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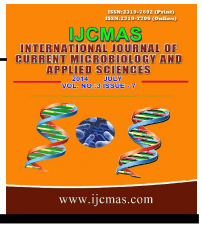

Original Research Article

Evaluation and efficacy study of Nitrifying Bacteria in freshwater Aquaculture system

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Keywords

Nitrifier, ammonia, nitrite, nitrate, aquaculture, prawn

A B S T R A C T

Nitrification is accomplished using biofilters in which the nitrifying bacteria usually coexist with heterotrophic microorganisms which metabolize biologically degradable organic compounds. An indoor study was conducted to determine the effect of two gram negative nitrifier and a gram positive nitrifier on ammonia nitrogen removal in freshwater giant prawns, *Macrobrachium rosenbergii* model system. Four sets of experiments in duplicate were conducted. Three sets of glass aquaria (10 L) were used for each two Gram negative nitrifiers and one Gram positive nitrifier and fourth set was used for control (without bacteria with prawn).The results indicated that nitrifiers treated group experienced 2-3 fold average decreased of ammonia and 1.2-4.5 fold of nitrite while 0.61-0.73 fold increased of nitrate as compared to the control groups. Similar nitrification benefits were noted in the production systems that received bacterial product. Further longer duration of experimentation (to improve nitrite-oxidation) will provide an indicator of nitrification of prawn culture system after incorporation of these microbial inoculums. Protein profiling was done to determine the molecular weight of these three isolates through SDS-PAGE analysis and it was found in the range of 17.5-100.6 kDa. This preliminary protein profile study could be used to obtain a more detailed identification and characterization of the organisms involved in the nitrification process.

Introduction

Aquaculture faces many challenges over the next decade, diseases and improvement of water-quality management. In aquatic environment, nitrogen is of primary concern in its various forms (Rabalais, 2002). The role of nitrifying bacteria in the process of mineralization is well known among aquaculturists, but the underlying bacteriology is still mysterious (Moriarty, 1997; Hagopian and Riley, 1998). The treatment of fish with chemotherapeutic agents could be disastrous in `aquaculture systems (Erondu and Anyanwu, 2005). So attention is paid to the paramount

biofiltration and recommendations are made for future recirculating aquaculture research (Hagopian and Riley, 1998; Malone and Pfeiffer, 2006). Today biological filters are commonly used for removal of ammonia in aquaculture systems (Malone et al., 2005; Kir, 2009). Aquaculture systems rely on nitrification to convert toxic ammonia to nitrite and then nitrate. This process is therefore, involved in the self-purification of aquatic systems because it prevents accumulation of potentially toxic or dangerous forms of nitrogen (non-ionized ammonia, nitrite) (Féray and Montuelle, 2002).

Nitrification is accomplished using biofilters in which the nitrifying bacteria usually coexist with heterotrophic microorganisms which metabolize biologically degradable organic compounds (Guerdat et al., 2010). Nitrifiers are known to survive for prolonged periods in anaerobic hypolimnions, wastewater reservoirs, and eutrophic sediments (Diab and Shilo, 1988; Smorczewski and Schmidt, 1991; Lusby et al., 1998). Nitrifiers possess survival mechanisms that enable them to be universally distributed in terrestrial and aquatic ecosystem. Nitrification proceeded at dissolved oxygen (DO) levels as low as 2.0 mg/ l and could even be interrupted with diurnal anaerobic cycles while still removing a significant amount of ammonia (Abeliovich, 1987), as some are bactericidal (Collins et al.,1976; Bower and Turner, 1982).

Freshwater prawn (*Macrobrachium rosenbergii*) is an important aquaculture industry in many Asian countries, contributes over 98% of the global freshwater prawn production. Indian freshwater prawn farming suffered severe difficulties in 2005–2007t leading to a reduced production of 27 -300t in 2007 (FAO, 2009). Development of novel ecofriendly processes based upon the utilization of biological systems is the interest of the present days of aquaculture. The present study aimed to reduce the inorganic nitrogen accumulating in freshwater prawn farming culture system by selecting the efficient nitrifiers for the process of nitrification. An investigation on the nitrification rate in water medium with prawn model, in different densities, associated nitrifiers and its important nitrification parameter such as ammonia, nitrite, nitrate was carried out. The whole cell protein profiles were studied through SDS-PAGE.

Materials and Methods

Sample collection and Isolation nitrifying bacteria

The samples of water were collected from the pond complex CIFA, Kausalyaganga, Bhubaneswar. Water samples were collected in sterile conical flasks (500ml) capacity from seven ponds brought to the laboratory for isolation and identification of nitrifiers.
The samples were inoculated in selective media Ammonium Oxidizing Bacterial (AOB) medium and Nitrite Oxidizing Bacterial (NOB) medium at 28°C in dark for a period of 45 days (Schneider and Rheinheirmer, 1988). Growth and development was indicated by change in color of AOB of medium (pink to orange). The conversion of ammonia into nitrite was indicated by the production of acid, which turned the colour of the indicator from orange to yellow. To be precise, this was evident from the change in colour of phenol red indicator pre-added into the medium.

Characterization and Identification of nitrifying bacteria

Identification of the isolates was performed according to their morphological, cultural

and physiological and biochemical characteristics by the procedure described in Bergey's Mannual of Systematic Bacteriology. The ammonia concentration in water samples and medium was analyzed by "Indophenol blue method (Strickland and Parsons, 1984). Development of blue colour after addition of the final reagent (Alkaline citrate: Sodium hypochlorite) was measured at 640 nm in the UV spectrophotometer (BIORAD). NO_2-N concentration has been determined by using two reagents i.e. sulphanilamide and NNED (N (-1-Napthyl) - ethylenediamine ditydrochloride). The nitrate-nitrogen concentrations in the water sample were estimated using phenol solution and Sodium hydroxide as the buffering agents and hydrazine sulphate and copper sulphate as the reducing agents. Colour developed through the diazotising reaction with the consecutive addition of acetone, NNED and sulphanilamide was measured at 543 nm (APHA 2005).

Enrichment for Ammonia Oxidizing Bacteria (AOB)

After adjusting the pH of the AOB medium to 7.6, 60 ml of media was poured into 8 conical flasks (500ml capacity) and was sterilized by autoclaving at (121°C/15min). When the media was in semisolid condition, these flasks were inoculated with 5 ml of pond water sample and a 1g of pond sediment. Four flasks were inoculated with water sample and rest three with sediment. Flasks were then incubated at 28°C in dark for a one and half month. Protocol was followed as described by Schneider and Rheinheirmer (1988). The conversion of ammonia into nitrite was indicated by the production of acid, which turned the color of the indicator from orange to yellow. To be precise, this was evident from the change in color of phenol red indicator pre-added into the medium.

Enrichment for Nitrite Oxidizing Bacteria (NOB)

Adjusting the pH of NOB broth to 8.9, it was sterilized by autoclaving. The liquid medium was inoculated with pond water samples at dilution 1:1000. Subsequently the flasks were incubated at 25-30°C in dark. The decreases in nitrite content within the cultivation flasks were tested as the conversion of nitrite into nitrate had been estimated so to denote the growth and development of NOB. Standard methods for estimation (APHA, 2005) were followed in this regard.

Effect of nitrifiers on ammonia/nitrogen removal in freshwater giant prawns, *Macrobrachium rosenbergii* culture system.

An indoor study was conducted to determine the effect of two gram negative nitrifier and a gram positive nitrifier on ammonia nitrogen removal in freshwater giant prawns, *Macrobrachium rosenbergii* culture system. In this study, four sets of experiments in duplicates were conducted. Three sets of glass aquaria (10 L) were used for each two Gram negative nitrifiers and one Gram positive nitrifier and fourth set was used for control (without bacteria with prawn). In each set 10 numbers of prawn (av. wt. 5 g) were kept for 5 days after acclimatization. The inoculums of 50 ml of DN-1, DN-4 and DN+3 each was charged in 10 L of water which corresponds to $56x\ 10^6$, $41x10^6$ and $42x10^6$ CFU/ml. The ammonia, nitrite and nitrate levels were estimated according to APHA protocols as discussed above.

Determination of Molecular Weight by SDS-PAGE

Sodium Dodecyl Sulphate Polyacrylamide

Gel Electrophoresis in discontinuous buffer system was carried out according to the method of Laemmli (1970) in order to determine the molecular weights of various proteins present in these nitrifying bacterial cell. The SDS-PAGE was carried out in the Bio Rad mini protein-II electrophoresis cell. Estimation of molecular weight of the protein bands in comparison to molecular weight standards was made from a minimum of two samples using AlphaEase®FC Imaging Software (Alpha Innotech Corp., USA) and expressed as mean. Samples and molecular weight markers were reduced by boiling in sample buffer containing 5% (v/v) 2-mercaptoethanol and were loaded into wells of a stacking gel of 5% above the separating gel of 10% acrylamide. Loaded samples were electrophoresed at 200 V for approximately 45 minutes. Then the gels were stained and the molecular weights of the bands were determined.

Results and Discussion

Isolation and characterization of nitrifying bacteria

The samples were inoculated in AOB medium and NOB broth, growth and development of change in color by AOB of the medium (pink and orange) and occurrences of turbidity in NOB broth. At the end of 45 days following inoculation, the color of AOB medium was changed into orange and turbidity was observed in NOB broth for almost all water and sediment samples.

The isolates of Gram negative nitrifiers (DN-1 and DN-4) and Gram positive nitrifying bacteria (DN+3) were isolated and identified based on the colony characteristics, growth and development on selective media, biochemical properties, staining characteristics, amino acid utilization, oxidase test, sugar fermentation and motility test.

Isolates DN-4 give negative result to biochemical test except oxidase test motility test and VP test, where it showed positive response. Regarding utilization of sugar, isolates DN-1and DN-4 showed positive response to glucose, lactose and trehalose. Isolates DN-1 and DN-4 gave positive result to xylose, while DN-1gave positive result to raffinose and saccharose.

DN+3 show negative response to ONPG test, lysine test, indole, molanate and urease test., but response to oxidase and motility test. Regarding utilization of sugar DN+3 gave negative response to glucose and lactose while positive to xylose and DN+4 saccharose.

Kinetic study of nitrifiers

Ammonia concentration in simulated ecosystem with prawn was found that decrease the concentration for all three isolates during that confirmed about the improved ammonia oxidation. Ammonia concentration in simulated ecosystem with prawn were 2.7 (DN-1), 2.9 (DN-4) and 1.8 (DN+3) fold decreased as compared to that of control (fig. 1). The nitrite concentration in DN-1 showed highest degree of oxidation (4.5 fold) while DN-4 and DN+3 show 1.19 and 3.09 fold decrease level of nitrite than control (fig. 2). Nitrate in treatment groups DN-1, DN-4 and DN+3 showed 0.6, 0.73 and 0.66 fold increase respectively as compared to the control (fig. 3).

Determination of molecular weight by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is one of the molecular techniques used for the characterization of bacterial macromolecules and is of significant importance (Vos et al., 1995).

Fig.1 Effect of nitrifiers on ammonia removal in simulated ecosystem with prawn. Data are expressed as mean±SE

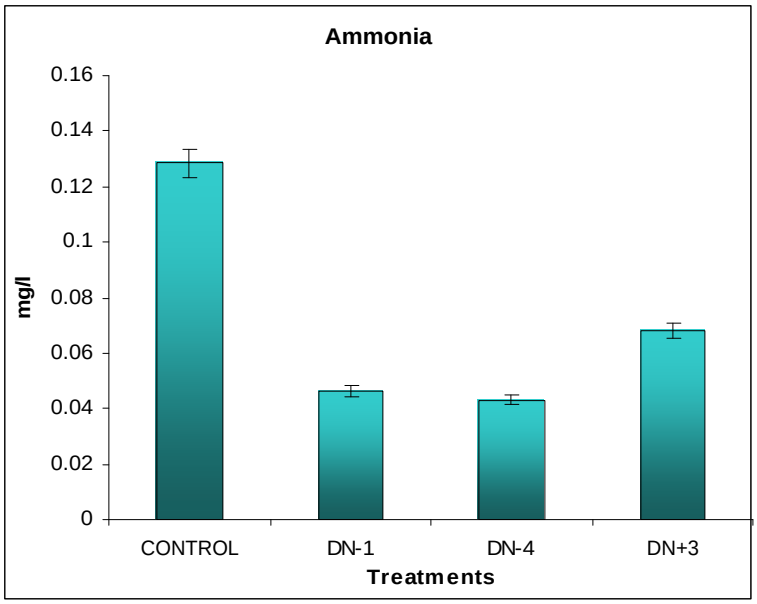


Fig.2 Effect of nitrifiers on nitrite removal in simulated ecosystem with prawn. Data are expressed as mean±SE

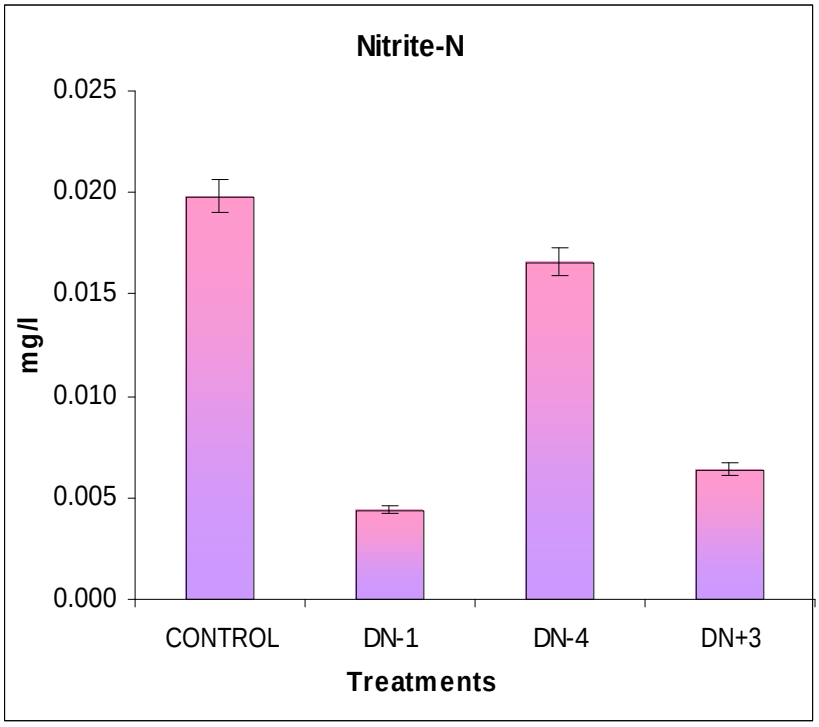


Fig.3 Effect of nitrifiers on nitrate enrichment in simulated ecosystem with prawn. Data are expressed as mean±SE

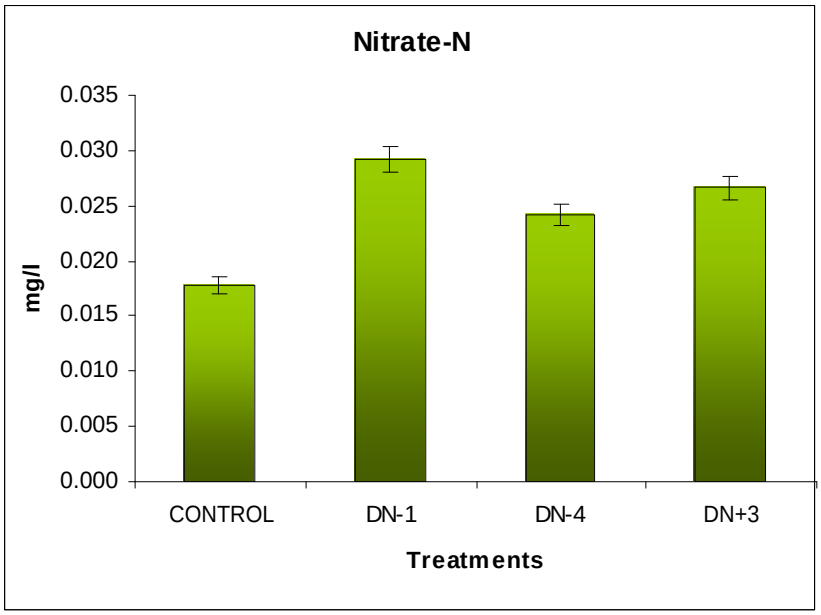
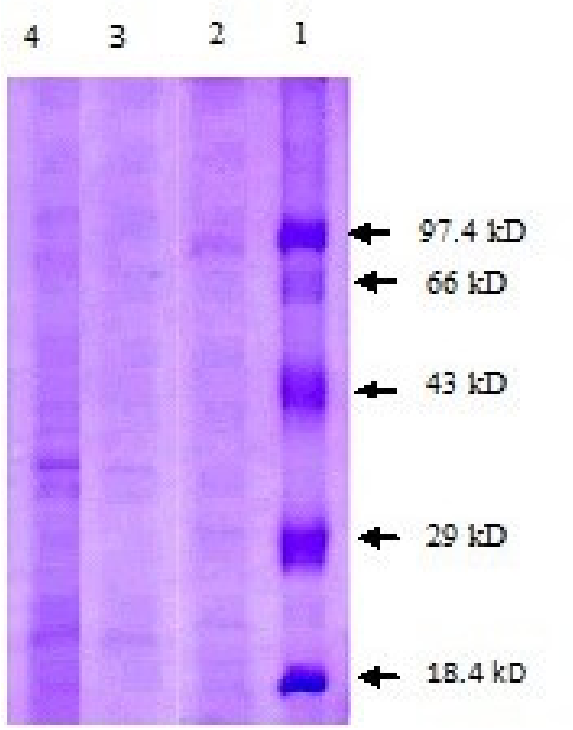


Figure.4 SDS-PAGE Analysis of whole cell lysates of nitrifiers in simulated ecosystem with prawn. Lanes (R-L) 1-molecular weight marker (18.4-97.4), 2- DN-1; 3- DN-4; 4-DN+3



It has the advantage of being fairly simple and rapid to perform (Durrani et al., 2008). In the current study, protein profiling was done to determine the molecular weight of gram negative and gram positive nitrifying bacteria through SDS-PAGE analysis (fig. 4). ECP of these nitrifiers produced 4-9 polypeptide bands ranging from 17.5-100.6 kDa. After electrophoresis 6 prominent bands was observed in DN-1 and molecular weight was found 17.5-95.4kDa, in DN-4 4 prominent bands with mol. wt 21.8-67.1kDa while in gram positive nitrifiers DN+3, 9 prominent bands was observed with molecular wt was ranged in between 22.1-100.6 kDa. (Fig.4). The bacterial protein profiles are a reflection of the genome of the strain; therefore, determination of the whole protein content plays an important role in classification, identification, typing, and comparative studies of bacteria (Kustos et al., 1998). The current protein profile study could be used to obtain a more detailed identification and characterization of the organisms involved in the nitrification process. Finally, it can be concluded that these bacterial species could be implemented successfully in production systems to rapidly promote efficient nitrification. Further longer duration of experimentation will provide an indicator of nitrification of prawn culture system after incorporation of these microbial inoculums.

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