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Exploration of three heterotrophic nitrifying strains from a tilapia pond for their characteristics of inorganic nitrogen use and application in aquaculture water

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Three heterotrophic nitrifying bacterial strains, HLf01, HBf01and HHf01, were isolated from a pond where genetically improved farmed tilapia (GIFT) (*Oreochromis niloticus*) was intensively cultured during the annual peak breeding period. Analysis of biochemical, morphological characteristics and sequences analysis of 16S rDNA identified strains HLf01 and HBf01 as *Pseudomonas* sp., and strain HHf01 as *Acinetobacter baumannii*. Further analysis of heterotrophic nitrifying medium inorganic nitrogen used showed that most of the ammonia nitrogen was removed after 48 h. Strains HBf01and HHf01 removed 67.9% and 76.7% of the total ammonia nitrogen after 24 h, recording the mass ratio of carbon to nitrogen used as 10.6 to 1 and 11.7 to 1, respectively. Strain HLf01 used a C:N ratio of 22.7 to 1 with a total of 43.8% ammonia nitrogen removed. In denitrifying media, strains HBf01 and HHf01 may have used nitrate and nitrite as the sole nitrogen source by strain HLf01. Generally, all the three strains used nitrogen in the order ammonia > nitrite > nitrate when all the three nitrogen forms were recorded present. Although the bacterial strains consumed dissolved organic carbon, it was limited when ammonia was the main source of nitrogen compared with other forms of inorganic nitrogen. Also, the test of the three strains on aquaculture water showed similar results of efficiency of ammonia nitrogen removal with the test of inorganic nitrogen use.

[**Key words:** Heterotrophic nitrifying bacteria; Ammonia nitrogen; Carbon/nitrogen ratio; Dissolved organic carbon; Nitrogen removal]

Nitrogen is one of the major causes of water pollution and eutrophication (1), and thus, removal of nitrogen from water plays a significant role in remediation (2,3). In intensively cultured fish ponds, bait and stool are the main sources of nitrogen in the water. Various studies have shown that the nitrogen conversion efficiency of the transformation process from bait to fish is 20%–50%, signifying that 50%–80% of the nitrogen in the bait is released into breeding ponds (4). This nitrogen is often in excess of that needed to meet the requirements of fish farming, and thus leads to eutrophication where inorganic components such as ammonia and nitrite may be toxic to living organisms.

A number of studies have investigated whether microbial functions could be enhanced to strengthen the internal conversion of nitrogen, for example, by using photosynthetic bacteria (5), bacillus (6) and other probiotics, thereby reducing the pollution load and toxic effects of nitrogen. Screening and studying of more efficient microbes at removing nitrogen has become an important quest in the development of biological nitrogen removal technologies. Heterotrophic nitrifying bacteria, with their fast growth, low dissolved oxygen requirements and tolerance of acidic environments, have shown themselves to be more applicable in environmental remediation process (7–9), and are of increasing interest as a potential tool for biological nitrogen removal (10,11). Some heterotrophic nitrifying bacteria also have aerobic denitrification functions, such as sulfur bacteria (*Thiosphaera pantotropha*) (12).

We isolated three strains of heterotrophic nitrifying bacteria (HLf01, HBf01 and HHf01) from the genetically improved farmed tilapia (GIFT) pond during its peak breeding phase, examined their characteristics of growth and inorganic nitrogen use, especially their efficiency of ammonia removal, and then tested their control effects on aquaculture water under laboratory conditions.

MATERIALS AND METHODS

Preparation of media Heterotrophic nitrification enrichment medium and heterotrophic nitrification medium was formulated according to the studies of Zhang et al. (13) with appropriate adjustments in, nitrate, nitrite and inorganic media preparations. The enrichment medium contained $(NH_4)_2SO_4$ (0.50 g/*l*), sodium succinate (2.17 g/*l*), and Vickers salt solution (50 ml/*l*). As for the Vickers salt solution, it is composed of $K_2HPO_4 \cdot 3H_2O$ (6.50 g/*l*), $MgSO_4 \cdot 7H_2O$ (2.50 g/*l*), NaCl (2.50 g/*l*), $FeSO_4 \cdot H_2O$ (0.05 g/*l*) and $MnSO_4 H_2O$ (0.04 g/*l*). The heterotrophic nitrification medium was similar to the enrichment medium, however the concentration of $(NH_4)_2SO_4$ was changed to 0.24 g/*l* for the solid medium of the heterotrophic nitrification medium, plus 2% agar. The differences of nitrate medium and nitrite medium with heterotrophic nitrification medium was that the nitrogen sources were changed to KNO_3 (0.36 g/*l* in nitrate medium) and KNO_2 (0.304 g/*l* in nitrite medium) respectively. Similarly, in inorganic medium, the nitrogen sources contained $(NH_4)_2SO_4$ (0.08 g/*l*), KNO_3 (0.12 g/*l*) and KNO_2 (0.10 g/*l*).

Once the media were prepared, they were sterilized in a TOMY SS-325 automatic autoclave at 121°C for 20 min. Test ponds of the Freshwater Fisheries Research

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Center of Chinese Academy of Fishery Sciences located in the Southern District No. GF01 with a spanned area of 1330 square meters and an average depth of 1.8 m were stocked on the 8th day of May 2013 with about 2300 fries (*Oreochromis niloticus*) having an average weight of approximately 100 g for the grow out stage. On the 29th day of July 2013, during the peak breeding phase of tilapia, water samples were collected using plexiglass mining water samplers for bacteria Isolation and water quality determination.

Preparation of water samples Water samples (1 ml) were collected and transferred by pipette to a sterilized 250-ml flask, to which 100 ml enrichment medium was added. The flask was placed in a constant-temperature shaker-incubator at 30°C and shaken for 24 h at 150 rpm. After natural sedimentation, the supernatant (5 ml) was added to another 250-ml flask along with 100 ml bloom enrichment medium and shaken continuously for 24 h. The supernatant was diluted by three different orders of magnitude (10^{-5}, 10^{-6}, and 10^{-7}), coated on heterotrophic nitrification medium agar plates and placed in an incubator at 30°C. The colonies grew out after 24 h, with colony characteristics emerging within 48–72 h. Three dominant heterotrophic nitrifying bacteria could initially be isolated according to the colony characteristics. Single colonies were picked and crossed on the plate, repeating the scribing step twice to obtain three types of characteristic uniform flat colonies after the colonies grew out. The three types of colonies were inoculated into the slant and cultivated at 30°C until the moss grew out and was named as HLf01, HBf01 and HHf01, respectively.

Measurement of some parameters According to the reference (14), a DIONEX ICS 3000 Ion Chromatograph was used to measure nitrate and nitrite; Nessler colorimetric assay was used to measure ammonia; The oxidation method was used to determine total organic carbon (TOC), using a GE Sievers InnovOx Laboratory TOC Analyzer. Dissolved organic carbon (DOC) was taken to be the TOC of water samples filtered through a 0.45-μm membrane. The test water in the experiment was treated with the Millipore Synergy ultra pure water instrument.

Identification of strains The single colony morphology corresponding to HLf01, HBf01 and HHf01 were observed through a magnifying glass, and the single colony characteristics were recorded. Three glass slides coated with corresponding gram-stained strains were observed under a Nikon Eclipse 90i Optical Microscope after Gram staining, and the strain characteristics were recorded and then photographed.

Biochemical analyses were performed using a bacterial biochemical tube (Hangzhou Tianhe Microorganism Reagent Ltd., China). Some biochemical parameters of HLf01, HBf01 and HHf01 were determined using these bacterial micro tubes. They are the metabolism capacity of amylase, sucrose, xylose, arabinose, glucose, hydrogen sulfide, urea, phenylalanine and citrate salt. And the VP-test was also performed.

The morphological identification and biochemical parameters were analyzed according to the reference (15).

For Analysis of 16S rDNA homology, UNIQ-10 column bacterial genomic DNA extraction kit (Shanghai Biological Engineering) was used to extract total genomic DNA of bacteria. Generating genomic DNA of the three strains processed by PCR amplification, for which, one pair of universal primers was used. The forward primer was 7F:5′-CAGAGTTTGATCCTGGCT-3′, and the reverse primer was 1540R:5′-AGGAGGTGATCCAGCCGCA-3′. The reaction volume was 25 μl, and the cycling conditions were as follows: 98°C for 3 min; 30 cycles of 98°C for 25 s, 55°C for 25 s, 72°C for 1 min; and then 72°C for 10 min. After the product was purified, Takara pMD18-T Vector Ligation Kit was used to link the T vector, after which it was transformed into the competent cells using calcium chloride. Following blue-white screening, the UNIQ-10 column plasmid extraction kit was used to extract the plasmid DNA, then sent to the Shanghai Biological Engineering Co. Ltd., to measure the sequence which used M13 ± primers: M13+(−47), AGGGTTTTCCCAGTCACG and M13−(−48), GAGCGGATAACAATTTCACAC. The sequencing results were submitted to GenBank and subjected to BLAST analysis. Several reference strains were chosen, and the phylogenetic tree was constructed based on the adjacent method using MEGA5.0 software system. The dendrogram was constructed using the neighbor-joining method and construction was confirmed by the maximum likelihood (16) and unweighted pair group method with arithmetic mean (UPGMA) methods (17) Confidence in the tree topology was determined by bootstrap analysis using 1000 resamplings of the sequences (18).

Determination of bacterial growth and use of inorganic nitrogen and DOC The three strains were separately inoculated in 250-ml flasks containing 100 ml heterotrophic nitrification medium. Then the flasks were placed in a shaking incubator at 30°C and 150 rpm. Cultivated bacterial broth (5 ml) in which the strains were in logarithmic growth phase was centrifuged at 10,000 rpm for 5 min by using a BBI HC-2518R frozen high-speed centrifuge. The supernatant was discarded, and the sterile ultrapure water was added into the centrifuge tubes to adjust the concentration of the organisms to the level of about 1×10^9 CFU ml^{-1}.

To establish growth and nitrogen use when ammonia was the only nitrogen source, 1 ml of each of the above three broths was separately inoculated in 500-ml flasks containing 300 ml heterotrophic nitrification medium and placed in a shaking incubator at 30°C and 150 rpm. Absorbance (OD 600) was measured at 6, 24, 30, 48, and 54 h after incubation; from 24 h onwards, changes in ammonia were measured by spectrophotometry simultaneously with nitrate and nitrite changes by ion chromatography. The TOC analyzer was used to determine changes in DOC.

For samples where nitrate or nitrite was the only nitrogen source, the medium was changed to a nitrate or nitrite medium, respectively, with the remainder of the procedure performed as previously.

For samples containing inorganic nitrogen, i.e., ammonia, nitrate and nitrite, the medium was changed to an inorganic medium with the remainder of the procedure performed as previously.

Application of the strains on the control of inorganic nitrogen in aquaculture water 12 round glass aquarium tanks (containers) with the diameter of 0.25 m and a height of 0.2 m were used. Each aquarium tank was filled with 5 L of sampled water obtained from the pond where the three strains were isolated and stocked with 5 fish (*O. niloticus*) having an average weight of 305.6 g. The aquariums were classified into 4 groups (one group was identified as the control group and the other 3 groups had the 3 distinguished strains). One small aeration pump with 12 airflow outlets was used to maintain the dissolved oxygen levels in each aquarium tank (the results of four random measurements during the period of the 10 days were all greater than 5 mg l^{-1}). Fish were cultured in the aquariums for 10 days, and were fed to apparent satiation once a day (about 3:30 PM). Three forms of inorganic nitrogen in each aquarium were determined at the time of 0, 2, 4, 6, 8, 9 and 10 days of the culture experiment. And at the time of the 8th day, the concentrations of DOC in each aquarium were determined; they were (8.31 ± 0.27) mg l^{-1} ($p > 0.05$). And then, 0.6 g sodium succinate was added into each aquarium, three of which were designated as the control group, and the other three groups in triplicate were classified as the treated groups that 2 ml broths of the three strains (as mentioned above) were added respectively.

RESULTS

Morphological identification of the three strains The HLf01 was observed to have a light blue, circular and moist colony, with a diameter of about 5 mm. The HBf01 had a white, circular, and wet colony with an intermediate projection, its diameter was about 3 mm, and the HHf01 had a light yellow, round, slightly moist colony with an intermediate bulge, the diameter about 2 mm or the strain characteristics, the HLf01 and the HHf01 strains were all rod-shaped, with length ranging between 0.2 and 0.5 μm for HLf01, and 0.1–0.5 μm for HHf01 whereas the HBf01 strain was globular, 0.15–0.2 μm in diameter (Fig. 1).

Biochemical parameters In the experiment, using bacterial micro tubes, the biochemical parameter tests for HLf01, HBf01 and HHf01 metabolism capacity plus the performed V. P test as per the methodology recorded positive effects in xylose for HBf01 and HHf01, in glucose for HLf01 and HBf01, in urea for the three strains, and in citrate salt for HLf01 and HHf01. The rest of the other biochemical reactions recorded negative effects.

Analysis of 16S rDNA homology for the three strains The sequences obtained with accession numbers KJ646021 for HLf01, KJ646022 for HBf01, and KJ646023 for HHf01were submitted to GenBank for BLAST analysis. Some strains were chosen as reference ones, among which the genus *Azomonas* and *Moraxella* were selected as two out groups belonging to the families *Pseudomonadaceae* and *Moraxellaceae*, respectively. The strains HLf01 and HHf01 showed high phylogenetic similarities with some strains belonging to the genus of *Pseudomonas* (*Pseudomonadaceae* family) (99% similarity), while the strain HBf01 appeared to show high similarity with the genus of *Acinetobacter* (*Moraxellaceae* family) (99% similarity) (Fig. 2).

Observed results indicated that strains HLf01 and HHf01 were *Pseudomonas* sp. and strain HBf01 was *Acinetobacter baumannii*.

Growth of the three strains with different sources of inorganic nitrogen With ammonia as the only source of nitrogen, the three strains showed a similar growth pattern; this was also the case when all three inorganic forms of nitrogen were present, however with nitrate or nitrite as the only nitrogen source, strain HLf01 was unable to grow (Fig. 3).

Use of inorganic nitrogen and DOC by the three strains Fig. 4a indicates that with ammonia as the only nitrogen source, the removal rate of ammonia by HLf01 was 43.84% within 24 h, while the removal rate of DOC was 83.51%.