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Original Research Article

Diversity and Abundance of Ammonia-Oxidizing Bacteria and Archaea in a Freshwater Recirculating Aquaculture System

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ABSTRACT

The role of ammonia-oxidizing bacteria and archaea was evaluated using *amoA* gene in a freshwater recirculating system. Broken earthen pot pieces (BEP) were used as filter bed material. Five archaeal and four bacterial operational taxonomic units were retrieved from *amoA* genes. Shannon-Weiner and Simpson indices were higher in archaeal *amoA* sequence compared with the bacteria. Subtype diversity ratio and subtype diversity variance were 0.522 and 0.008, respectively, for archaea and 0.403 and 0.015, respectively, for bacteria. In archaea, 50% *amoA* sequences showed 99%–100% similarity with the known sequences of ammonia monooxygenase subunit A of uncultured archaeon clones and thaumarchaeote. In bacteria, 84% sequences showed 99% similarity with *amoA* sequences of different uncultured bacterial clone and Nitrosomonadaceae. Absolute quantification showed that the abundance of archaea was 12-fold higher compared with bacteria. In this recirculating system, ammonia-oxidizing archaea and bacteria played a major role; BEP supported the growth of these ammonia-oxidizing microorganisms.

1. Introduction

Recirculating aquaculture system is based on the reutilization of waste water involving microorganisms as a tool that reduces the nitrogenous wastes in the culture system. In intensive aquaculture, the main problem is the accumulation of toxic nitrogenous metabolites derived from excess, uneaten feed and excreta. Removal of these nitrogen metabolites is carried out by the process of biofiltration with the help of ammonia-oxidizing microbes. Furthermore, the treatment is completed by nitrite-oxidizing bacteria; denitrifying bacteria releases the free nitrogen into the atmosphere, else absorbed by the plants as nutrients. It is a great challenge for the aquaculturists to remove these toxic metabolites from the culture systems. Ammonia-oxidizing bacteria (AOB), two narrow clades of beta- and gamma-proteobacteria are considered to be the only members that play major role in the global nitrogen cycle (Prosser 1989; Prosser and Nicol 2008). The molecular study shows that members of the Crenarchaeota (within the archaeal domain) also play an important role in nitrification (Hansel *et al.* 2008; Könneke *et al.* 2005; Tourna *et al.* 2008). *Nitrosopumilus maritimus*, a strain of marine ammonia-oxidizing archaea (AOA), has been isolated from nitrifying filtration unit of the Shedd Aquarium (Könneke *et al.* 2005). AOA play an important role in nitrification and their *amoA* genes serve as marker for diversity and abundance (Pester *et al.* 2012). The *amoA* gene is a function-specific target for the detection of AOB (Kowalchuk and Stephen 2001).

The success of recirculating system is determined by stable and reliable performance of its biofilter. The maintenance of biofilter is most important for a start-up recirculating system as well as for a functional one (Badiola *et al.* 2012). The selection of proper substrate for biofilter is the next critical step as it influences the efficiency of water treatment and operational cost (Summerfelt 2006). A perfect biofilter should be easily available, non-poisonous, non-reactive and cost-effective. It should remove all the nitrogenous metabolites from the effluent and support the growth of dense populations of nitrifying microbes. Study shows that no biofilter has all these characteristics; each has its own advantages and disadvantages (Michaud 2007; Rusten *et al.* 2006). Various biological filters like trickling filters, rotating biological contactors, granular substrate biofilters, submerged fixed substrate biofilters (static bed), mobile substrate biofilters (moving bed) are used in recirculating system. The static bed filter has been used in the present study. This filter supports excellent volumes of water with good purification rate. A wide variety of substrates viz. rocks, shells, sand, corals, ceramic, expanded clay, plastic bio balls, etc. are commercially available as biofilter substrates (Malone and Pfeiffer, 2006).

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Replacement of such commercial products with traditional materials will significantly reduce the operational cost of the recirculating system. In the present study, broken earthen pot pieces (BEP) are used as filter bed material. The capability of these pieces to support the growth of ammonia-oxidizing microbes has been evaluated using *amoA* gene as marker.

2. Materials and Methods

2.1. Experimental set-up

The recirculating system consisted of three rectangular fish culture tanks (500 L, each) and a filtration unit (600 L). This recirculating system was kept inside the Aquahouse and the temperature was uniformly maintained inside this house. In the filtration unit, a 20-cm thick layer of BEP (7 × 3.5 cm) were used as substratum for the growth of microorganisms. These BEP are easily available, indigenous, cheaper and non-toxic to fish. These are porous in nature with large surface area which facilitates the growth of microbes. Indian major carp catla *Catla catla* fingerlings (55 ± 5 g) were cultured in this recirculating system. The stocking density was 20 fish/tank. The duration of circulation was 6 h/d. The water temperature in the filter bed was 25°C ± 1°C during the study period. Dissolved oxygen was monitored using portable oxygen meter (Intellical LDO101, Hach, USA) and the level was maintained at 5 mg/L with the help of aerator. The water quality of the fish culture tanks after treatment in the filtration unit were as follows: temperature 25.2°C ± 1°C, dissolved oxygen 6.17 ± 0.20 mg/L. pH was measured with probe (Intellical PHC101) and ranged from 7.8 to 8.25. Ammonia, nitrite and nitrate levels were 0.15 ± 0.01, 0.55 ± 0.02 and 0.11 ± 0.04 mg/L, respectively, during the culture period. Ammonia was measured with Orion Ion Analyzer (Thermo Scientific, Massachusetts, USA). Nitrite and nitrate were analysed following the standard methods (APHA 1998). The whole experiment was conducted following the guidelines of the Institutional Animal Ethics Committee (565/GO/ReBi/S/02/CPCSEA).

2.2. Batch activity test

Twelve pieces of BEP were collected from the filter bed of the recirculating system and were equally distributed in three beakers (1 L) containing 500 mL of synthetic wastewater (Table 1). The synthetic wastewater was prepared following the method of Munz *et al.* (2011). The beakers were partially closed with aluminium foil. Water sample from each beaker was collected daily and ammonia, nitrite and nitrate levels were measured. The rate of change in concentration of nitrogenous materials confirmed the nitrification process.

2.3. DNA extraction, quantification of DNA and PCR amplification

For the recovery of microbial cells, the BEP were collected from the filter bed. The outer layer of broken earthen pot was scrapped, ground to powder and used for DNA extraction. DNA was isolated from 10 g of BEP powder using PowerMax DNA Isolation Kit (MO BIO Inc., Carlsbad, CA, USA) with slight modifications as per the manufacturer's recommendations. A 1.2 mL of the solution containing sodium dodecyl sulfate (SDS) and other disrupting agents were added to the tube and vortex for 30 s to lyses the cell membrane by breaking down the associated fatty acids and lipids. These tubes were placed to the half-filled plastic bottles and kept at the incubator shaker, shaking at the maximum speed (300 rpm) for 45 min at 60°C and the rest of the protocol was followed as per the kit's manual.

Table 1. Composition of synthetic wastewater used for the batch activity study

Composition	Quantity (mg/L)
Beef extract	90
Yeast extract	90
$MnSO_4$	1.22
$FeSo_4$	10.1
KCl	3.125
K_2HPO_4	87.6
$NaHCO_3$	163.5
$CaCl_2$	1055
$MgSO_4$	10.88
NH_4Cl	1055

The isolated DNA was checked through 0.8% agarose gel. The concentration was measured using NanoDrop 1000 (Thermo Scientific). Amplification of DNA was performed with peqSTAR 2X Double block thermocycler, peqlab, using the following conditions. A total of 50 μL reaction mixture was used with 25 μL 2X Master Mix (Thermo Scientific Lithuania, California, USA), 25 ng DNA templates, 2.5 μL forward (FW) and 2.5 μL reverse (RV) primers of bacterial *amoA*—1F: 5′GGGGTTTCTACTGGTGGT 3′and 2R: 3′CCCCTCKGSAAAGCCTTCTTC 5′ (Rotthauwe *et al.* 1997) and archaeal *amoA* A—5′ STAATGGTCTGGCTTAGACG 3′ and R: 3′ GCGGCCATCCATCTGTATGT 5′ (Francis *et al.* 2005). The rest volume was made up with nuclease free water. The amplification programmes of archaeal *amoA* was 95°C for 4 min; 30 cycles consisting of 94°C for 45 s, 53°C for 45 s and 72°C for 60 s; and 72°C for 15 min. The amplification programme of bacterial *amoA* was little modified from the method of Rotthauwe *et al.* (1997). Initial denaturation was at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s and extension at 72°C for 30 s; final extension was at 72°C for 10 min. All polymerase chain reaction (PCR) results were confirmed with 1.2% (w/v) agarose gel electrophoresis.

2.3.1. Cloning, sequencing and phylogenetic analysis

The triplicate PCR products were pooled and purified using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The purified product was cloned to construct library using PBase-TA Cloning Kit (The Gemini, Singapore). The recombinant plasmids were transformed and positive colonies were randomly selected and sequenced (ABI PRISM 3730xl Genetic Analyzer, Applied Biosystems, Foster City, USA) with the help of 1st BASE (The Gemini, Singapore). The raw sequences obtained from the sequencer were screened for vector contamination using NCBI's VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen/VecScreen.htmL). Whereas, operational taxonomic units (OTUs) were extracted by clustering sequences, using CD-HIT Suite (http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit-est). Phylogenetic analysis was performed by aligning the sequences with *Clustal X* (version 2.1) (Conway Institute, University College Dublin, Ireland, UK), and the neighbour-joining tree was constructed (Jukes and Cantor 1969) with the reference sequences of known/previously reported sequences of archaeal and bacterial *amoA* and clone sequences derived from BEP biofilter using MEGA 7 (The Pennsylvania State University, Philadelphia, USA) (Pester *et al.* 2012; Purkhold *et al.* 2000; Zhou *et al.* 2016). The ecological indices like Shannon-Weiner index (H′; Shannon 1948) and Simpson index (D; Simpson 1949) were calculated using the number of OTUs.

The *amoA* gene sequences were submitted to the NCBI. These are the accession numbers: KP272121—KP272128 and KP259843—KP259869.

2.4. Absolute quantification of *amoA* genes

Copy numbers of archaeal *amoA* and bacterial *amoA* genes were determined in triplicate for both samples by quantitative real-time PCR (ViiA 7 Real-Time PCR System, Applied Biosystems). The assay was performed in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystem, USA) with optical adhesive cover. New sets of primers of archaeal *amoA* (F: 5CATCCTAGAGCGGCAAAGGT3′ and R: