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# Characterization of novel *Bacillus* strain N31 from mariculture water capable of halophilic heterotrophic nitrification–aerobic denitrification

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**The development of an intensive aquaculture industry has been accompanied by increasing environmental impacts, especially nitrogen pollution. In this study, a novel halophilic bacterium capable of heterotrophic nitrification and aerobic denitrification was isolated from mariculture water and identified as *Bacillus litoralis* N31. The efficiency of ammonium, nitrite and nitrate removal by N31 were 86.3%, 89.3% and 89.4%, respectively, after a 48-h cultivation in sole N-source medium with initial nitrogen approximately 20 mg/L. However, ammonium was removed preferentially, and no obvious nitrite accumulated during the simultaneous nitrification and denitrification process in mixed N-source media. The existence of *hao*, *nap*A and *nir*S genes further proved the heterotrophic nitrification–aerobic denitrification capability of N31. The optimal conditions for ammonium removal were 30°C, initial pH 7.5–8.5, C/N ratio 5–20 and salinity 30–40‰, and the nitrification rate of N31 increased with increasing initial $NH_4^+$–N from 10 to 250 mg/L. Biosecurity assessment with shrimp indicated that strain N31 could be applied in the marine aquaculture industry safely for culture water remediation and effluent treatment.**



[**Key words:** Halophilic bacterium; *Bacillus litoralis*; Heterotrophic nitrification–aerobic denitrification; Ammonium removal; Biosecurity]

In recent years, the development of intensive aquaculture industry has brought a rapid expansion of fishery production such as shrimp farming (1). However, this successful industry has been accompanied by an increase in environmental impacts. Nitrogen pollution is one of the major environmental concerns because of the continuous accumulation of nitrogenous compounds, including both organic and inorganic (ammonia, nitrite and nitrate) nitrogen, which mostly originate from uneaten protein rich feed, excretion and other metabolic wastes of aquatic animals in the production process (2,3). For instance, nitrogen is provided in high concentrations in shrimp feed but most of it added to ponds (60–80%) is not retained as shrimp biomass, and 30–60% of the nitrogen added to ponds is discharged into the effluents (3–5). Nevertheless, the discharge of nitrogen-rich effluents from culture farms into adjacent surroundings without prior treatment can lead to eutrophication and oxygen depletion in the receiving water bodies, threatening human or animal health (2–4). Furthermore, the deterioration of water quality caused by the accumulation of nitrogenous compounds (particularly ammonium-N) may increase the occurrence of pathogenic bacteria and introduce invading pathogen species, resulting in diseases outbreak and huge economic losses to farmers (2,4). Therefore, appropriate treatments for aquaculture water and effluent to decease the accumulation of nitrogen in aquatic ecosystems become increasingly necessary for maintaining sustainable fishery production and reducing the amount of nitrogen released into the environment.

The biological method without second-pollution and residues is the best way to remove nitrogen in aquaculture practice (2,3,5). The conventional biological nitrogen removal processes include autotrophic nitrification, which oxidizes ammonium via nitrite to nitrate under aerobic conditions, and heterotrophic denitrification, which converts nitrite and nitrate to nitrogen gas under anaerobic conditions; meanwhile, aerobic autotrophic nitrifiers and anaerobic heterotrophic denitrifiers play important roles in this process (2,3,5). However, these nitrifiers and denitrifiers have some disadvantages in the removal of nitrogen from wastewaters because of their distinct differences in physiology and biochemistry. The growth of nitrifiers depends on oxygen, which is toxic to the denitrifiers, and the nitrifiers are sensitive to organic matter, which is necessary for the denitrifiers (6–8). Whereupon, their application will be restricted in aquaculture practice such as the intensive aquaculture systems of shrimp which always contain high levels of dissolved oxygen and organic compounds. Recently, aerobic heterotrophic nitrifying–denitrifying microorganisms have received more attention, because they achieve the vision of simultaneous nitrification and denitrification in one system owing to the tolerance for oxygen and the utilization of organic substrates agreeing with each other, compared with the traditional nitrifiers and denitrifiers (8,9). Therefore, aerobic heterotrophic nitrifying–denitrifying bacteria may be more suitable for decreasing the accumulation of nitrogen compounds in aquaculture industry.

In the last few years, more and more heterotrophic nitrification–aerobic denitrification bacteria have been reported, such as

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*Pseudomonas stutzeri*, *Bacillus subtilis*, *Bacillus methylotrophicus*, *Klebsiella pneumoniae*, *Acinetobacter junii* and *Pseudomonas tolaasii* (10–15). However, the current aerobic heterotrophic nitrifying–denitrifying bacteria, including these mentioned above, are almost all from sludge or fresh water and may not perform efficiently when treating high salinity wastewater such as marine aquaculture effluent (16,17). Moreover, most studies have merely examined the nitrification and denitrification characteristics of isolates under a high level of nitrogen corresponding to municipal wastewater or sediment (6,12). The nitrogen removal capabilities of these isolates may be challenged in mariculture water or effluent containing relatively low total nitrogen (TN) and carbon to nitrogen ratio (C/N) and high salinity compared to municipal wastewater (18,19). More importantly, few isolates applied in the treatment of polluted water are really suitable for the aquaculture industry in consideration of their ecological characteristics and unknown potential pathogenicity (20). Thus, autochthonous strains that are capable of performing heterotrophic nitrification–aerobic denitrification in aquatic water may be more effective and secure for application in aquaculture industry.

*Bacillus* bacteria such as *Bacillus coagulans*, *Bacillus licheniformis* and *B. subtilis*, as putative probiotics, have been extensively applied in aquaculture industry to facilitate growth, prevent diseases and improve water quality (20–23). But few studies have detailed the heterotrophic nitrifying–aerobic denitrifying characteristics of *Bacillus* strains isolated from aquacultural environments and their safety for aquatic animals. In this study, a novel halophilic *Bacillus* strain, N31, capable of heterotrophic nitrification and aerobic denitrification was isolated from shrimp mariculture water. Its heterotrophic nitrification, aerobic denitrification and simultaneous nitrification and denitrification abilities were examined in sole N-source and mixed N-source media. Given that the ammonia excreted by the cultivated organisms is the main source of dissolved nitrogen and is toxic to aquatic life in aquatic systems (2,4,5), the key environmental factors affecting the ammonia removal performance of the isolated strain N31 were therefore investigated. In addition, this study approximately evaluated the biosecurity of strain N31, which might have impacts on the growth and immunity of shrimp. This study may provide an alternate, effective and safe microbial resource for nitrogen removal treatment of marine aquaculture water and effluent, and it also may promote the development of bioremediation methods for water quality control in shrimp cultivation systems.

## MATERIALS AND METHODS

**Sample and medium** The water sample was collected from intensive shrimp mariculture ponds in the Modern Fishery Demonstration Area of Dongying (Shandong, China) for isolation of halophilic heterotrophic nitrification and aerobic denitrification bacteria. The basic screening medium (BSM) used for bacteria isolation was provided with the following components (g/L): sodium succinate 6.5, $(NH_4)_2SO_4$ 0.25, $K_2HPO_4 \cdot 3H_2O$ 1.5, $KH_2PO_4$ 0.45, $MgSO_4 \cdot 7H_2O$ 0.05, $FeSO_4 \cdot 7H_2O$ 0.01, $MnSO_4 \cdot 4H_2O$ 0.01, NaCl 30 and agar 20. To study the ammonium removal ability of isolates, heterotrophic nitrification medium (HNM) consisted of the following components (g/L): sodium succinate 1.2, $(NH_4)_2SO_4$ 0.096, $K_2HPO_4 \cdot 3H_2O$ 1.0, $KH_2PO_4$ 0.3, $MgSO_4 \cdot 7H_2O$ 0.05, $FeSO_4 \cdot 7H_2O$ 0.01, $MnSO_4 \cdot 4H_2O$ 0.01 and NaCl 30. The two denitrification media used for nitrite and nitrate reduction investigations contained (per liter) $NaNO_2$ 0.10 g (DM-1) and $NaNO_3$ 0.122 g (DM-2), respectively; the other two media used for determining simultaneous nitrification and denitrification performance of isolates in mixed N-source contained (per liter) $(NH_4)_2SO_4$ 0.048 g, $NaNO_2$ 0.05 g (SNDM-1) or $NaNO_3$ 0.061 g (SNDM-2); and other components in the DM and SNDM media were the same as the HNM medium. The media mentioned above had initial pH 7.5 and salinity 30‰. Luria–Bertani medium (LB) contained (per liter) 10 g beef extract, 10 g tryptone, 5 g NaCl and pH 7.5. All chemicals in the experiments were of analytical grade.

**Isolation and identification of *Bacillus* strain** Water samples (2 mL) were added to three conical flasks (250 mL capacity) containing 98 mL sterile liquid BSM medium (excluding agar) for enrichment of halophilic heterotrophic nitrification–aerobic denitrification bacteria. After incubated at 28°C on a rotary shaker at 160 rpm for 48 h, they were treated in 85°C water bath for 15 min. Afterwards, 0.1 mL of gradient dilutions ($10^{-1}$ to $10^{-5}$) of the enrichment suspensions were spread on solidified BSM medium and incubated at 28°C for 3 days under aerobic conditions. Separate colonies were picked and purified by repeatedly streaking on fresh BSM medium plates. Gram staining was then performed, and a total of eight gram-positive isolates were individually cultivated in liquid BSM medium at 28°C, 160 rpm for 24 h to determine their nitrification ability. Strain N31 with the highest nitrification ability was selected, and then it was suspended in 25% glycerol solution and stored at −80°C for further study. Physiological and biochemical characteristics were tested using API 20 NE and API ZYM strips (bioMérieux, French) following the manufacturer's instructions. After cultivation in LB medium overnight, genomic DNA of the isolated strain N31 was extracted with a TIANamp Bacteria DNA Kit (TianGen Biotech, China). The isolated strain was identified by 16S rDNA analysis following described methods (17).

**Assessment of heterotrophic nitrification and aerobic denitrification ability of strain N31** After cultivation for 36 h at 28°C and 160 rpm in LB medium, 6 mL of pre-culture strain N31 was harvested by centrifugation at 4000 ×*g* (4°C, 15 min). The pellet was washed with 6 mL sterile distilled water (pH 7.4) three times to purify the cell suspension. Then, 1 mL bacterial suspension was inoculated into 250 mL conical flasks containing 100 mL sterile HNM, DM-1, DM-2, SNDM-1 and SNDM-2 medium, respectively. These conical flasks were incubated aerobically at 28°C and 160 rpm. Different medium samples were taken from the flasks periodically to determine the cell density ($OD_{600}$), $NH_4^+$–N, $NO_2^-$–N, $NO_3^-$–N and TN. For analyzing the nitrogen balance of heterotrophic nitrification and aerobic denitrification by strain N31, samples of sole and mixed N-source media at 48 h were centrifuged at 4000 ×*g* (4°C, 15 min) to determine the intracellular and soluble organic nitrogen content. The supernatant was used for analysis of the final soluble TN, $NH_4^+$–N, hydroxylamine ($NH_2OH$–N), $NO_2^-$–N and $NO_3^-$–N. The intracellular nitrogen level was calculated by subtracting the final soluble TN from the final TN of non-centrifuged medium. The soluble organic nitrogen level was calculated by subtracting $NH_4^+$–N, $NH_2OH$–N, $NO_2^-$–N and $NO_3^-$–N from the final soluble TN level (11). All the experiments were performed in three biological replicates.

**Amplification of nitrification and denitrification enzyme genes** To determine whether strain N31 contained enzyme genes involved in heterotrophic nitrification and aerobic denitrification, the presence of the *hao* gene, *nap*A gene and *nir*K or *nir*S gene was confirmed. The *hao* gene encoding hydroxylamine oxidase, which oxidizes hydroxylamine to nitrite, is always regarded as a marker gene of nitrification. Primers ($haoF_1$ and $haoR_3$) were used for *hao* gene amplification with the reaction conditions (13). The *nap*A gene encodes periplasmic nitrate reductase, which reduces nitrate to nitrite, and the *nir*K and *nir*S genes encode nitrite reductase, which reduces nitrite to nitric oxide during denitrification process; the three genes are generally amplified to verify the aerobic denitrification by isolates (14). Primers ($NAP_1$ and $NAP_2$) were used for *nap*A amplification in terms of the conditions described by a previous study (17). The *nir*K and *nir*S genes were amplified with primers nirK1F/nirK5R and nirS1F/nirS6R, respectively, according to a previous description (21). The PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Bands were visualized by UV excitation and photographed using an electrophoresis image analysis system.

**Effects of different factors on heterotrophic ammonium removal performance of strain N31** The ammonium removal performance of strain N31 was examined under different culture conditions, including temperature, C/N ratio, initial pH, salinity and ammonium concentration. For all the single-factor experiments, strain N31 was inoculated into 100 mL sterile HNM medium (initial $NH_4^+$–N 20 mg/L, C/N ratio 10, initial pH 7.5 and salinity 30‰) and then incubated at 28°C, 160 rpm for 48 h with the single factor being adjusted according to the experimental design. To study the effects of culture temperature, initial pH and salinity on ammonium removal by strain N31, the temperature was set at 15°C, 20°C, 25°C, 30°C, 35°C and 40°C; the initial pH was adjusted to 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 by addition of 0.5 mol/L NaOH or 0.5 mol/L HCl; and the salinity was set at 5‰, 10‰, 20‰, 30‰, 40‰ and 50‰ by changing the NaCl content in the HNM medium. The effects of C/N ratio on ammonium removal by strain N31 were examined by varying the carbon source (sodium acetate) content in the HNM medium to adjust the C/N ratio to 0, 2, 5, 10, 15 and 20 with a fixed concentration of $NH_4^+$–N 20 mg/L. For ammonium concentration experiments, initial $NH_4^+$–N levels were adjusted to 10, 50 and 250 mg/L by altering the $(NH_4)_2SO_4$ content in the HNM medium; meanwhile sodium acetate content was adjusted accordingly to maintain a settled C/N ratio of 10. All the experiments were carried out in three biological replicates with an inoculation size of 1% (v/v), and non-seeded samples were conducted as controls.

**Assessing the biosecurity of strain N31 for shrimp** *Bacillus* strain N31 was cultivated in LB medium using a rotary shaker at 28°C for 36 h. Then, the medium was centrifuged at 4000 ×*g* (4°C, 15 min), and the pelleted bacteria were resuspended and washed three times in sterile saline solution (0.9% NaCl, pH 7.5) after discarding the supernatant. The bacterial suspension density was calculated by using a spectrophotometer at 600 nm and correlated to colony-forming units (CFU) by a spread-plate method (23). The bacterial suspensions (approximately $10^{11}$ CFU $mL^{-1}$) were kept at 4°C and immediately added to the rearing tanks.

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