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ISOLATION, IDENTIFICATION OF NITRIFYING BACTERIA FROM SHRIMP CULTURE POND SOIL AND EFFECT OF PH AND TEMPERATURE ON THEIR NITRIFYING EFFICIENCY

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ABSTRACT

Two efficient strains (AOB-6 and NOB-4) of ammonia oxidizing bacteria and nitrite oxidizing bacteria have been isolated in pure culture by liquid enrichment techniques from shrimp culture pond bottom soil. Both were identified as gram negative, rod shaped and similar to the genera Nitrosomonas (AOB-6 KT231978) and Nitrobacter (NOB-4 KT231980) by 16s RNA sequencing. The both strains of AOB-6 and NOB-4 were studied on bioconversion of ammonium to nitrite and nitrite to nitrate at different pH and temperature respectively. The strain AOB-6 has been recorded highest ammonium oxidation to nitrite pH 8 and Temperature 30˚C. The strain NOB-4 also recorded maximum oxidation of nitrite to nitrate at pH 8 and at Temperature 30˚C.

KeyWords : Enrichment-Nitrosomonas-Nitrobacter-Nitrification

INTRODUCTION

Aquaculture is an important food producing sector of many coastal line countries. However, poor water quality and disease are the main problems to aquaculture production and in that way affect financial status of native people in several countries. The latest technique of probiotic application has improved the water quality and shrimp production in aquaculture (Gomez-Gil et al., 2000; Verschuere et al., 2000; Rao, 2001; Shariff et al.,2001; Irianto and Austin, 2002; Balcázar, 2003; Ali,2006; Lakshmanan and Sounderpandian, 2008; Sreedevi and Ramasubramanian, 2010; Dimitroglou et al., 2011; Iribarren et al., 2012). Uneaten food material, organic wastes, Prawn faeces and dead animals are the sources for water pollution mainly by accumulation of ammonium in pond water there by affecting the survival of aquatic species and leading to massmortality in production.Ammonia is toxic to fish and shrimp in aquatic environment. Ammonia oxidized to nitrate is harmless to the shrimp by ammonium oxidizing bacteria and nitrite oxidizing bacteria (Nitrosomonas sp and Nitrobacter sp).

Nitrosomonas is one of the five genera of the ammonia-oxidizers. This genus includes all species having ellipsoidal to rod-shaped cells with extensive intra cytoplasmic membranes, arranged as flattened vesicles in the peripheral cytoplasm (Koops et al 1991). Nitrosomonas is a gram negative rod or oval shaped chemolithotrophic bacteria which obtains all of its energy for growth from the oxidation of ammonia (NH3) and can obtain all its carbon for biomass from CO_2. Ammonia is oxidized to nitrite (NO_2) and CO_2 is assimilated by ribulosebiphosphate carboxylase and the reductive pentose phosphate cycle (Calvin cycle). Recently Nitrosomonas used as a probiotic, played a major role in maintaining optimum water quality parameters (especially dissolved oxygen, ammonia and phosphates, bacterial loads and zooplankton) throughout the culture period which resulted in better growth, survival and disease resistance in the culture fish (K. Sunitha and P. Padmavathi. 2013). The isolation of nitrifying bacteria in pure culture was first accomplished by Winogradsky (1890); it has been identified for some years, before that nitrification was carried out by living organisms, but efforts to isolate the organisms by the common bacteria isolation methods had failed (Frankland and Frankland, 1890). The present study aimed to isolate effective ammonium oxidizing bacteria and nitrite oxidizing bacteria from shrimp culture pond sediment soil in Parangipetai and studied the effect of pH and temperature on bioconversion of ammonia (NH_3) to nitrite (NO_2)to nitrate(NO_3)by using nitrifying isolate.

Materials and Method

Sample collection and soil Enrichment

Soil samples were collected from shrimp cultivation pond bottom of Vellarbasin in Parangipettai in sterile containers and aseptically brought to Microbiology laboratory of Department of Microbiology, Annamalai University.

About ten grams of fresh soil was enriched in 100 ml mineral salt solution A as described by Goldberg and Gainey (1955) and slightly modified by Lewis and Pramer (1958) and 100 ml mineral salt solution B.The inoculated growth medium was incubated for 18 to 20 days at 25˚C in a rotary shaker at 120 rpm.

Solution A

Na_2HPO_4-13.5g, KH_2PO_4-0.7g, $MgSO_4.7H_2O$-0.1g, $NaHCO_3$-0.5g $(NH_4)_2$-SO_4-2.5g, $FeCl_3.6H_2O$-14.4mg, $CaCl_2.2H_2O$-18.4mg, Distilled water 1000 ml, final pH-7.2 + 0.2.

Solution B

Na_2HPO_4-13.5g, KH_2PO_4-0.7g, $MgSO_4.7H_2O$-0.1g, $NaHCO_3$-0.5g $FeCl_3$.6H2O 0.014g, CaCl2.2HO 0.18g,$NaNO_2$5g, Distilled water 1000 ml, pH 7.2 + 0.2

Isolation of nitrifying bacteria

Three replications of 100 ml growth medium A and B were prepared in 250 ml conical flasks and adjusted the pH to 7.2 ± 0.2 with 0.5% of aqueous calcium carbonate and 0.5 % w/v aqueous sodium bicarbonate. Each 10 ml of the soil enrichment A and B (Nitrifying Bacterial enrichment) were pipetted out aseptically and added in to the conical flask containing growth medium A and B respectively. Mostly nitrifying bacteria are obligate aerobes and have more oxygen demand; therefore incubation was on vigorous agitation at 120 rpm on rotary shaker at 28˚C for 28 days. The same process was repeated to have second and subsequently third enrichment of nitrifying bacteria during which it was expected to remove the heterotrophs from the culture. The pH was checked at every seven days interval and

maintained at a pH 7.2 ± 0.2 with 0.5% of aqueous calcium carbonate and 0.5 % w/v aqueous sodium bicarbonate.

Growth monitor

Throughout the incubation of first, second and third enrichment, all the flasks were tested for
1.presence of nitrite
2.presence of nitrate
3.Number of heterotrophs
4.Number of nitrifying bacteria AOB(Ammonium Oxidizing Bacteria) and NOB (Nitrite Oxidizing Bacteria).

Nitrite

One drop of enrichment A was mixed with one drop of sulfuric acid (2%) and three drops of Trommasdroff's reagent for presence of nitrite test then further confirmation by adding Nessler's reagent.

Nitrate

One drop of enrichment B was mixed with two drops of sulfuric acid (2%) and three drops of diphenylamine.

Heterotrophs

One ml of aliquot was serially diluted up to 10^6 and poured one ml of 10^5 and 10^6 dilutions on nutrient agar medium and incubated for 24 hrs.

Nitrifying Bacteria

One ml of aliquot from dilution 105 and 106 was transferred on salt solution agar medium A and B and then transferred purified bacterial colony on same agar slant.

Effect of pH and Temperature on bioconversion of ammonia to nitrite

100 ml mineral salt solution A was prepared in 250 ml Erlenmeyer flask with different pH ranging from 6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5. Each flask was inoculated with 1ml of AOB isolate and was incubated at room temperature in a rotary shaker at 120 rpm for seven days.

Another 100 ml mineral salt solution A was prepared in 250 ml Erlenmeyer flask. Each flask was inoculated with 1ml of AOB isolate and was incubated at room temperature in a rotary shaker at 120 rpm at different temperature ranging from 20, 25, 30, 35 and 40˚C for seven days. The flasks were covered with black color paper to avoid penetration of light. After incubation, the flasks were tested for the presence of nitrite by UV Spectrophotometer. About 2.5 ml of aliquot mixed with 0.2 ml of sulfanilamide solution and followed by 0.2 ml of NNEQ (n-(1-napthyl) ethylene diamine-2HCl). It was mixed thoroughly and allowed to stand for 30 min. The color of the solution changed to a vivid violet color. The absorbance of the samples was measured at 540 nm calculated by using standard curve. The nitrite standard was made by 10 mg of sodium nitrite mixed in 1000 ml distilled water. 8 test tubes were arranged with distilled water as 4.5 ml in the first tube and as others 2.5ml. About 0.5 ml of standard solution was diluted in first tube and mixed well then from the 1st tube transfer 2.5 ml in to the tube 2 this procedure repeated up to 7^{th} tube. Tube 8 serves as blank. All the tubes were measured at 540 as

above mentioned.

Effect of pH and Temperature on bioconversion of nitrite to nitrate

100 ml mineral salt solution B prepared in 250 ml Erlenmeyer flasks at different pH ranging from 6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5. Each flask was inoculated 1ml of NOB isolate and was incubated at room temperature in a rotary shaker at 120 rpm. Another 100 ml mineral salt solution B was prepared in 250 ml Erlenmeyer flask and each flask was inoculated with 1ml of NOB isolate. All the flasks were incubated at room temperature in a rotary shaker at 120 rpm at different temperature ranging from 20, 25, 30, 35 and 40˚C for seven days. The flasks were covered with black color paper to avoid penetration of light.

After incubation the flasks were tested for the presence of nitrate by UV Spectroscopy. About 0.25ml of aliquot was mixed thoroughly with 0.8 ml of salicylic acid solution (5% (w/v) salicylic acid in concentrated H_2SO_4). Then after allowed to stand for 20 minutes at room temperature, 19 ml of 2N NaOH was added to raise the pH above 12 and cooled to room temperature. The absorbance was measured at 410 nm and calculated by using standard curve. Nitrate standard was prepared by 1.805 g of potassium nitrate dissolved in 1 litter of distilled water. Six 50ml flasks containing 0.0, 0.05, 0.10, 0.15, 0.20 and 0.25ml of standard solution to a final volume of 0.25ml with distilled water were prepared and measured at 410 nm as above mentioned.

Identification of the isolate

Select bacterial isolates were studied by microscopic, morphological and biochemical analysis using standard techniques. Slides containing bacterial isolate smears were examined by gram staining for cell morphology and motility. Standard biochemical tests such as Catalase, Oxidase, Urease, Ammonium Utilization and Nitrate Reductase tests were performed according to Bergey's Manual (Half et al 1994).

16s RNA sequencing

The strain AOB-6 and the strain NOB-4 were analysed using 16S rRNA done by using predetermined universal primers of 16S r RNA. RNA sequences were compared with already submitted sequence in database BLAST software. Further, most similar sequences were aligned by Clustal W and Clustal X software and Phylogenetic tree was drawn using PHYLIP software (Fig. 5 & 6).

Results and Discussion

Nitrifying bacteria were enriched, enumerated and isolated by serial transfer method from the shrimp culture pond soil of Vellar basin in Parangipettai(Table-1&2). Totally ten isolates were isolated as five of ammonium oxidizing bacteria were screened for nitrite production (AOB-2, AOB-4 AOB-6, AOB-7 and AOB-8) (Table-3) and five of nitrite oxidizing bacteria were screened for nitrate production (NOB-1, NOB-3, NOB-4, NOB-6 and NOB-9) (Table-4). All the isolates were subjected to effect of pH and temperature on ammonium oxidation and nitrite oxidation.

Table.1 Enrichment of ammonium oxidizing bacteria

S.NO	Soil Enrichments	No of Heterotrophic Bacteria cfu /10^6	No of Ammonium Oxidizing Bacteria cfu /10^6	Nitrite
1	First Enrichment	8	12	+
2	Second Enrichment	5	20	+
3	Third Enrichment	2	32	+

Table.2 Enrichment of nitrite oxidizing bacteria

S.NO	Soil Enrichments	No of Heterotrophic Bacteria cfu /10^6	No of Nitrite Oxidizing Bacteria cfu /10^6	Nitrate
1	First Enrichment	10	9	+
2	Second Enrichment	6	18	+
3	Third Enrichment	3	28	+

Table.3 screening of nitrite production

S.NO	Strains	Nitrite production
1	AOB-1	-
2	AOB-2	+
3	AOB-3	-
4	AOB-4	+
5	AOB-5	-
6	AOB-6	+
7	AOB-7	+
8	AOB-8	+
9	AOB-9	-
10	AOB-10	-

Table.4 screening of nitrate production

S.NO	Strains	Nitrate production
1	NOB-1	+
2	NOB-2	-
3	NOB-3	+
4	NOB-4	+
5	NOB-5	-
6	NOB-6	+
7	NOB-7	_
8	NOB-8	-
9	NOB-9	+
10	NOB-10	-

Effect of pH and temperature on bioconversion of ammonia to nitrite

Nitrifying bacteria are pH-sensitive organisms (Villaverde et al., 1997). The value of pH is an important factor affecting the process of nitrification. The highest conversion level was obtained in AOB-6 with pH 8 (306.664 mg/l) and followed by pH 7.5(217.99) and 8.5(174.55) (Fig.1). Previously authors have reported that optimum pH for nitrification ranged between 7.0 and 8.0 (Jones and Paskins, 1982; Painter and Loveless, 1983; Antoniou et al., 1990). The ammonia conversion to nitrite at different temperatures is shown in Fig. 2. Maximum conversion level recorded in AOB-6 at temperature 30˚C (297.13) followed by temperature 25˚C (176.53). The result indicated that ammonia conversion to nitrite was significantly accelerated when the temperature was increased from 20˚C to 30˚C. The results coincided with the effect of temperature (20˚C vs. 30˚C) on the nitrification rate coefficients (Yamaguchi et al., 1996). The results indicated that temperature had a greater impact on nitrification of suspended bacteria in low concentrations than at high concentrations of ammonia nitrogen (Bae et al., 2002).

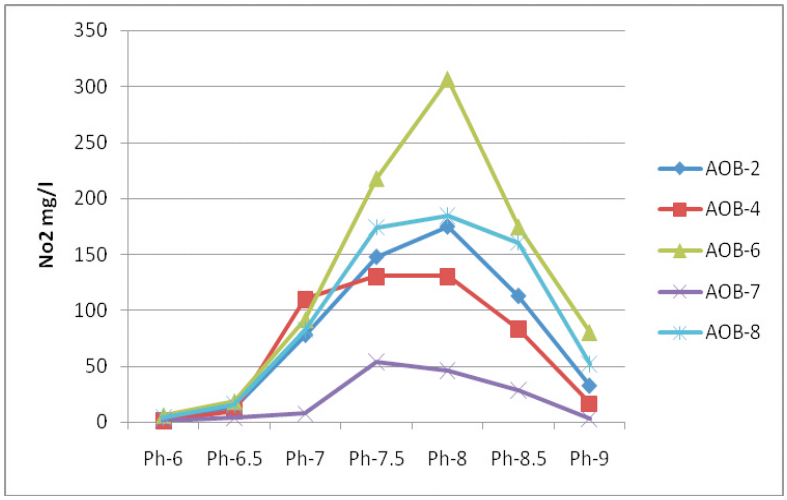


Fig.1 effect of pH on bioconversion of ammonia to nitrite.

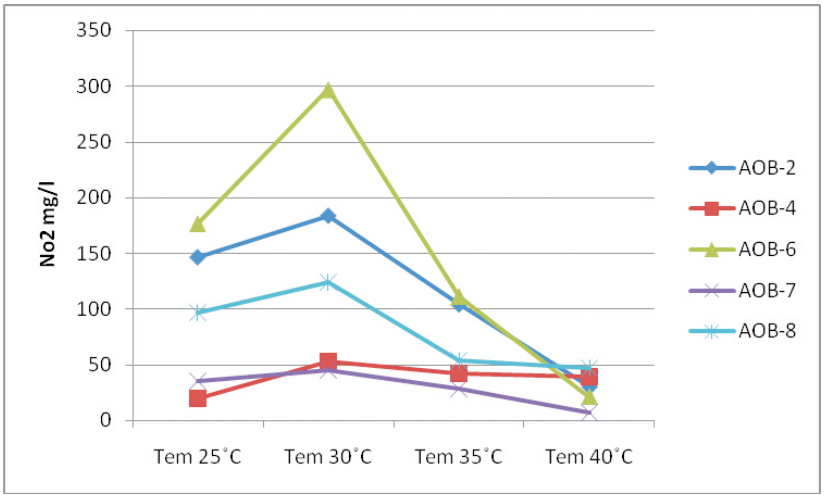


Fig.2 Effect of temperature on bioconversion of ammonia to nitrite.

Effect of pH and temperature on bioconversion of nitrite to nitrate.

The effect of pH value on nitrite oxidation by NOB isolates were studied at various pH conditions. The results indicated that when the initial pH value was set at 6 and 6.5 there was no considerable difference in NO_2-N changes. When the initial pH values were 7 to 8.5 similar results were obtained in NOB-4 with higher nitrification efficiency at pH 8 (121.06) (Fig.3). The pH value is an important factor for nitrite oxidation, since it affects the nitrification process either by directly infiuencing the bacterial growth or by indirect involvement in the ionization of NO_2/HNO_2or NH_4+/ NH_3(Jimenez et al., 2011).The optimal pH value was found to be 7.9 for Nitrobacter by Grunditzm, 2001 Between the temperature range of 20˚ to 40˚C, nitrification activity was indicated to increase in a temperature dependent manner as showed the highest nitrification activity in NOB-4 at 30˚C (112.97) (Fig.4). Temperature was found to be one of the most important factors affecting the nitrification activity and population structure of (NOB) nitrite oxidizing bacteria. The activity of Nitrobacter was optimal at the temperature range of 25–28˚C, (Antoniou et al., 1990 and Sorokin et al., 1998) while in the present study the optimal conditions for NOB-4 were around 25–30˚C.

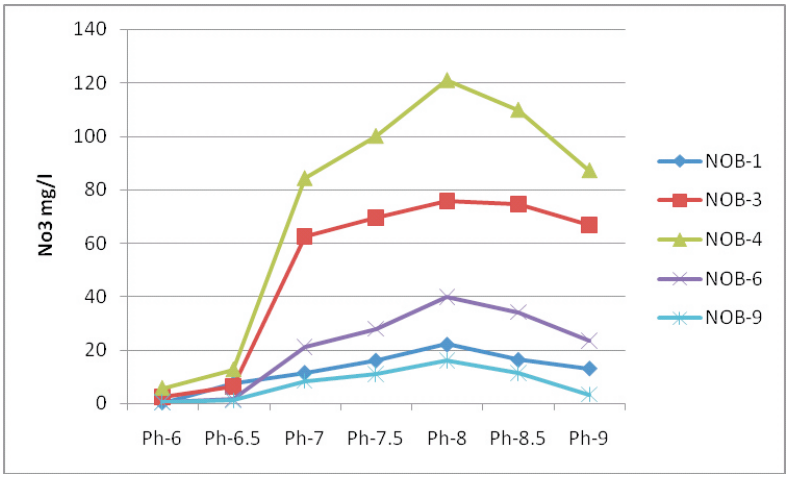


Fig.3 effect pH of bioconversion of nitrite to nitrate.

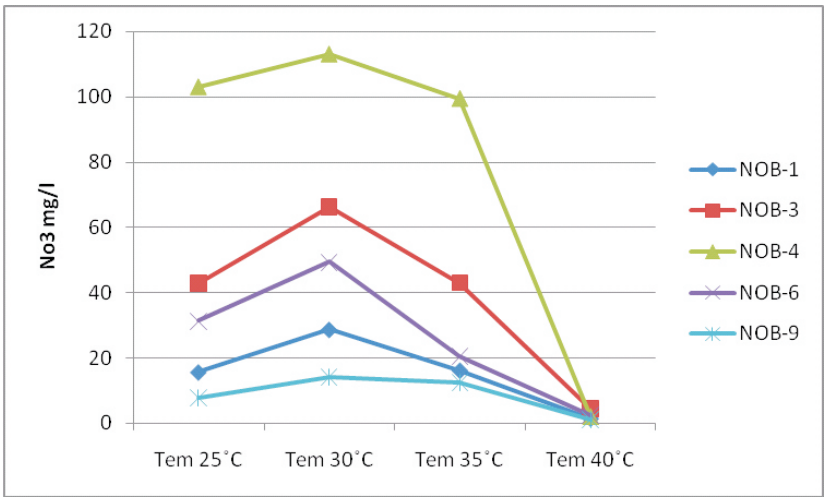
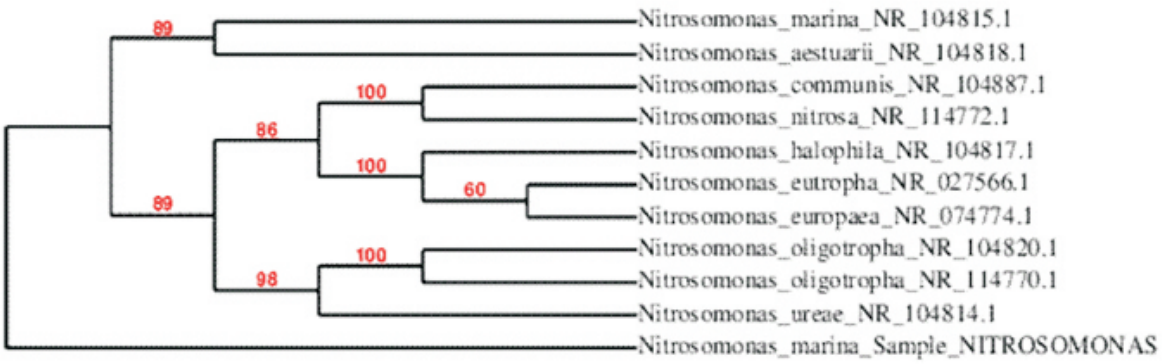


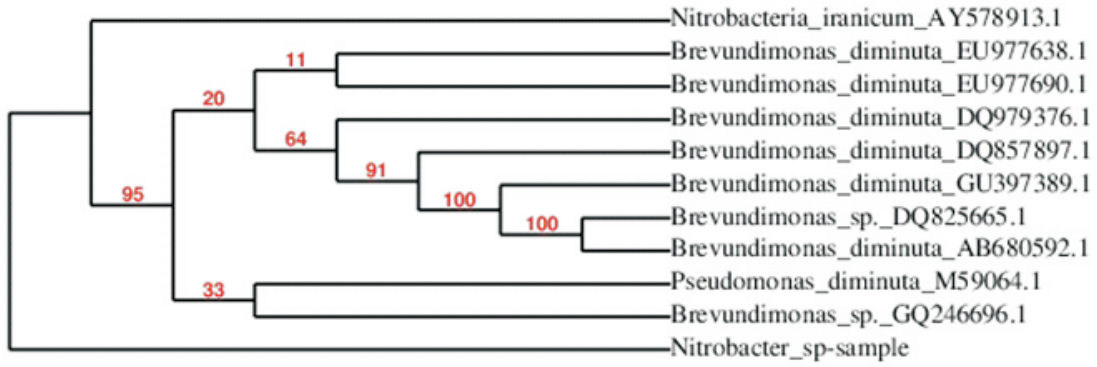
Fig.4 effect temperature of bioconversion of nitrite to nitrate.

Identification of bacteria

Isolate AOB-6 and NOB-4 were found to be gram negative rods with cell length and breadth of 1.0-1.5 µm × 0.5-0.9 µm and1.2–1.8 × 0.6–0.9 µm respectively. AOB-6 and NOB-4 were identified as Nitrosomonas-907R KT231978 and Nitrobacter-KT231980 respectively based on the 16s RNA sequencing.



Phylogeny tree of sample Nitrosomonas



Phylogeny tree of sample Nitrobacter

CONCLUSION

In the present study, AOB-6 and NOB-4 strains were isolated from the shrimp ponds of Vellaru basin, Parangipettai and identified as Nitrosomonas sp and Nitrobacter sp which showed high nitrification efficiency. Complete nitrification can be achieved in a wide range of temperatures and pH values. The study also showed that the pH value had greater impact on ammonia oxidation rate constants than temperature. These characteristics can be of great importance for application of AOB-6 and NOB-4 in deferent field for bioremediation such as aquaculture, water treatment dying effluent treatment etc.

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