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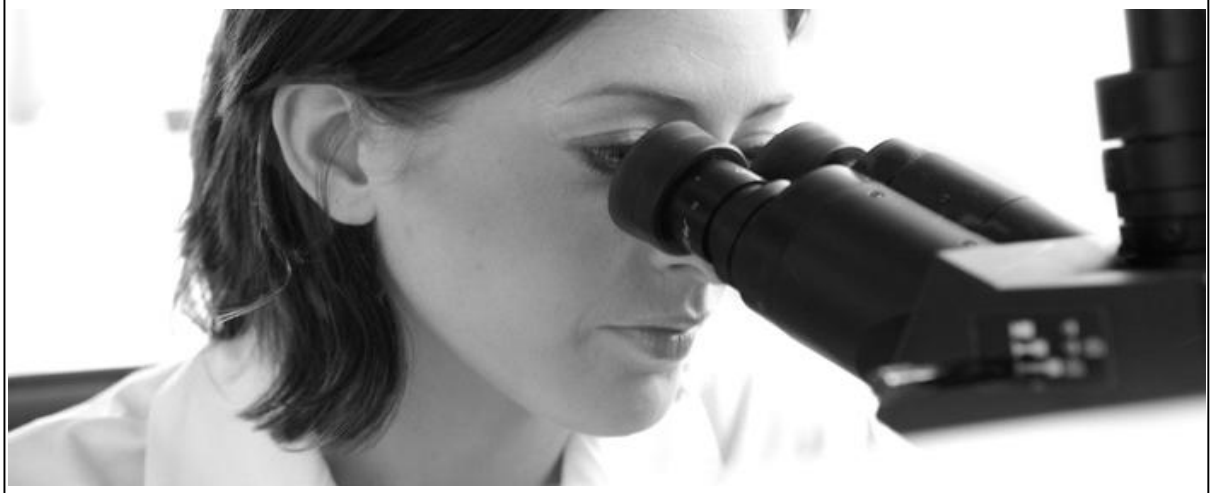

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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF DENITRIFYING BACTERIA FROM SHRIMP CULTIVATION POND SOIL IN PARANGIPETTAI

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ABSTRACT

Denitrifying bacteria were isolated from shrimp cultivation pond soil in vellar basin Parangipettai. Totally ten isolates were obtained and screened for denitrification activity. Four denitrifying isolates were selected (DNB2, DNB3, DNB5 and DNB6) and subjected to different pH and Temperature on denitrification activity. Highest nitrate removal was recorded in DNB3 at pH 7 (78.71 %) followed by pH 7.5 (76.92%), and pH 8(73.25%) with minimum nitrite accumulation of 18.41 mg/l, 21.21mg/l and 26.19mg/l respectively where as the effect of temperature on nitrate removal was recorded in DNB3 as 75.36% at 30°C and 67.8% at 35°C with minimum nitrite accumulation of 20.38 mg/l at 30°C and 93.0 mg/l at 35°C respectively.

INTRODUCTION

Aquaculture creates large amount of wastes, comprising of metabolic by-products, residual food, faecal matter and residues of prophylactic and therapeutic inputs, leading to the deterioration of water quality and disease outbreaks (Antony and Philip, 2006). The water quality parameters that affect and influence shrimp behavior and health (Thuyet *et al.,* 2012). Ammonia present in the waste is a highly toxic to shrimp and other aquatic organisms (Nathan Stone *et al.,* 2013).

Ammonia plays an important part within the nitrogen cycle particularly nitrification and denitrification process of any aquatic environment. The nitification is the oxidative process in which ammonia is first converted into nitrite (NO_2) by naturally occurring *Nitrosospira* and *Nitrosomonas* bacteria in the water (Cuijuan *et al.,* 2013). The nitrite is still toxic to shrimp and other aquatic organisms but encourages the growth and colonization of *Nitrobacter* to convert it to the less toxic nitrate form (NO_3) (Regina Nogueira and Luis F. Melo, 2006). The nitrate is then taken up by aquatic plants, algae and denitrifying bacteria through denitrifying process in aquaculture systems thus completing the nitrogen cycle (Mia Kim *et al* 2004). Denitrification is the dissimilative reduction of nitrate (NO_3) to nitrogen gas (N_2), through the production of nitrite (NO_2) and gaseous nitric oxide (NO) and nitrous oxide (N_2O) intermediates.

$$\longrightarrow NO_3NO_2 \longrightarrow NO\ (g) \longrightarrow N_2O\ (g) \longrightarrow N_2\ (g)$$

This process is performed by heterotrophic bacteria under anoxic conditions and uses Nitrate as a terminal electron acceptor in the presence of a carbon and energy source. The process involves the enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR), and it is commonly anticipated that the fitness of denitrifying bacteria depends on their ability to regulate the process to avoid accumulation of toxic intermediates and to maximize energy conservation(Linda Bergaust *et al.,* 2010). Denitrification was considered to be an anaerobic process by previous investigators as the enzyme system was inhibited under certain oxygen concentrations (Apel and Turick, 1993; Ferguson, 1994). However, with the increasing number of reports on denitrifying bacteria, aerobic denitrification attracted a lot of attention for its easier operation and higher denitrification rate than anaerobic denitrification. There are recent reports of aerobic denitrifying species isolated from canals, ponds, soils, and activated sludge that can simultaneously utilize oxygen and nitrate as electron acceptors. These include *Paracoccus* (Lukow and Diekmann, 1997), *Pseudomonas* (Kesser *et al.,* 2003), *Bacillus* (Kim *et al.,* 2005), *Alcaligenes* (Robertson and Kuenen, 1983) etc. As a part of aquaculture management on water quality and pond bottom soil, the knowledge on denitrifying bacteria is inevitable. Hence the present study focused on the isolation and

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identification of denitrifying bacteria from shrimp cultivation pond bottom soil in Parangipettai.

MATERIAL AND METHOD

Sample collection

Soil sample collected from shrimp cultivation pond bottom of Vellaru basin in Parrangipettai in sterile container left to microbiology laboratory in the Department of Microbiology, Annamalai University.

Enrichment and Isolation of bacteria

About ten gram of fresh soil was enriched in 250 ml Erlenmeyer flask containing 100 ml Nitrate rich (NR) medium (Glycerol-10.0g, KNO_3-10.0g, Yeast extract-3.0g, $(NH_4)_2SO4$ 1.5g, $K_2HPO_4 \cdot 3H_2O$-0.8g, $MgSO_4 \cdot 7H_2O$-0.5g, KH_2PO_4-0.2g, $CaCl_2$ 0.1g, distilled water 1 litter, pH was adjusted to 7.2 ± 0.2). The flask was incubated at 25°C in rotary shaker at 150 rpm for 7 days. After the incubation period one ml of enrichment was transferred in to the fresh NR medium and incubated for seven days. The procedure was repeated for one more time with fresh NR medium. After the final enrichment, one ml of enrichment mixture was serially diluted from 10^1 to 10^6. One ml aliquot from 10^6dilutions was poured in Petri plates containing NR solid medium. Plates were replicated and incubated for 24 hours and selected Colonies were purified on same agar medium.

Screening of bacteria

All isolates were individually inoculated in test tube containing 5 ml of denitrification broth with Durham's tube for the gas production. All the test tubes were incubated at 25°C for 24 hours. After the incubation, observed for the gas production and presence of nitrite. One drop of aliquot from the test tube was mixed with one drop of sulfuric acid (2%) and three drops of Trommasdroff's reagent for presence of nitrite then further confirmation by adding Nessler's reagent. Then another one drop of enrichment has mixed with two drop of sulfuric acid (2%) and three drops of diphenylamine for presence of nitrate.

Effect of pH and Temperature on denitrification

The denitrification medium prepared at different pH ranging from 6, 6.5, 7, 7.5, 8, 8.5, and 9 was dispersed in 100 ml quantities in 250 ml Erlenmeyer conical flasks and replications were maintained in each pH range. Each flask was inoculated 1ml of DNB (Denitrifying Bacteria) isolate and was incubated at room temperature in a rotary shaker at 120 rpm and all the treatments were replicated. Another one set of denitrification medium was prepared and dispersed in 100 ml quantities in 250 ml Erlenmeyer conical flasks. Each flask was inoculated 1ml of DNB (Denitrifying Bacteria) isolate and was incubated at different temperature in a rotary shaker at 120 rpm and all the treatments were replicated.

Nitrate assay

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.

Nitrite assay

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 and 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 transferred 2.5 ml in to the tube 2, this procedure repeated up to 7th tube. Tube 8 served as blank. All the tubes were measured at 540 nm as above mentioned. Selected isolate was characterized by microscopic and biochemical analysis performed according to Bergey's Manual (Half *et al* 1994)

RESULT AND DISCUSSION

Denitrifiying bacteria were isolated and identified from the shrimp cultivation pond bottom soil from Parangipettai. Four isolates were obtained in primary screening based on the nitrate reduction and gas production (Jutharat *et al* 2015, Nagarajan *et al* 2015). These isolates were subjected to effect of pH and temperature on denitrification activity. Out of ten isolates obtained, four isolates (DNB2, DNB3, DNB5 and DNB6) showed denitrifying activity by way of reducing nitrate. In addition to the positive response for the denitrification activity, these isolates also showed gas production (Table-1).

Table-1 Screening of Denitrifying Bacteria

S.No	Isolates	Nitrate	Nitrite	Gas production
1	DNB1	+	-	-
2	DNB2	-	+	+
3	DNB3	-	+	+
4	DNB4	+	-	-
5	DNB5	-	+	+
6	DNB6	-	+	+
7	DNB7	+	-	-
8	DNB8	+	-	-
9	DNB9	+	-	-
10	DNB10	+	-	-

In this study, isolates were subjected to deferent levels of pH on denitrification activity and higher level of denitrification activity was recorded at pH 7 (78.71 %) followed by pH 7.5(76.92%), and 8(73.25%) with minimum nitrite accumulation of 18.41 mg/l, 21.21mg/l and 26.19mg/l respectively (Fig.1 & 2).

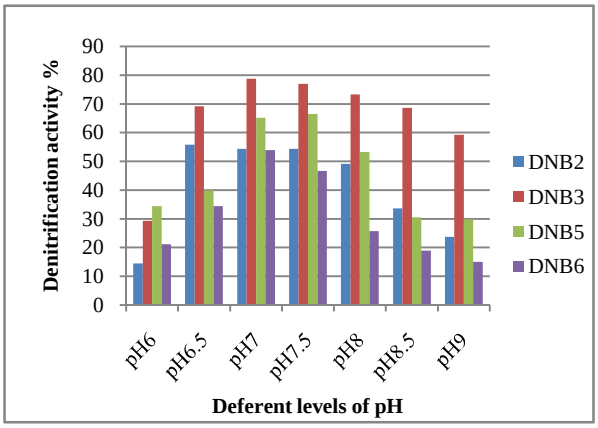

Fig.1. Effect of pH on Denitrification

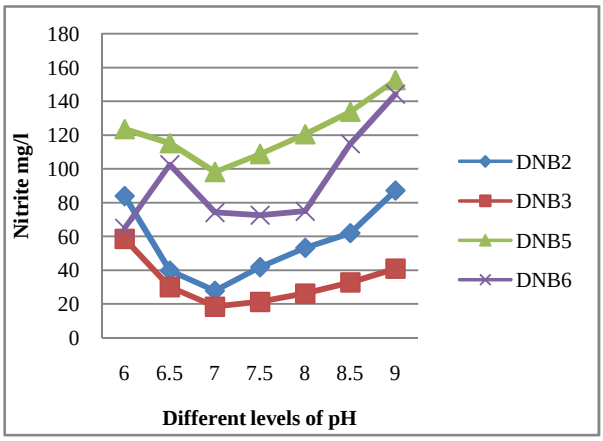

Fig.2 Effect of pH on Denitrification (Precence of Nitrite)

Saleema *et al* (2009) also reported that the NO_3 utilization and NO_2 accumulation were measured between 2 and 8 h in *P. mandelii* cultures grown at pH 6, 7, and 8. Knowles (1982) and Thomas, *et al* (1994) reported that denitrification activity was found to be in the range of pH 7.0 to 7.5 in pure cultures of *Pseudomonas* species.

In our study the effect of temperature on denitrification activity (25°C to 40°C) was experimented. The maximum denitrification was recorded at 30°C(75.36%) with minimum nitrite accumulation of 20.38 mg/l followed by 35°C (67.8 %) (Fig.3 & 4). Stanford *et al* (1975) previously established that the denitrification activity was limited within a temperature range of 15°C to 35°C, and the denitrification rate was increased for every 10°C increase in temperature in soil. Saleema *et al* (2009) reported that NO_3 utilization, NO_2 accumulation, and denitrification activity differed among different temperature treatments and higher level of denitrification was recorded at 30°C at 10 h than in *P. mandelii* cells grown at 10°C and 20°C. Among the five isolates subjected to different pH and temperature treatment, DNB3 was recorded maximum denitrification followed by DNB5 and DNB2. Low denitrification was observed in DNB6 (Fig 1, 2, 3&4).

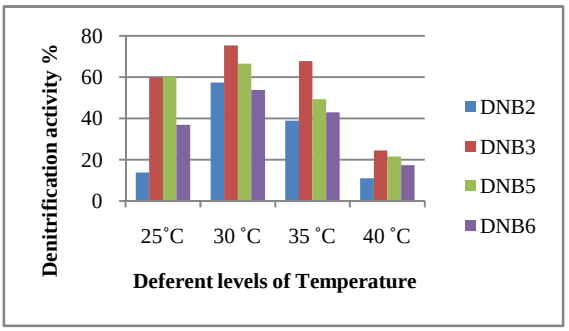

Fig.3 Effect of Temperature on Denitrification

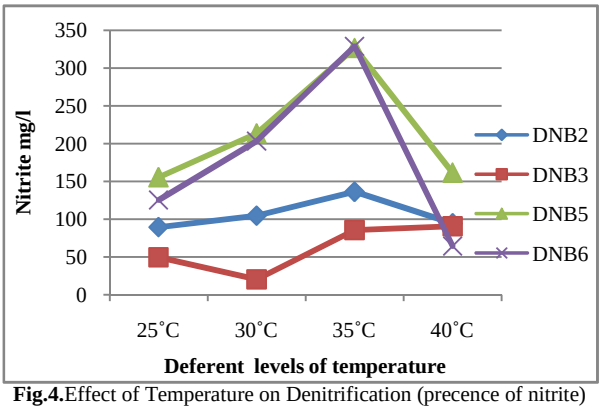

Fig.4. Effect of Temperature on Denitrification (precence of nitrite)

Identification of denitrifying bacteria

The isolate DNB3 was more efficient denitrifying bacteria among all the isolates characterized by microscopic and biochemical analysis according to Bergey's manual (Half *et al* 1994) (Table.2). Based on the characterization studies, highest nitrate reducing bacterial strain DNB3 was identified as the genus *Pseudomonas* sp.

Table-2 Biochemical characterization of DNB3 strain

S.No	Test	Result
1	Gram reaction	-
2	Spore formation	-
3	Motility	+
4	Catalase	+
5	Oxidase	-
6	Urease	-
7	Nitrate reduction	+
8	Indole	-
9	Citrate utilization	+
10	Starch hydrolysis	+
11	Gelatin hydrolysis	-
12	Casein hydrolysis	+
13	Lipid hydrolysis	+
14	Glucose	+
15	Fructose	+
16	Sucrose	+
17	Xylose	+
18	Mannose	+
19	Sorbitol	+

CONCLUSION

The bacterium, *Pseudomonas* sp (DNB3) was isolated from aquatic environment with higher denitrification activity (78.71 % from 1000 mg of initial nitrate) it is understood that the aquatic environment contains denitrifying bacteria and

successive denitrification activity from the experiments. As the continues cropping of fish and shrimp culture system resulted in culture water pollution and higher nitrate toxicity, the application of denitrifying bacteria may be followed or practiced for reducing the nitrate toxicity and to improve the culture pond water quality as well as bottom soil conditions.

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