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Isolation and characterization of *Pseudomonas stutzeri* and its potential application in biological nitrification

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Abstract

Ammonia-oxidizing bacteria (AOB) were isolated from sediment samples of fishponds with an aim to use them for application in biological nitrification of water. Isolation of AOB was done in an inorganic medium and nitrite-producing bacterial isolates were selected. These isolates were further screened by polymerase chain reaction using specific primers for AOB. Out of 119 nitrate positive isolates, only 12 showed positive amplification and yielded a PCR product of ~465bp. Treatment of aquaculture pond and river water with one of the bacterial isolate (HC-5) resulted in lowering of soluble ammonia level from 3.50 to 0.05 mgl^{-1} and 7.5 to 0.01 mgl^{-1}, respectively. Partial 16S rRNA gene sequencing of isolate HC-5 identified the microorganism as *Pseudomonas stutzeri*.

Key words

Ammonia-oxidizing bacteria, Aquaculture ponds, Biological nitrification, *Pseudomonas stutzeri*

Introduction

Aquaculture is a rapidly growing food-producing sector that has registered an average growth rate of 8.9% per year since 1970, as compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat-production systems (FAO, 2006). With the rapid development of aquaculture and continuous employment of intensive management, water is being polluted severely in culture ponds. It is estimated that approximately 25-30% of nitrogen applied in feeds is transformed in the flesh of the cultured fish while the rest is converted into NH_3 that enters the aquatic ecosystem (Boyd, 1990; Boyd and Tucker, 1995). The nitrogenous compounds generally get accumulated due to uneaten feed, faces and death of planktons and may be toxic to aquatic animals and could lead to eutrophication of water bodies (Ying *et al*., 2008). Nitrification, the oxidation of ammonia to nitrate via nitrite, is central to the cycling of nitrogen in the environment and, when coupled with denitrification, alleviates eutrophication through removal of nitrogen to the atmosphere as nitrous oxide or dinitrogen (N_2) gas. Therefore, nitrifiers such as ammonium-oxidizing and nitrite-oxidizing bacteria (AOB and NOB) play an important role in the management of water quality by oxidizing ammonium to nitrite and nitrate, respectively (Jun and Tongbing, 2000). Chemoautotrophic bacteria are typically identified as the main agents of nitrification, but many heterotrophic bacteria and fungi can also act as nitrifiers (De Boer and Kowalchuk, 2001). Ammonia from organic wastewaters from various sources like food processing industries, aquaculture, domestic wastes *etc* can be removed by a variety of physico-chemical and biological processes (Metcalf and Eddy, 2002) but biological processes are preferred because they are usually more cost-effective (USEPA, 1993). Removal of nitrogen from wastewater is possible by combination of nitrification and denitrification. *P. stutzeri* is one of the bacteria involved both in nitrification and denitrification processes as well as degradation of environmental pollutants. Wastewater treated with *P. stutzeri* strains has been used successfully for aquaculture in many developed countries (Su *et al*., 1997, 2001; Diep *et al*., 2009). *P. stutzeri* is heterotrophic nitrifying- as well as denitrifying-bacteria (Papen and von Berg,1998; Matsuzaka *et al*., 2003; Joo *et al*., 2005; Wen *et al*., 2010) which is present in different natural environments. An attempt was made to isolate the nitrifying bacteria and to evaluate

its role in removal of nitrogenous wastes from different freshwater aquatic ecosystems.

Materials and Methods

Samples : Fishpond sediment sampling was carried out from five ponds. Size of the fishponds varied from 0.10-0.35 ha with a stocking density of Indian major carps of 600-2100 fingerling/ha. The fish were fed with supplementary diet of rice bran and mahua oil-cake @ 2-4% of body weight.

Isolation of AOB : Isolation of AOB was done in inorganic medium containing $(NH_4)_2SO_4$ 0.6 g; KH_2PO_4 0-2 g; $CaC1_2.2H_2O$ 0.04 g; $MgS0_4.7H_2O$ 0.04 g; Fe (as ferric citrate or Fe-EDTA chelate) 0-5 mg and phenol red 0.5 mg in 1 l distilled water (Soriano and Walker, 1968; Kumari *et al*., 2011). After sterilizing at 120°C for 15 minutes, pH of the medium was adjusted to 7.5-8.0 by adding a sterile aqueous 5% sodium carbonate solution. One gram of sediment sample from fishpond was added to100 ml liquid medium in 250 ml Erlenmeyer flasks and the mixture was incubated at 25ºC for at least 10 days or until nitrite was produced. Production of nitrite was checked by addition of 1 ml Greiss reagent to 5 ml incubated culture media. Appearance of red colour indicated formation of nitrite. From the positive samples, sub-cultures were performed five times at weekly intervals by adding 5 ml of culture broth to fresh inorganic medium. Cultures were diluted in normal saline solution before spreading on inorganic medium solidified with 1.5% agar. The plates were incubated at 30ºC until colonies appeared. Representative colonies from individual plates were randomly selected and purified for further characterization.

16S rRNA amplification and screening of AOB by nested PCR : Bacterial 16S rRNA of the selected isolates were amplified by PCR using the combination of universal bacterial primer 27F and universal primer 1492R. The PCR reaction was performed in a thermal cycler with the following programme preheating at 94ºC for 5 minutes, and 29 cycles of 94ºC for 1 min, 52ºC for 30s, 72ºC for 2 min and a final extension of at 72ºC for 10 min. For screening of AOB, the first PCR product was re-amplified with primers specific for AOB, namely CTO189F and CTO654R. The PCR programme was the same as described by Kowalchuk *et al.* (1997) and PCR reaction products were run and visualized on 1.5% agarose gel electrophoresis.

Potential application of selected AOB for biological nitrification : Visually clear surface water samples, collected from fishpond and river Gomti, were used to assess the efficacy of PCR positive AOB isolates for reduction of total soluble nitrogen. The isolates were individually inoculated using 4% inoculum from 5 day - old broth culture into separate conical flasks containing 5 l river as well as 5 l pond water and incubated at room temperature. The control flasks containing only river and pond water (without bacterial culture) were also simultaneously incubated. Water quality parameters (ammonia, nitrite and nitrate) were recorded at regular intervals for experimental and control flasks. All the experiments were carried out in completely randomized design with 2 replications. All the statistical analysis was carried out using SPSS version 10.0 for Windows.

Ammonia-nitrogen (NH_3-N) : Concentration of ammonia in the samples was estimated by phenate method. Accordingly, 25 ml of each water sample was added to 50 ml volumetric flask followed by 1 ml phenol alcohol solution in the sample. Thereafter, 1 ml sodium nitroprusside and 2.5 ml oxidizing solution was added to the flask and shaken. The flask was covered with paraffin film and colour was allowed to develop for 30 min before measuring absorbance at 640 nm. A series of standard solutions covering the concentrations of 1000, 100, 10, 1 and 0.1 mg NH_3-N l^{-1} were prepared by making decimal dilutions of stock NH_4Cl solution (3.819 g l^{-1}) with water and processed as above. The standard curve was made by plotting absorbance readings of standards against NH_3-N concentrations of standards. The mg NH_3-N of unknown sample was read from the standard curve and the value was divided by volume in liter to obtain the concentration in mg NH_3-N l^{-1}.

Nitrite-nitrogen (NO_2-N) : Concentration of NO_2-N in water samples was measured by standard colorimetric method. 2 ml each of sulfanilamide solution (1%) and N-(1-Naphthyl)-ethylene-diamine-dihydrochloride solution (0.1%) were added to 40 ml of sample in a 50 ml volumetric flask and filled to the mark with deionized water. After 10 min of incubation at room temperature, the absorbance was measured at 540 nm. The standard curve of sodium nitrite-N ranging from 1 to 50 µg was used to estimate the amount of NO_2-N in the water samples. The absorbance values of samples were read from the standard curve and extrapolated to obtain the concentration in mg NO_2-N l^{-1}.

Nitrate-nitrogen (NO_3-N) : Concentration of NO_3-N in water samples was measured by hydrazine reduction method (Clesceri *et al*., 1998). The standard curve of sodium nitrite-N, ranging from 1 to 10 mgl^{-1}, was used to estimate the amount of NO_3-N in the water samples. The absorbance values of samples were read from the standard curve and extrapolated to obtain the concentration in mg NO_3-N l^{-1}.

Molecular identification of bacteria by 16S rRNA sequencing: Most promising AOB isolate (HC-5) showing potential of biological nitrification was identified by 16S rRNA gene sequencing. Bacterial 16S rRNA gene was amplified by PCR using the combination of universal bacterial primer 27F and universal primer 1492R. The amplified products (~1500 bps) were excised from 1% agarose gel and eluted with the gel elution kit. The eluted PCR product was sequenced using 27F as sequencing primer on an automated ABI sequencer. The resulting consensus sequences (~500 bp) were compared with those available in RDP 10 database by use of the BLAST programme to

determine 16S rRNA gene sequence similarities with its nearest neighbours.

Results and Discussion

Isolation and identification of bacteria from freshwater fishpond : A total of 54 fishpond water samples were processed for isolation of nitrifiers in three different seasons (summer, rainy and winter) of the year. Out of these, 33 samples (61.1%) were positive for production of nitrite. From these positive samples, 119 bacterial isolates were selected on basis of colony morphology which were round-shaped, motile, short-rod and gram-negative. Of the total 119 isolates, only 12 isolates (10.08%) showed specific PCR amplification (~465-bp) with CTO189F and CTO654R primers and were presumed to be ammonia-oxidizing bacteria. The frequency of isolation of AOB in rainy and winter season was higher (~11%) as compared to summer season (5.2%) (Table 2).

Evaluation of potential application of AOB for biological nitrification : All the12 PCR positive AOB isolates were tested for biological nitrification activity, out of which only two isolates (HC-5 and HC-2) showed maximum potential. The nitrification activity of both the isolates was almost equal. Therefore, the results of only HC-5 are being shown in the present paper. In river water samples inoculated with HC-5, the ammonia-nitrogen level decreased from 7.5 to 0.01 mgl^{-1} in 5 days of the treatment (Fig. 1) as compared to control which showed a marginal decrease in ammonia level from 7.5 to 6.45 mgl^{-1} in 5 days. Similarly, nitrate and nitrite levels also declined steadily in treatment group as compared to control (Fig. 2, 3).

Ammonia, nitrite and nitrate concentration in fishpond water treated with HC-5 decreased from 3.5 to 0.05 mgl^{-1}, 1.12 to 0.001 mgl^{-1} and 6.4 to 2.8 mgl^{-1}, respectively (Fig. 1, 2, 3). These results indicate that the isolate HC-5 had the ability of oxidation of ammonia as well as reduction of nitrite and nitrate.

Table 1 : Biochemical tests for *Pseudomonas stutzeri* (HC-5)

Biochemical test	HC-5 (Present study)	NS-2 (Su *et al.*, 2001)
Oxidase	+	+
Catalase	+	N.D
Gelatin hydrolysis	-	-
Amylase	+	N.D
Indole	-	-
Urease	+	-
Tween 80	+	+
ONPG	-	-
Arginine dihydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-

(+) Positive, (-) Negative

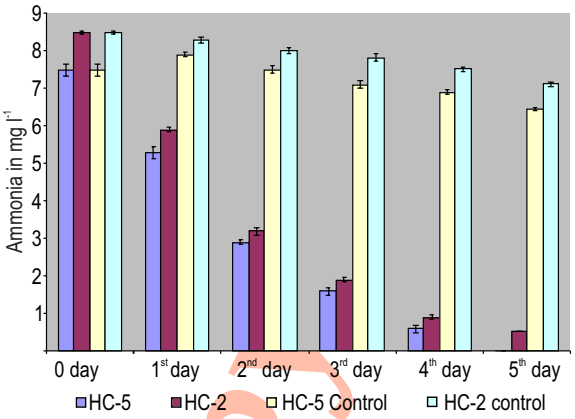

Fig. 1 : Effect of *Pseudomonas stutzeri* on ammonia concentration in river and pond water

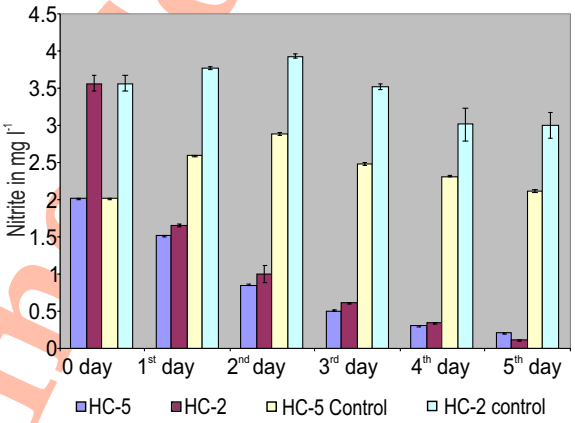

Fig. 2 : Effect of *Pseudomonas stutzeri* on nitrite concentration in river and pond water

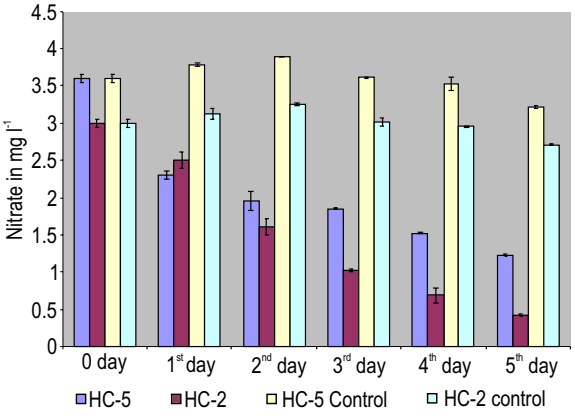

Fig. 3 : Effect of *Pseudomonas stutzeri* on nitrate concentration in river and pond water

Table 2 : Details of samples processed for isolation of nitrifiers from fishponds during different seasons

Fish pond No.	Summer				Winter				Rainy			
	No. of samples processed	Nitrate positive samples	Nitrate positive isolates	PCR positive nitrate isolates	No. of samples processed	Nitrate positive samples	Nitrate positive isolates	PCR positive nitrate isolates	No. of samples processed	Nitrate positive samples	Nitrate positive isolates	PCR positive nitrate isolates
1	4	2	8	-	4	3	11	1	4	3	12	1
2	4	2	10	1	4	4	12	2	4	3	10	2
3	4	2	9	1	4	2	5	1	4	2	6	1
4	3	1	6	-	3	2	8	-	3	2	5	-

Molecular identification of HC-5 : On the basis of partial 16S rDNA sequencing of HC-5, the isolate (Accession No. GU358072) was found to be closely related (99%) with *P. stutzeri.* This isolate was also characterized as *P. stutzeri* on the basis of biochemical tests (Su *et al*., 2001) (Table 1).

In the present study, *P. stutzeri* was isolated from fishpond and used as potential agent for biological nitrification to remove the total nitrogen wastes *i.e*. ammonia, nitrite, and nitrate present in the water. Diep *et al*. (2009) also isolated *P. stutzeri* from wastewater of catfish pond and demonstrated its application in wastewater treatment. Those isolates were effective in lowering soluble N (NH_4, NO^-_2 and NO^-_3) levels in fishpond water from 10 mgl^{-1} to negligible amount after 4 days. Recently, *Pseudomonas stutzeri* and *Candida utilis* have also been used as probiotic to enhance production and increase survival of *Artemia* larvae (Abdelkarim *et al*., 2010). *P. stutzeri* has been used in treatment plant to improve the total nitrogen removal efficiency and the loss was found to be 40.1% (Wen *et al*., 2010). Zala *et al*. (1999) applied bioreactor packed with alginate beads containing *Pseudomonas stutzeri* and *Comamonas testosteroni* for removal of nitrate - nitrogen (NO_3-N) from industrial effluent within 12 hr. *P. stutzeri* strains possess strong ability to oxidize ammonia as well as reduce nitrite and nitrate in piggery wastewater (Su *et al*., 2001). All these studies show that *P. stutzeri* has potential application in removal of soluble nitrogen from water.

Previously, denitrification was considered to be an anaerobic process as nitrate, nitrite and nitrous oxide reductase activities were inhibited at certain O_2 concentrations. However, certain organisms such as *Alcaligenes faecalis* (van Niel *et al*., 1993) and *P. denitrificans* can perform denitrification under aerobic conditions also. *P. stutzeri* is also one of the heterotrphic nitrifying bacteria capable of removing nitrogen from activated sludge or wastewater (Su *et al*., 2001).Yan *et al.* (2008) explained the presence of ammonium monooxygenase gene (*amo*) in the genome of *P. stutzeri. Pseudomonas putida* is also a heterotrophic nitrifier and contain ammonium monooxygenase gene (Daum *et al*., 1998).

Nitrifying bacteria play vital role in maintaining water quality of the ponds. Fish culture ponds are generally provided with high inputs of nutrients and mineralization of organic substances results in increase in the ammonia concentration which is harmful to fish. Ammonia-N is toxic to commercially cultured fish at concentrations above 1.5 mg N^{-1}. In most cases, the acceptable limit of unionized ammonia in aquaculture systems is only 0.025 mg (Chen *et al*., 2006). According to Barnabe (1994) un-ionized ammonia concentration 0-0.01 mg N-NH_3l^{-1} caused damage to freshwater species, 0-0.02 mg N-NH_3 l^{-1} for salmonids and 0-0.05 mg l^{-1} for sea fish. However, the ammonia toxicity threshold depends strongly on the species, size, fine solids, refractory organics, surface-active compounds, metals and nitrate (Colt, 2006). To reduce ammonia concentration in wastewater, oxygenation of water is done which changes ammonia to nitrate. However, nitrifying and denitrifying bacteria species in environment also facilitate nitrogen removal by promoting ammonia oxidation to nitrite followed by nitrite reduction to nitrate and finally reduction of nitrate to nitrogen gas. The results showed that *P. stutzeri* (HC-5) has the capability to oxidize ammonia concentration as well as decrease the concentration of nitrite and nitrate from fishponds and can be employed in a bioreactor. The results suggest that *P. stutzeri* could play an important role in oxidation of ammonia as well as reduction of nitrite and nitrate in fish ponds as immobilized cells.

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