colony of the strain was transferred into 100 mL LB liquid medium and cultured in a rotary shaker with 200 rpm at 28 °C for 24 h. The solution of bacteria was centrifuged at 4500× *g* for 10 min at 4 °C, followed by removing the supernatant, the sediment was washed three times with phosphate buffer. After washing, the bacteria were suspended with phosphate buffer, and then 2.5 mL of the bacterial solution was added into 250 mL DM-1, DM-2 and DM-3 medium.

The initial concentrations of nitrate in DM-1, ammonia in DM-2, and nitrite in DM-3 were 306.9, 165.49, and 200 mg·L^{-1}, respectively. The conical flasks were incubated in a rotary shaker to supply oxygen with 200 rpm at 28 °C. The medium without inoculation was used as a control. The 10 mL samples were taken from the flasks every 12 h and centrifuged at 6500× *g* for 10 min at room temperature. The supernatant was taken to determine the optical density at 600 nm (OD_{600}) to measure the concentrations of NH_4^+-N, NO_3^--N, and NO_2^--N. All of the assays were performed in triplicate. The formula of nitrogen removal rate was $(C_0 - C_n)/t$, where t was the total time for strain SC16 treatment, C_0 was the initial concentrations of NH_4^+-N, NO_3^--N, and NO_2^--N, as well as C_n was the final concentrations at time t.

To study the NO_2^--N tolerance of strain SC16, $NaNO_2$ was added into the DM to make initial nitrite concentrations of 0.1, 2, 50, 100, 200, 250, 300, 500, and 1000 mg·L^{-1}. SC16 was inoculated into these media, followed by incubating with 200 rpm at 28 °C using a rotary shaker. The samples were collected as mentioned above, and the removal percentage was determined as follows: removal percentage (%) = 100 × (initial total nitrite − final total nitrite)/initial total nitrite.

2.4. Enzyme Assay

After cultivation in DM-1 medium for 24 h, the medium was centrifuged at 3000× *g* for 10 min to obtain bacteria. Enzyme extracts were prepared by breaking bacterial cells applying ultrasonication as described by previous study [20]. (1) NAR activity trial: 20 mL reaction mixture contained 0.2 mM

NADH, 10 mM potassium phosphate buffer, 10 μM $NaNO_3$, and enzyme extracts; (2) NiR activity trial: 20 mL reaction mixture contained 0.2 mM NADH, 10 mM potassium phosphate buffer, 10 μM $NaNO_2$, and enzyme extracts; (3) Ammonia monooxygenase (AMO) activity trial: 20 mL reaction mixture contained 10 mM Tris/HCl, 0.2 mM NADH, 10 μM NH_4Cl, and enzyme extracts. All of the trials were incubated at 28 °C for 15 min with three repetitions, and a constant shaking of 120 rpm was used for the AMO activity trial. The enzyme activities of NAR, NIR, and AMO were determined by the reduction of $NaNO_3$, $NaNO_2$, and NH_4Cl, respectively, according to previous study [20]. The Lowry method was used to determine the protein concentration in the bacteria extract [21]. The specific activity (U mg^{-1}) was expressed as the amount of enzyme that catalyzed the transformation of 1 μmol of substrate per minute by the amount of protein in milligrams [22].

2.5. Amplification of the Functional Genes

Fragments of the NIR encoding gene *nirK* (or *nirS*) were amplified using the primer pairs nirK1F-nirK5R and nirS1F-nirS6R (in supplementary, Table S1). PCR amplification was performed in a 25 μL reaction mixture containing 2.5 μL 10 × PCR buffer for KOD-Plus-Neo, 1.5 μL dNTP mixture (2 mM), 1.5 μL $MgSO_4$ (25 mM), 1 μL F-primer (10 μM), 1 μL R-primer (10 μM), 10-100 ng DNA template, 1 U KOD-Plus-Neo (Toyobo, Japan), and 15 μL ddH_2O. The PCR program included the following steps: 94 °C for 4 min; 35 cycles at 98 °C for 10 s, annealing temperature of 55 °C for 30 s, 68 °C for 30 s; and final extension of 68 °C for 7 min. The PCR products were sent to BGI Genomics Co., Ltd., (Shenzhen, Guangdong, China) for sequencing.

2.6. Quantitative Real-Time PCR (QRT-PCR) Assay

The expression levels of nitrite reductase genes in strain SC16 incubated with different nitrogen sources (DM-1, DM-2, and DM-3) were detected using QRT-PCR. Five milliliter samples were collected after 0, 12, 18, 24, 36, 48, 60, and 72 h in DM-1 post cultivation. Similarly, 5 mL samples were collected after 0, 6, 12, 18, 24, 36, and 48 h in DM-2 and DM-3 post cultivation. Media with no nitrogen sources were used as controls. Total RNA of each sample was extracted with TRIZOL reagent according to the manufacturer's instructions (Cat. No.15596-026; Invitrogen) [23]. Then, the first complementary DNA (cDNA) was synthesized with M-MLV Reverse transcriptase (Promega) and oligo (dT) primer. A 20 μL reaction mixture containing SYBR Green Real-time PCR Master Mix (Toyobo, Japan), cDNA, ddH_2O, and gene-specific primer pairs *nirK*$^{\#}$-F/*nirK*$^{\#}$-R was prepared for QRT-PCR. Reaction mixtures with no reverse transcription were employed as negative controls. The 16S rRNA (in supplementary, Figure S1) was used as the reference gene to normalize for differences of cDNA between samples. QRT-PCR was run on an iCycler iQ instrument (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The PCR protocol was as follows: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 60 °C for 15 s, 72 °C for 20 s; and a gradient from 65 °C to 95 °C during 10 min with continuous detection. The cross-point values were analyzed using the iCycler software version 1.0 (Bio-Rad, Hercules, CA, USA). Three replications were carried out for the experiment under identical experimental conditions.

2.7. The Performance of Strain SC16 in Aquaculture Water

The water was collected from another intensive aquaculture pond in Guangzhou. The concentrations of NH_4^+-N, NO_3^--N, and NO_2^--N in the aquaculture water were 8.35 mg·L^{-1}, 0.46 mg·L^{-1}, and 0.02 mg·L^{-1}, respectively, with a pH of 6.60. A total of 84 L water was distributed into six 20 L tanks, with each tank containing 14 L water. The six tanks were randomly divided into treatment group and control group, and each group had three tanks. The treatment group was added with 140 mL of strain SC16 inoculum, and the control group was added with 140 mL autoclaved culture medium without any bacteria. The tanks were equipped with an aeration pump (XK32, Xinke Co., Ltd., Shanghai, China) to supply oxygen, and the water temperature was maintained at 28 °C during the experiment.

2.8. Analytical Methods

The bacterial density was detected using spectrophotometry (EPOCH, BioTek® Instruments. Inc., Winooski, VT, USA) with a wavelength of 600 nm (OD_{600}). Culture samples were filtered through a 0.45-μm membrane filter after a centrifugation at 10,000× *g*. The concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N were detected with methods of the phenol disulfonic acid photometry, Nessler's reagent photometry, and N-(1-naphthalene)-diaminoethane photometry, respectively [24].

2.9. Statistical Analysis

All of the data shown in the tables and figures were expressed as the mean values (± standard deviation (SD)). The data of QRT-PCR were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed with independent-samples T tests and one-way ANOVA analysis using a statistical analysis system software package (SPSS 13.0, IBM, New York, NY, USA). Results were considered statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**).

3. Results

3.1. Isolation and Identification of Strain SC16

Denitrifying bacteria showed blue colonies on the BTB plates due to the reaction caused by the increasing pH during the denitrification process. In the preliminary screening study, 19 strains isolated from the sludge samples with blue colonies on solid BTB were selected for nitrogen removal tests. The strain SC16 (GDMCC, accession number: 60942) demonstrated the highest nitrogen removal ability. The colony of SC16 was oyster white, convex, opaque, and had neat edges, and the surface was smooth, moist, and shiny. The SC16 was a gram-positive strain with morphological characteristics of short rod-shaped single cells, and the body size of SC16 was about 2.0 μm × 0.5 μm (length × width) (Figure 1).

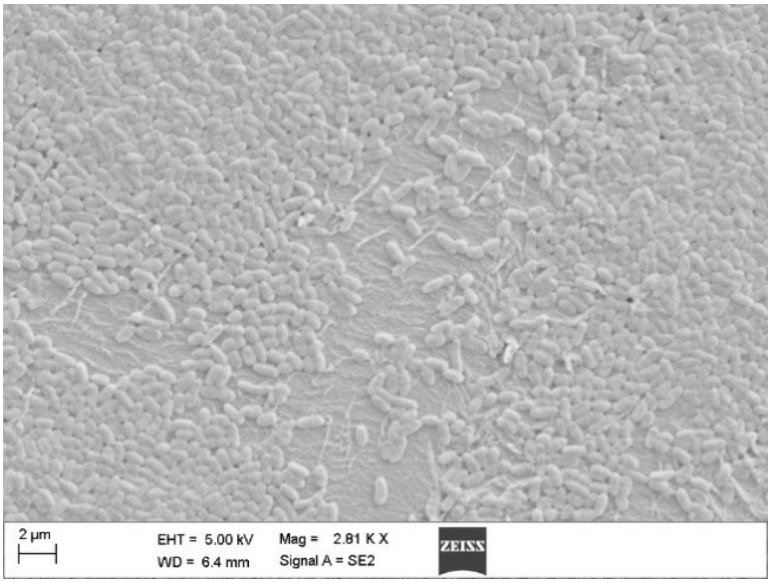

Figure 1. Single cell morphology of strain SC16 observed by SEM.

The obtained sequence of 16S rRNA gene was compared with other homologous sequences deposited in the NCBI database. The phylogenetic tree constructed using MEGA 6.0 (MEGA software development team, Phoenix, AZ, USA) is shown in Figure 2. Strain SC16 was clustered into a group with *Bacillus velezensis* QT-101 and showed 99% homology with *B. velezensis* QT-101. The partial 16S rRNA nucleotide sequence was submitted to GenBank with the accession number MN620386.

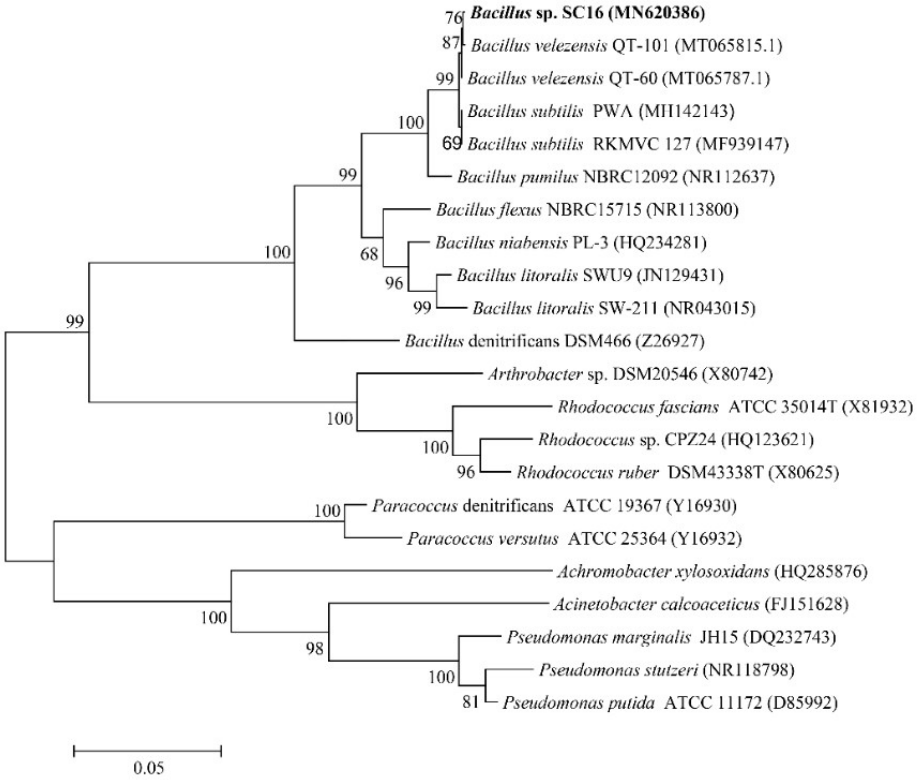

Figure 2. Phylogenetic tree of 16S rRNA of strain SC16 and other strains was constructed using the maximum-likelihood method. Bootstrap values were calculated by 1000 repetitions; 0.02 denotes the genetic distance. The accession numbers of the GenBank sequences are shown in parentheses.

3.2. Evaluation of Nitrogen Removal Ability of Strain SC16

When NO_3^--N was used as the nitrogen source (Figure 3a), the concentration of NO_3^--N decreased significantly, from 306.9 to 46.99 mg·L^{-1}, with a removal rate of 20.50 mg·L^{-1} h^{-1} after 12 h of cultivation. Then, an obvious lag period was observed from 24 h to 36 h. The final removal efficiency of nitrate was 97%, with a removal rate of 4.15 mg·L^{-1} h^{-1} at 72 h post cultivation. The maximum concentration of NO_2^--N was 0.63 mg·L^{-1}, with a time to peak concentration of 24 h, and then it decreased gradually from 24 h to 72 h. OD_{600} increased slowly during the first 36 h, then increased exponentially from 36 h to 48 h and subsequently entered into a stationary phase. The concentration of ammonium remained at a relatively low level during the trial.

When NH_4^+-N was used as the nitrogen source, the concentration of NH_4^+-N decreased from 165.49 to 104.85 mg·L^{-1} in the initial 12 h, with a removal rate of 5.05 mg·L^{-1} h^{-1} and a removal efficiency of 36.6% (Figure 3c). The concentration of NH_4^+-N maintained the same level from 12 h to the end of the incubation. The concentrations of NO_3^--N and NO_2^--N reached maximum values of 1.93 mg·L^{-1} at 12 h and 0.31 mg·L^{-1} at 24 h, respectively; subsequently they decreased gradually to 0 mg·L^{-1} at 48 h. The OD_{600} increased from 0.045 at 0 h to the maximum value of 0.320 at 48 h.

When nitrite was used as sole nitrogen source, the trend of cell growth was similar to that in the ammonium culture condition (Figure 3e). After 24 h incubation, the concentration of nitrite decreased significantly from 200 mg·L^{-1} to 0.039 mg·L^{-1}, with the removal efficiency of 99.9% and the removal rate of 8.33 mg·L^{-1} h^{-1}. The concentration of NO_3^--N increased to 28.36 mg·L^{-1} during the first 12 h, and then it decreased to 5.66 mg·L^{-1} at 48 h. No NH_4^+-N was detected during the experiment.

Based on the single-factor experiments for the aerobic denitrification performance, the optimal culture conditions of strain SC16 were at pH of 7.0, carbon source of citric acid, C/N ratio of 8, and temperature of 28 °C (in supplementary, Figure S2).

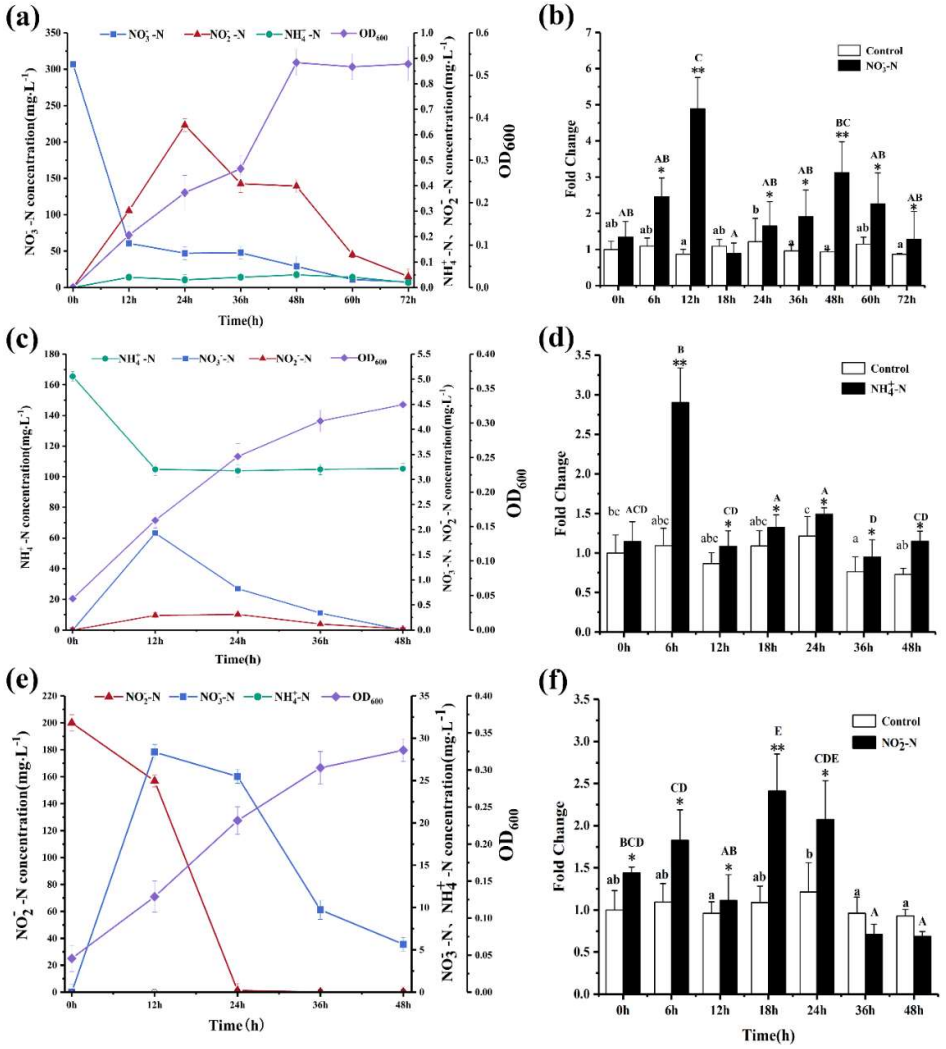

Figure 3. Nitrogen removal efficiency (**a**,**c**,**e**) and transcriptional analysis (**b**,**d**,**f**) of the *nirK* gene in denitrification medium (DM)-1, DM-2, and DM-3: (**a**,**b**) in DM-1 containing 306.9 mg/L of NO_3^--N; (c and d) in DM-2 containing 165.49 mg/L of NH_4^+-N; (**e**,**f**) in DM-3 containing 200 mg/L of NO_2^--N. All values are presented as the means ± SD (n = 3). The values that are significantly different from the individual controls are present with asterisks (* $p < 0.05$ and ** $p < 0.01$). Values with different capital (lowercase) letters are significant differences among the experimental group (control group).

3.3. Assessment of Nitrite Removal Performance of SC16 under Different Nitrite Concentrations

A series of concentrations of NO_2^--N ranging from low concentrations of 0.1, 2, and 50 mg·L^{-1} to high concentrations of 100, 200, 250, 300, 500, and 1000 mg·L^{-1} were added into DM to assess the nitrite removal performance by strain SC16. The unutilized nitrite and the OD_{600} were measured every 12 h after SC16 was cultured with NO_2^--N. As shown in Figure 4, SC16 in the nitrite concentrations of 0.1, 2.0, 50, and 100 mg·L^{-1} showed high growth rates under aerobic conditions. The OD_{600} in the nitrite concentration of 100 mg·L^{-1} reached 1.0. The growth rate decreased significantly with the increasing of initial nitrite concentration; the OD_{600} values in the groups at concentrations of 200 mg·L^{-1} and 250 mg·L^{-1} were 0.63 and 0.29, respectively. The strain SC16 showed no growth when the initial nitrite concentration was higher than 300 mg·L^{-1}.

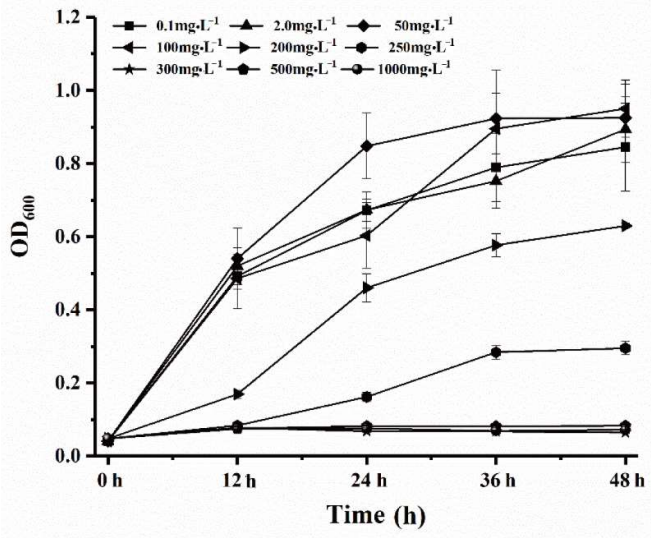

Figure 4. The growth curves of strain SC16 under different nitrite concentrations.

The nitrite utilization by SC16 showed a positive dose-response when the nitrite concentration ranged from 0.1 to 200 mg·L^{-1}, and it demonstrated a negative dose-response when nitrite concentrations were higher than 200 mg·L^{-1} (Table 1). The removal rate increased from 90% at a concentration of 0.1 mg·L^{-1} to 99.99% at a concentration of 200 mg·L^{-1}, while it decreased from 57.29% at a concentration of 250 mg·L^{-1} to 6.78% at a concentration of 1000 mg·L^{-1} (Table 1).

Table 1. The nitrite removal characteristics of *Bacillus* sp. SC16 under different nitrite concentrations.

Time (h)	Nitrite Concentration (mg·L^{-1})								
	0.1	**2**	**50**	**100**	**200**	**250**	**300**	**500**	**1000**
0	0.091 ± 0.03	1.98 ± 0.088	49.52 ± 0.29	99.71 ± 0.19	199.76 ± 6.39	248.57 ± 3.29	297.25 ± 5.58	499.46 ± 17.26	992.96 ± 11.17
12	0.031 ± 0.05	0.043 ± 0.027	0.2 ± 0.15	0.71 ± 0.19	156.84 ± 4.35	200.48 ± 5.89	249.65 ± 9.19	438.24 ± 18.19	934.81 ± 14.07
24	0.02 ± 0.05	0.043 ± 0.058	0.1 ± 0.17	0.071 ± 0.17	1.29 ± 1.19	176.78 ± 1.58	246.89 ± 7.13	435.2 ± 16.52	933.62 ± 13.32
36	0.015 ± 0.02	0.04 ± 0.02	0.02 ± 0.01	0.07 ± 0.05	0.11 ± 0.18	138.76 ± 3.19	245.53 ± 6.73	434.34 ± 9.8	932.42 ± 12.3
48	0.01 ± 0.03	0.03 ± 0.02	0.015 ± 0.011	0.04 ± 0.02	0.039 ± 0.02	106.78 ± 2.79	240.89 ± 9.87	430.25 ± 11.23	932.23 ± 10.02
Removal [a]	0.09 ± 0.03	1.97 ± 0.02	49.985 ± 0.011	99.96 ± 0.02	199.98 ± 0.02	143.22 ± 2.79	59.11 ± 9.87	69.75 ± 11.23	67.77 ± 10.02
Removal percentage (%) [b]	90 ± 0.3	98.5 ± 0.01	99.97 ± 0.00	99.96 ± 0.00	99.99 ± 0.00	57.29 ± 0.01	19.7 ± 0.03	13.95 ± 0.02	6.78 ± 0.01

[a] Removal = initial nitrite N − final nitrite N. [b] Removal percentage (%) = 100 × (initial nitrite N − final nitrite N)/initial nitrite N.

3.4. Enzyme Assay

In order to further characterize the possible pathways involved in the process of nitrogen removal, the activities of AMO, NAR, and NIR of strain SC16 were detected under aerobic conditions. NIR demonstrated the highest activity of 0.0271 ± 0.006 U/mg protein, followed by NAR with an activity of 0.026 ± 0.008 U/mg protein and AMO with an activity of 0.0061 ± 0.002 U/mg protein.

3.5. Amplification of the Denitrification Genes

nirK gene (GenBank accession number: MN918716) was cloned from the SC16 genome using the primers of nirK1F and nirK5R, while no fragment was cloned using the primers of nirS1F and nirS6R.

3.6. Effects of Nitrogen Sources on the Expression Level of Nitrite Reductase

The transcriptional level of the *nirK* gene during the process of nitrogen removal was investigated to determine whether this gene was involved in denitrification. When nitrate was used as the nitrogen source, the mRNA expression of *nirK* was significantly up-regulated at 6 h post incubation ($p < 0.05$), and it reached the peak (4.89-fold) at 12 h post-incubation (Figure 3b). The expression of *nirK* decreased notably ($p < 0.05$) and demonstrated no significant difference compared with that of the control group at 18 h ($p > 0.05$). Interestingly, the expression of *nirK* increased again from 24 h to 48 h and reached a second peak at 48 h.

When SC16 was exposed to ammonium or nitrite, the mRNA expression pattern of *nirK* was similar to that of the nitrate treated group (Figure 3d,f). Both ammonium and nitrite resulted in a significant ($p < 0.05$) up-regulation of *nirK* gene expression, with double peaks during the 48 h of incubation. The maximum mRNA expression levels of *nirK* in the ammonium and nitrite treatment groups were 2.9-fold at 6 h (Figure 3d) and 2.4-fold at 18 h (Figure 3f).

3.7. The Application of Strain SC16 in Aquaculture Water

The treatment of strain SC16 on aquaculture water was conducted to investigate the practical application of the strain. As shown in Figure 5a,b, the initial pH of the water was 6.6 ± 0.1, and this increased gradually to 8.2 ± 0.1 and 8.3 ± 0.05 in the treatment group and control group, respectively. In the treatment group, the concentration of NH_4^+-N decreased significantly from 8.35 to 4.56 mg·L^{-1} with a removal efficiency of 45.3%, and there was almost no accumulation of NO_2^--N at the end of experiment. In the control group, the concentration of NH_4^+-N kept at a fluctuating level, and the concentration of NO_2^--N increased to 0.14 mg·L^{-1}.

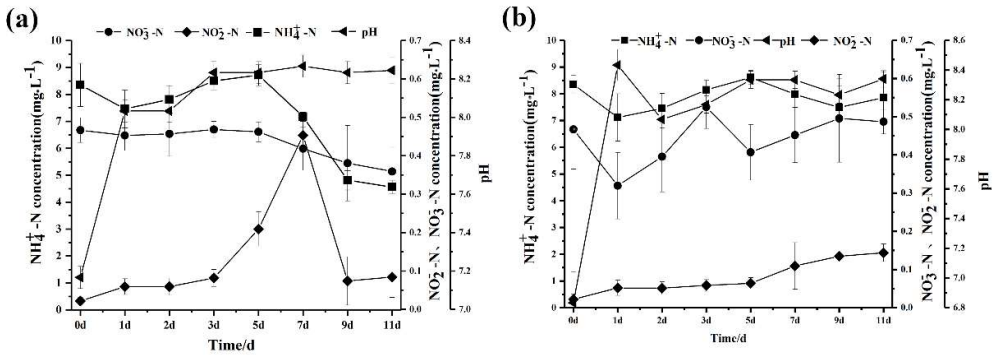

Figure 5. The nitrogen removal performance by strain SC16 in aquaculture water ((**a**): the treatment group; (**b**): the control group).

4. Discussion

Aerobic denitrifying bacteria with the capability of eliminating nitrogen have mainly been isolated from industrial and municipal wastewater, and they are usually used to treat wastewater [12,22]. Previous studies indicated that some aerobic denitrifying isolates had been successfully applied to treat aquaculture freshwater [13]. In this study, an aerobic denitrifying bacterium with high removal efficiency on nitrogen was isolated from an aquaculture pond. The strain belonged to the genus *Bacillus* according to the phylogenetic tree analysis. Moreover, the strain was a gram-positive bacterium with short rods. Thus, the isolated strain was identified as *Bacillus* sp. SC16.

When ammonia, nitrite, or nitrate were used as the sole nitrogen sources, strain SC16 showed a high removal efficiency and fast removal rate for all three inorganic nitrogen forms. The final removal rate of NO_3^--N by strain SC16 was 4.15 mg·L^{-1} h^{-1}, higher than that by *Pseudomonas tolaasii* Y-11 (1.99 mg·L^{-1} h^{-1}) [25] and *Klebsiella pneumoniae* CF-S9 (2.2 mg·L^{-1} h^{-1}) [26], and similar to that by *Ochrobactrum anthropi* LJ81 (4.16 mg·L^{-1} h^{-1}) [17]. The final removal rate of ammonium by strain SC16 was 5.05 mg·L^{-1} h^{-1}, higher than that of some other denitrifiers such as *P. stutzeri* T13

(3.98 mg·L^{-1} h^{-1}) [27] and *O. anthropi* LJ81 (3.84 mg·L^{-1} h^{-1}) [17]. In addition, the removal rate of nitrite (8.33 mg·L^{-1} h^{-1}) was higher than those for nitrate and ammonium when SC16 was incubated with the three nitrogen sources, and the removal rate of nitrite by SC16 was also higher than those of *Pseudomonas mendocina* TJPU04 (1.85 mg·L^{-1} h^{-1}) [28] and *Arthrobacter arilaitensis* Y-10 (0.19 mg·L^{-1} h^{-1}) [29]. Moreover, the majority of nitrogen was removed during the logarithmic phase of cell growth, and the maximal removal rates of nitrate, ammonia and nitrite achieved 20.50 mg·L^{-1} h^{-1} at 12 h, 5.05 mg·L^{-1} h^{-1} at 12 h, and 8.33 mg·L^{-1} h^{-1} at 24 h, respectively. The nitrogen removal rates decreased significantly after the logarithmic phase. This phenomenon was in agreement with the previous reports [17]. The reason might be that the nitrogen removal rates were inhibited by products released from bacteria, so that the concentrations of these inhibitors increased rapidly during the logarithmic phase due to the rapid growth of the bacteria, resulting in significant decreases in the removal rates. The removal efficiencies of nitrate and nitrite by SC16 reached 97% and 99.99%, respectively, under aerobic conditions. Thus, the high removal performance of nitrogen by SC16 indicated that the strain had promising potential in aquaculture for the remediation of wastewater.

The characteristics of heterotrophic nitrification and aerobic denitrification had been demonstrated in other *Bacillus* spp., such as *Bacillus methylotrophicus* L7 [30], *Bacillus litoralis* N31 [31], and *Bacillus subtilis* A1 [12]. However, it has rarely reported in freshwater aquaculture. In the present work, the elimination of ammonia, nitrite, and nitrate suggested that nitrification and denitrification co-existed in strain SC16 under aerobic conditions. The following assay was conducted to investigate the activities of ammonia monooxygenase (AMO), nitrate reductase (NAR), and nitrite reductase (NIR). AMO was reported to be involved in the oxidation of ammonia to hydroxylamine. NAR and NIR are the key enzymes in the conversion of nitrate into nitrite and nitrite into nitric oxide, respectively. The present results indicated that AMO, NAR, and NIR coexisted in strain SC16, thus providing additional evidence of heterotrophic nitrification and aerobic denitrification by this strain.

As is well known, nitrite is harmful to aquatic animals due to its damage to the immune system and its interference with oxygen carrying in hemoglobin and hemocyanin [13]. In this study, SC16 grew well under a concentration of 200 mg·L^{-1} NO_2^--N, with a high nitrite removal efficiency of 99.99% during 48 h. The strain even survived under a concentration of 250 mg·L^{-1} NO_2^--N, with a removal efficiency of 57.29%. The results revealed that SC16 had a strong tolerance to nitrite. This phenomenon is consistent with the claims that aerobic denitrifiers usually tolerate high concentrations of nitrogen [32]. Moreover, there was almost no nitrite accumulation in DM-1 or DM-2. Similar results were also obtained for *Acinetobacter* sp. Y16 [33] and *P. stutzeri* D6 [34], which were no nitrite accumulation in *Acinetobacter* sp. Y16 treated group [33] and only trace accumulation of nitrite in *P. stutzeri* D6 treated group [34]. The concentration of NO_2^--N in SC16 was extremely lower than that in *P. mendocina* TJPU04 [28]. These results indicated the advantageous features of the SC16 strain in eliminating nitrite. Therefore, SC16 is a potential candidate biocontrol agent for eliminating nitrite.

Nitrite can be converted into nitrogenous compounds via three pathways: nitrite oxidation ($NO_2^- \rightarrow NO_3^-$) catalyzed by nitrite oxidoreductase in nitrification, reduction of nitrite to nitric oxide ($NO_2^- \rightarrow NO$) catalyzed by nitrite reductase in denitrification, and reduction of nitrite to ammonium ($NO_2^- \rightarrow NH_4^+$) catalyzed by nitrite reductase in dissimilation [35]. It has been reported that different pathways of removing nitrite are involved in different bacteria [25]. For example, *A. faecalis* No. 4 isolated from activated sludge could not remove NO_2^--N and NO_3^--N at the same time [36]. However, in the present study, NO_2^--N and NO_3^--N were present in the DM-2, indicating that NH_4^+-N was converted into NO_2^--N and NO_3^--N during nitrogen removal; NO_2^--N and NH_4^+-N were detected in DM-1, and their concentrations decreased gradually, indicating that NO_3^--N was converted into NO_2^--N and subsequently degraded to N_2 and NH_4^+-N; NO_3^--N was accumulated in DM-3, indicating that NO_2^--N was converted into NO_3^--N. These results indicated that the nitrogen removal pathway in strain SC16 included $NO_2^- \rightarrow NO_3^-$, $NO_2^- \rightarrow NO$, and $NO_2^- \rightarrow NH_4^+$. Therefore, the complete nitrification–denitrification process in strain SC16 was further confirmed as follows: $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. In addition, the removal rate of nitrite

was much higher than that of nitrate, and the accumulation of nitrite was lower than that of nitrate during the removal of ammonia, meaning that there might be partial nitrification–denitrification, i.e., $NH_4^+ \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ [17].

The reduction of nitrite by NIR is the rate-limiting process of denitrification [37]. There are two entirely different NIR enzymes in terms of structure and the metal ion: one with heme cd1 (cd1-NIR) encoded by the *nirS* gene and the other containing copper (Cu-NIR) encoded by the *nirK* gene [38]. In this study, the *nirK* gene rather than the *nirS* gene was amplified from the *Bacillus* sp. SC16 genome. The result was consistent with the phenomenon that the two types of NIR do not co-exist in most denitrifiers [39]. The Cu-NIR is distributed extensively in gram-negative denitrifying bacteria, and its molecular structures have been well studied. However, Cu-NIR in gram-positive *Bacillus* sp. are rarely reported. Gao, et al. [40] reported molecular and structural analyses of Cu-NIR in gram-positive bacteria of *Bacillus firmus* GY-49. The present study provides further evidence for the existence of *nirK* in *Bacillus* sp.

The denitrification properties of bacteria are closely correlated to the functional gene encoding NIR, and there are many reports on the genes related to NIR [41]. The *nirS* gene has been demonstrated that it was more likely involved in aerobic denitrification, such as in *Bacillus* sp. YX-6 [19] and *P. stutzeri* ZF31 [42]. Recent studies have shown that the *nirK* gene was also related with denitrification [43] and detoxification [18]. Therefore, the quantitative PCR and enzyme assays were carried out to investigate whether NIR encoded by *nirK* gene was involved in denitrification in strain SC16. The results indicated that the expression levels of the *nirK* gene in sole nitrogen media were significantly higher than in the controls with no nitrogen source, demonstrating that the *nirK* gene was involved in the process of denitrification. However, the change in *nirK* expression did not maintain a pattern similar to the change in concentration of nitrite during the present tests. Previous work had shown that nitrogen resources could be captured into the pathway of assimilation in the process of denitrification [44]. It was speculated that some of the nitrogen resource could be assimilated into organic compounds. Additionally, the highest expression level of *nirK* was 4.89-fold in the nitrate medium, while the highest expression levels of *nirK* were 2.41-fold in nitrite medium and 2.90-fold in ammonia medium. These results indicated that nitrate was the more potent inducer for *nirK* gene expression.

Interestingly, a unique bimodal distribution of the expression of *nirK* was detected in sole nitrogen source media. The phenomenon was in agreement with the expression pattern of the *nir* gene in *Marinobacter hydrocarbonoclasticus* RAD-2 [45]. A suggested explanation was as follows: the expression level of *nirK* was up-regulated by nitrogen induction and reached its first peak during the process of nitrogen removal in DM-1, DM-2, and DM-3 media. In time, SC16 translated enough nitrite reductase to catalyze the conversion of substrates, and the excess nitrite reductase played a negative regulatory role on *nirK* gene expression. With the decrease of nitrite reductase, the expression of *nirK* was upregulated again and reached a second peak. The transcriptional pattern of the *nirK* gene was identical with the elimination of nitrate, ammonium, and nitrite in the process of denitrification. The similar expression pattern of the *nirK* gene in the presence of ammonium, nitrite, or nitrate as sole nitrogen sources indicated that both ammonium and nitrate were converted into nitrite, and then nitrite was converted into NO by NIR, demonstrating again the existence of HN-AD in this strain.

5. Conclusions

In this study, a heterotrophic nitrifying-aerobic denitrifying bacterium, *Bacillus* sp. SC16, was isolated from aquaculture ponds. Strain SC16 exhibited an enhanced ability remove nitrate, ammonia, and nitrite with high removal rates. The strain showed high tolerance against nitrite, and it grew well under the concentration of 250 $mg \cdot L^{-1}$ NO_2^--N. It was demonstrated that adding strain SC16 to aquaculture wastewater could effectively reduce the nitrite. The *nirK* gene plays an important role in the elimination of residual nitrogen in water. Therefore, *Bacillus* sp. SC16 possesses highly practical application value in aquaculture water quality improvement.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/12/3559/s1, Table S1. The related primers for corresponding genes. Figure S1. The agarose gel electrophoresis of the qRT-PCR amplification product of *nirK* gene (lane 1) and 16S rRNA gene (lane 2) from strain SC16. Figure S2. Effects of carbon source (**a**), C/N ratio (**b**), pH (**c**) and temperature (**d**) on the growth and nitrogen removal of strain SC16 under 60 h cultivation.

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