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Research article

Biopotentiality of High Efficient Aerobic Denitrifier *Bacillus megaterium* S379 for Intensive Aquaculture Water Quality Management

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ABSTRACT

Excessive nitrite accumulation is a very tough issue for intensive aquaculture. A high efficient aerobic denitrifier *Bacillus megaterium* S379 with 91.71 ± 0.17% of NO_2^--N (65 mg L^{-1}) removal was successfully isolated for solving the problem. Denitrification of S379 showed excellent environment adaptation that it kept high nitrite removal ratio (more than 85%) when temperature ranged from 25°C to 40°C and pH varied between 7.0 and 9.0, and could endure as high as 560 mg L^{-1} of NO_2^--N. Immobilization of S379 could enhance denitrification even when NO_2^--N adding amount got to 340 mg L^{-1}. Immobilized cells also showed well pollutants removal performance in aquaculture wastewater treatment. Moreover, S379 possessed positive hydrolase activities for starch, casein, cellulose and fat and bore more than 60 ppt of salinity. Totally, all the results revealed significant potentiality of immobilized S379 applied in aquaculture water quality management.

1. Introduction

Marine biodiversity is being destroyed due to the over-developed marine natural biological resource, which brings a serious of threat to natural fishery resource (Cao et al., 2015). In this case, aquaculture industry has been rapidly developed. Intensive culture system is most commonly used in aquaculture industry because of its higher yield than other systems (Crab et al., 2007). For intensive aquaculture, rapid nitrite (NO_2^--N) accumulation in pond, mainly comes from uneaten high protein feed and feces degradation in the proceeding, is a very tough issue (Barman et al., 2017). Many studies confirmed nitrite damaged immune system and induced the conversion of hemoglobin into a form incapable of carrying oxygen for aquatic animals (Grguric et al., 2000; Idi et al., 2015; Zhang et al., 2012). Cultivators periodically exchange the high nitrogen water with fresh seawater and directly discharged the nitrogen-enriched wastewater into adjacent coastal water resulting in eutrophication with adverse consequences for marine ecology and fishery.

In the past two decades, many techniques had been applied to remove nitrogen from wastewater, including rotating biological contactors, trickling filters, bead filters, fluidized sand biofilters and so on (Shan et al., 2016). Comparing with physical and chemical processing, biological method may be more cost-effective for nitrogen removal. Traditionally, water purifying bacteria remove nitrite through anoxic denitrification which limits their application in open water body. The finding of *Thiosphaera pantotropha* realized the aerobic denitrification (Robertson et al., 1988). Hence, nitrite removal becomes simplicity in open environment because denitrification can happen at oxygenic conditions. In recent years, more and more aerobic denitrifers were screened and characterized, such as *Alcaligenes faecalis* (Anderson et al., 1993), *Microvirgula aerodenitrificans* (Patureau et al., 1998), *Pseudomonas* sp. (Zhang et al., 2011), *Rhodococcus* sp. (Chen et al., 2012), *B. methylotrophicus* (Zhang et al., 2012), *Agrobacterium* sp. (Chen and Ni, 2011). Nevertheless, aerobic denitrifers with good properties, such as high nitrogen removal efficiency, low temperature resistance, metal tolerance and so on, are still in urgent need (He et al., 2016; Idi et al., 2015). Furthermore, limited reports on aerobic denitrifier specific for intensive aquaculture water quality management are available so far.

Immobilization of bacteria onto matrices has been found efficient for wastewater treatment due to their high operational stability, strong environmental tolerance, fast growth rate and enhanced cell densities on the matrices (Hill and Khan, 2008; Lee and Cho, 2010). In recent years, researchers applied immobilized bacteria into water quality management of intensive shrimp farming and achieved good results (Manju et al., 2009; Shan et al., 2016). In this sense, immobilized microorganism technology may also have very positive potentiality applied in the intensive aquaculture fields. Autochthonous bacteria generally face no acclimation problem for application in corresponding wastewater treatment. Therefore, immobilization of indigenous microbes with well pollutants removal ability may be a good choice for *in-*

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situ solving eutrophication of intensive aquaculture.

Generally, most microbes isolated from *Bacillus* genera have the advantages of safety, fast growth, wide environment adaptation, easy production and high treating efficiency, additionally, some of them have good probiotic effect via producing antimicrobial peptides to inhibit certain pathogenic bacteria of fishery (Aly et al., 2008; Zhao et al., 2012). In this work, an autochthonous *Bacillus* bacterium with efficient aerobic denitrification was isolated from intensive aquaculture ponds and evaluated its nitrite removal capability in synthetic wastewater with different pH, temperature and NO_2^--N content. Meanwhile, the performance of immobilized cells in synthetic wastewater and actual aquaculture wastewater treatment was analyzed. All the above attempts would elucidate the applied potential of *B. megaterium* S379 in in-situ intensive aquaculture water management.

2. Materials and methods

2.1. Media

The media used for aerobic denitrification bacteria isolation were enrichment medium (EM), solid bromothymol blue (BTB) medium (BTBM) and second screening medium (SSM). The EM (pH 7.0-7.2) included the following components per liter: glucose 8 g, $NaNO_2$ 0.5 g, KH_2PO_4 1.36 g, yeast extract 2.4 g and $MgSO_4$•$7H_2O$ 0.19 g. BTB and agar were added into the synthetic wastewater (SW) to form solid BTBM. The ingredient of solid BTBM was as follows per liter: agar 20 g, CH_3COONa 5.388 g, $MgSO_4$•$7H_2O$ 0.912 g, KCl 0.36 g, $NaNO_2$ 0.4 g, K_2HPO_4 0.766 g, KH_2PO_4 0.3 g, trace element 1 mL and BTB regent (1% in alcohol) 1 mL. The trace element (per liter) was composed of EDTA 99.922 g, $ZnSO_4$•$7H_2O$ 4.4 g, $CaCl_2$•$2H_2O$ 16.361 g, $MnCl_2$•$4H_2O$ 10.118 g, $FeSO_4$•$7H_2O$ 9.98 g, $Na_2Mo_7O_{24}$•$2H_2O$ 3.073 g, $CuSO_4$•$5H_2O$ 3 g and $CoCl_2$•$6H_2O$ 3.213 g. The SSM was the SW without adding agar and BTB. LB (Luria-Bertani) medium containing 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract and 10 g L^{-1} of NaCl was used for seed preparation and culture preservation. All of the media were sterilized at 121°C for 20 min. BTB regent was added after sterilization.

2.2. Bacteria isolation and identification

The sources for aerobic denitrifier isolation were water samples from intensive aquaculture ponds (Dagang district, Tianjin, China). Samples (at a depth of 0.4 m) were collected with 1 L sterile bottles from different pools. 50 mL samples and 100 mL EM were transferred into 500 mL Erlenmeyer flasks and shaken at 150 rpm, 30°C for 3d to enrich denitrifying microflora. 5mL culture was taken into a test tube and incubated at 90°C for 10 min to kill non-spore bacteria. Afterwards, 0.5 mL of above suspension was transformed into 4.5 mL EM and incubated at 30°C for 2d. The enrichment of the culture was incubated at 90°C for 10 min again and performed gradient dilution. 0.1 mL diluted solution was placed on solid BTB and incubated at 30°C for 2d. Blue cloudy colonies were isolated and purified further. The purifying was inoculated into flask with SSM and incubated at 30°C, 150 rpm for 7d to test nitrite removal ability for second screening.

Genomic DNA of the bacterium was extracted using Wizard genomic DNA extraction kit (Solarbio, Beijing, China). The 16S rRNA gene was amplified using two primers: 27-F and 1429-R. Polymerase chain reaction (PCR) conditions were set as follows: 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 58°C, 1.5 min at 72°C and 10 min at 72°C for initial denaturalization, denaturalization, annealing, extension and final extension respectively. The PCR products were visualized on 0.8% agarose gel electrophoresis using Goldview as stain, and then purified and sequenced with both the forward and the reverse primers. The initial nearest neighbor sequences was made by the online BLAST program to the NCBI database. Sequences were aligned using the Clustal-X program, and phylogenetic tree was constructed with MEGA 5.0 program.

2.3. Amplification of denitrification related genes

Periplasmic nitrate reductase encoding gene *napA* and nitrite reductase encoding gene *nirK* (or *nirS*) were checked in the isolated bacterium for confirming the aerobic denitrification further. The *napA* was amplified with the napAF/napAR primers according to the condition described (Kong et al., 2006). The PCR condition was set up at 94°C for 5 min, 35 cycles of 94°C for 30s, 59°C for 30 s and 72°C for 1 min followed by the final extension at 72°C for 5 min. The nirK1F/nirK5R and nirS1F/nirS6R were separately used to amplify *nirK* and *nirS* conducted as described (Braker et al., 1998). The PCR condition was same as that for *napA* in addition to the annealing temperature was 50°C. All the PCR products were run on 0.8% agarose gel and sequenced further.

2.4. Nitrite removal ability assay

Nitrite removal capacity was separately analyzed under different pH, temperature and nitrite quantities. In the pH influence test, exponential phase bacterial seeds (OD_{600} = 6, approximately 0.36 g dry cells weight per liter medium) (10%, v/v) were added into 200 mL of synthetic wastewater (SW) with various pH (5.0, 6.0, 6.5, 7.0, 8.0, 9.0, 11.0 and 12.0). Similar to the above test, bacterial seed (10%, v/v) was incubated at different temperature (15, 20, 25, 30, 35, 40 and 45 °C) in the temperature influence test. In the NO_2^--N quantities influence test, initial NO_2^--N concentration was adjusted to 65, 90, 150, 265, 340, 465 and 560 mg L^{-1}. For the above tests, the exponential phase bacterial seeds were centrifugated (6,000 × g, 10 min) at room temperature for triplicate and resuspended with the SW before inoculation, and the original incubation parameter was set at 30°C, 150 rpm and pH 7.0. All the experiments were carried out for more than 5 days.

2.5. Nitrite removal kinetic assay

The kinetics model used in this study is based on the experimental results which showed no accumulation of NH_4^+-N during denitrification. The reaction rate constant k and saturation constant K_m are derived based on Michaelis-Menten kinetics relationship as seen in Eq. (1). Similar derivation where also reported elsewhere (Aslan and Kapdan, 2006).

$$Rx_i = \frac{R_{max} S}{Km + S} \tag{1}$$

where R_{max} is the maximum substrate removal rate and S is the substrate concentration. The initial substrate concentrations at time "t" and the corresponding substrate removal rates (Rx_i) are considered in batch system. Therefore, the Eq. (1) takes the following form :

$$Rs_t = \frac{Rm_t S_t}{Km + S_t} \tag{2}$$

where $Rm_t = k \cdot X_t$ is the maximum substrate removal rate, S_t is the corresponding substrate concentration. Eq. (2) can then be written as:

$$Rs_t = \frac{kX_t S_t}{Km + S_t} \tag{3}$$

where k is the reaction rate constant and X_t is the biomass at time "t". Dividing both terms of Eq. (3) by X_t gives the specific substrate removal rate (μ_t).

$$\mu_t = \frac{Rs_t}{X_t} = \frac{kS_t}{Km + S_t} \tag{4}$$

Eq. (4) can be linearized to form Eq. (5).

$$\frac{S_t}{\mu_t} = \frac{Km}{k} + \frac{S_t}{k} \tag{5}$$

In batch kinetics, the substrate removal rates (Rs_t) is determined as the slope of time versus nitrogen concentration at time "t" as described