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Evaluation for Nutrient Utilization Pattern of *Bacillus* Spp. Isolates in Response To Water Quality Parameters In vitro

Rajashree Jena[1,2], Chiranjiv Pradhan[3], Nikhil Kumar Maiti[2] and Prasanta Kumar Choudhury[1,2*]

[1]*School of Agricultural and Bioengineering, Centurion University of Technology and Management, Odisha, India*
[2]*Microbiology Unit, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar-751002, Odisha, India*
[3]*Department of Aquaculture, Kerala University of Fisheries and Ocean Studies, Panangad, Kochi, Kerala, India*

ABSTRACT

In the current investigation efforts have been made to study the effect of Bacillus spp. on different water quality parameter in in vitro. A total of 16 (RJ1 to RJ16) bacterial strains comprising of B. subtilis (n=08), B. cereus (06) and, B. pumilis (02) were subjected to grow in synthetic pond water to evaluate the nutrient cycling and utilization pattern with respect to ammonia, nitrite, nitrate and phosphorus verses their growth rate for a 16 hrs study individually. After incubation the strains showed luxuriant growth in the media with turbidity increasing upto12 hrs and after 14hrs stationary growth was observed in most of the isolates. All the strains showed increase in ammonia concentration (NH_4-N). Highest ammonia concentration up to 50% level was observed with RJ15 and remaining isolates also increased ammonium ion concentration with the lowest range producer was RJ3 with 4%. All the strains showed nitrate reduction activity. Strain RJ12 reduced nitrate (NO_3-N) concentration (30%) from 12.04 to 8.37 mg/L. RJ5 was the lowest nitrate reducer with activity of 2%. Six strains showed reduction in nitrite concentration including RJ1 and RJ5 where the highest nitrite reduction with 38% activity was observed. All other isolates showed increased in nitrite concentration and highest was observed with RJ11 (47%). In phosphorus metabolism RJ2 was found to be utilize highest phosphorus in synthetic pond water with 32% decrease in phosphorus concentration whereas most of the strains showed no change in activity. This study showed that Bacillus spp. are ammonia producers whereas they have the potential to reduce nitrate, nitrite and utilize phosphorous in water environment.

KEY WORDS: AMMONIA, BACILLUS SPP., NITRATE, NITRITE, PHOSPHOROUS.

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*****Corresponding Author: choudhury.microbio@gmail.com
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INTRODUCTION

The genus *Bacillus* represents a major microflora in many natural biotopes, where they play an important role in ecosystem development and transform many nutrients that supports the quality of water (Lalloo et al., 2007). Water environment is connected with the accumulation of organic and nitrogenous wastes viz. ammonia, nitrite and a heavy amount of organic matter (Hlordzi et al., 2020). Increasing concentration of these wastes can be toxic to aquaculture leading to stress and mortality of fish health (Loh, 2017). Total ammonia nitrogen ($NH4^+$-N), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N) and phosphorous are utilized by many microorganisms for their metabolism, leading to nitrogen removal from the water column (Martínez Córdova et al., 2015). Nitrogenous substances mainly ammonia, nitrites and nitrates are typical water pollutants connected with aquatic environments and have drawn attention of many investigations (Boopathy et al., 2015; Liang et al., 2015).

Nevertheless, fish culture follows in a heavy load of these wastes; that require careful measures to improve the water quality. Nitrification, denitrification, ammonification, and phosphate utilization are the major steps involved in the nutrient cycling of water ecosystem. The different initial forms of nitrogen include dead plants materials, animals or their released waste organic products. This organic nitrogen present are finally converted to ammonium (NH_4+) and ammonia (NH_3) by different fungal and bacterial species counting, *Bacillus* species in ammonification process (Hlordzi et al., 2020). The biologically available nitrogen and returning it to the atmosphere from nitrification and denitrification from ammonia by *Nitrosomonas* and *Nitrobacter* species mostly (Bernhard, 2010). However, *Bacillus* species present in aquaculture ecosystems (Mohanty et al., 2011) play a unique role in the nitrogen cycle through ammonification (Hui et al., 2019), nitrification (Rout et al., 2017), and denitrification (Verbaendert et al., 2011) as well as nitrogen fixation (Yousuf et al., 2017).

This activity is dissimilar to the processes govern by Nitrosomonas and Nitrobacter that are primarily associated in nitrification and denitrification (Liu et al., 2020). As far as *Bacillus* species is concerned B. amyloliquefaciens DT has observed to be able to covert organic nitrogen into ammonium (Hui et al., 2019) and *Bacillus* cereus PB8 removed NO_2-N from wastewater as reported by Barman et al., 2018. This signifies that *Bacillus* species have the potential to eliminate the different forms of toxic nitrogen waste from aquaculture ecosystem. Furthermore, *Bacillus* sp. proved to be a budding probiotic applicant which can improve aquaculture water quality and digestibility of feed (Dat et al., 2019). Keeping in view towards the improvement of water quality and application of these bacteria as probiotic candidate in aquaculture, the study was undertaken to observe the utilization pattern of soluble nutrients in freshwater ecosystem by application of *Bacillus* spp. *in vitro*.

MATERIALS AND METHODS

Procurement of *Bacillus* spp. isolates: A total of 16 isolates designated as RJ1 to RJ16b were taken into the investigation, already isolated from the same study conducted at Fish Health management Division, ICAR-Central Institute of Freshwater Aquaculture, Odisha, India. All the isolates were revived from 10% glycerol stock and maintained at Tryptone Soya Broth (TSB, Himedia, Mumbai) as broth culture for further use in the experiment. The purity and activity were checked microscopically as per standard microbiological procedures.

Preparation of standard curves: Standard curve for ammonia ($NH4^+$-N): For the preparation of standard curve for ammonia, ammonium chloride was taken as a standard curve reagent. Ammonium chloride was oven dried at 100 overnight and 382.07mg (100 mg $NH4^+$-N/L) was taken and dissolved in 1L of double distilled water (DDW). From this solution 20 mL was taken and added to 480 mL of DDW to make final conc. of 4 mg/L. This ammonium solution was used for preparation of different conc. of $NH4^+$-N mg/L. Different concentrations of the solution 0.00, 0.04, 0.08, 0.2, 0.4, 0.6, 0.8, 1, 1.4, 1.8, 2 mg/L were made by diluting ammonium chloride solution with DDW to make volume of 1 ml in eppendorf tubes in triplicates.

In eppendorf tubes to every dilution 40 µL of phosphate buffer (5% Na_3PO_4 solution), was added using a micropipette and then mixed well. Then 100 µL of ammonia reagent A [40 mg sodium-nitroprusside in 30 mL stock phenol solution (11.1 mL of liquefied phenol with 95% of v/v ethyl alcohol/ 100 mL] followed by 50 µL of ammonia reagent B (Equal volume of) sodium hypochlorite and 27% sodium hydroxide) was added and mixed properly. Then the tubes were transferred to dark place for 1 hr. After 1hr the absorbance was measured against blank (DDW) at 635 nm in an UV visible spectrophotometer (6050 UV/VICS spectrophotometer, Jenway). Then standard curve was plotted for the absorbance of each of the standard solutions against its concentration of NH4- N mg/L.

Standard curve for nitrate (NO_3-N): For the preparation of standard curve for nitrate, potassium nitrate was taken as a standard curve reagent. Potassium nitrate was oven dried at 100 overnight and 722.21mg (100 mg NO3-N/L) was taken and dissolved in 1L of DDW. From this solution 20 mL was taken and added to 480 mL of DDW to make final conc. of 4 mg/L. This nitrate solution was used for preparation of different conc. of NO3-N mg/L. Different concentrations of the solution 0.00, 0.04, 0.08, 0.2, 0.4, 0.6, 0.8, 1, 1.4, 1.8, 2 mg/L were made by diluting potassium nitrate solution with DDW to make volume of 1 mL in eppendorf tubes in triplicates. In eppendorf

tubes to every dilution 40 µL of 0.5 N HCl was added using a micropipette and then mixed well. After 30 min the absorbance was measured against blank (DDW) at 220 nm in an UV visible spectrophotometer (6050 UV/VICS spectrophotometer, Jenway). Then standard curve was plotted for the absorbance of each of the standard solutions against its concentration of NO3-N mg/L.

Standard curve for nitrite (NO_2– N): For the preparation of standard curve for nitrite, sodium nitrite was taken as a standard curve reagent. Sodium nitrite was oven dried at 100 overnight and 492.85 mg (100mg NO2-N/L) was taken and dissolved in 1L of DDW. From this solution 20 mL was taken and added to 480 mL of DDW to make final conc. of 4 mg/L. This nitrate solution was used for preparation of different conc. of NO2-N mg/L. Different concentrations of the solution 0.00, 0.04, 0.08, 0.2, 0.4, 0.6, 0.8, 1, 1.4, 1.8, 2 mg/L were made by diluting sodium nitrite solution with DDW to make volume of 1 mL in eppendorf tubes in triplicates. In eppendorf tubes to every dilution 40 µL of colour reagent (160 mL of distilled water was taken and 20 mL of 85% H3PO4 was added and mixed. Then 2 gm of sulphanilamide and 0.2 gm of N (1-napthyl) ethylene diamine dihydrochloride (NEDD) was added and final volume was made up to 200 mL with distilled water) was added using a micropipette and then mixed well. After 10 min the absorbance was measured against blank (DDW) at 543 nm in an UV visible spectrophotometer (6050 UV/VICS spectrophotometer, Jenway). Then standard curve was plotted for the absorbance of each of the standard solutions against its concentration of NO2-N mg/L

Standard curve for phosphorus (PO4-P): For preparation of standard curve for phosphorus, potassium dihydrogen phosphate was taken as a standard curve reagent. Potassium dihydrogen phosphate was oven dried at 100 overnight and 439.42 mg (100 mg PO4 -P/L) was taken and dissolved in 1L of DDW. From this solution 20 mL was taken and added to 480 mL of DDW to make final conc. of 4 mg/L. This phosphorus solution was used for preparation of different conc. of PO4-P mg/L. 2. Different concentrations of the solution 0.00, 0.04, 0.08, 0.2, 0.4, 0.6, 0.8, 1, 1.4, 1.8, 2 mg/L were made by diluting potassium dihydrogen phosphate solution with D. D.W to make volume 1 mL in eppendorf tubes in triplicates. In eppendorf tubes to every dilution 40 µL ammonium molybdate reagent (6.5 gm ammonium molybdate was mixed with 43 mL of distilled water and 62.5 mL of conc.

H_2SO_4 was mixed with 100 mL distilled water and, final volume was made up to 250 mL) was added using a micropipette and then 40 µL of SnCl2 (5gm $SnCl_2$ was mixed with 200 mL of glycerol) was added and mixed well. After 10 min but before 12 min the absorbance was measured against blank (DDW) at 590 nm in an UV visible spectrophotometer (6050 UV/VICS spectrophotometer, Jenway). Then standard curve was plotted for the absorbance of each of the standard solutions against its concentration of phosphorus mg/L.

Nutrient cycling parameters assay: The study was conducted according to Lalloo et al., 2007. The isolated *Bacillus* sp. were pre-incubated in TSB at 30 for 24 hrs for luxuriant growth. On next day the cultures were centrifuged at 10,000 rpm for 10 min and the supernatant was decanted. The pellets formed were suspended in 10 mL sterile NSS and washed twice and were resuspended in 10 mL sterile NSS. Synthetic pond water [KNO_3-0.0085, $NaNO_2$-0.006, $(NH_4)_2SO_4$-0.0093, H_3PO_4-0.0038, Yeast extract- 0.1, Glucose-0.1 and final pH was adjusted to 7.0 ± 0.2, suspend the constituents in 1L distilled water and adjust the pH with liquor ammonia (25%), then filter the media using 0.22 µm membrane filters] was prepared and 300 mL was filtered to sterile flasks through 0.22 µm membrane filter (Millipore). About 300 µL (1% v/v) of the culture was inoculated in the filtered media and incubated in water bath at 100 rpm/min maintaining the temperature at 30.

Initial sample was collected, and after in every 2 hr the culture medium was taken out and the supernatant was used to see specific growth rate and determine the respective ions concentrations in the synthetic pond water up to the entry of stationary phase, when no change in optical density of cell biomass was observed. The rate of increase or decrease in ion concentrations (ammonia, nitrate, nitrite and, phosphorus) by each *Bacillus* sp. in synthetic pond water was determined by using the standard curve methods mentioned above and calculated using the straight-line curve equation. Data Analysis: The data of the experiments were represented in terms of concentration of ammonia (NH4-N), nitrate (NO3-N), nitrite (NO2-N) and phosphorous (PO4-P) in mg/L verses incubation time. The growth (biomass production) optical density was presented in tertiary axis in relation to hrs of incubation for all the 16 *Bacillus* sp. isolates.

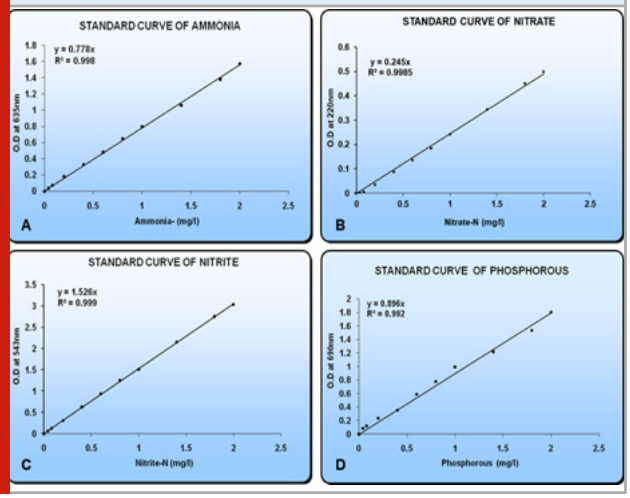
RESULTS AND DISCUSSION

Microbial culture and propagation: All the procured culture isolates were observed to be Gram positive, rod shaped either in single or in a chain as observed microscopically. All are able to grow aerobically and showed endospore formation. The growth propagation showed luxurious growth in TSB media, which are further used for nutrient utilization assay in synthetic pond water.

Standard curve preparation: The standard curve for NH4+-N, NO_3-N, NO_2-N and PO_4-P prepared were depicted in Fig. 1 [A], [B], [C] and [D] respectively. All

the graphs showed R2 value of more than 0.99, showing a potential procedure of preparation of standard curve that can be applied for estimation these water quality parameters. Standard curve of NH4+-N showed y=0.778x (Fig. 1A), NO3-N showed 0.245x (Fig. 1B), NO2-N showed y=1.526x (Fig. 1C) and PO4-P showed y=0.896x (Fig. 1D) respectively. The amount of these nutrients present in the synthetic pond water were after incubation of *Bacillus* spp. isolates were estimated using the graph equations.

Figure 1: Photograph showing different standard curves for Ammonia nitrogen (mg/L) [A], Nitrate nitrogen (mg/L) [B], Nitrite nitrogen (mg/L) [C] and Phosphorous (mg/L) respectively.



Nutrient cycling parameters: The nutrient cycling parameters of NH4+-N, NO_3-N, NO_2-N and PO4-P with respect to incubation period were depicted in Fig. 2[A], [B], [C] and [D]; Fig. 3[A], [B], [C] and [D]; Fig. 4[A], [B], [C] and [D]; fig. 5[A], [B], [C] and [D] for all the 16 *Bacillus* sp. isolates. The results showed that all the isolates attended stationary phase after 14 hrs of growth in synthetic pond water as observed in plateau in biomass production curve with the supplemented 1% inoculum presented in X axis of all the figures. All the isolates were observed to be ammonifiers as increase in NH4+-N, was found after incubation of 8 hrs in most of the isolates. Highest ammonia concentration up to 50% level was observed with RJ15 and remaining isolates also increased ammonium ion concentration with the lowest range producer was RJ3 with 4%. All the strains showed nitrate reduction activity. Strain RJ12 reduced nitrate (NO3-N) concentration (30%) from 12.04 to 8.37 mg/L whereas RJ5 was the lowest nitrate reducer with activity of 2%. Six of all the strains showed reduction in nitrite concentration including RJ1 and RJ5 where the highest nitrite reduction with 38% activity was observed. All other isolates showed increased in nitrite concentration and highest was observed with RJ11 (47%).

This study was supported with the findings of Lalloo et al. (2007, but a number of studies stated that B. amyloliquefaciens (Xie et al., 2013); B. subtilis (Cha et al., 2013; Zokaeifar et al., 2014) and B. megaterium (Hura et al., 2018), to reduce ammonia level in aquaculture practices. A study in carp rearing water after the addition of *Bacillus* sp. total ammonia nitrogen compared to the control was found to be reduced although nitrate level as found to increased (Naderi Samani et al., 2016). In connection to these findings Reddy et al. (2018) also recorded decreased ammonia nitrogen and illustrates that *Bacillus* spp. have the capacity to mineralize nitrogenous wastes through nitrification and denitrification (Nimrat et al., 2012).

Figure 2: Nutrient utilization pattern for ammonia, nitrate, nitrite and phosphorous of the isolates RJ1, RJ2, RJ3 and RJ4 in synthetic pond water depicted in A, B, C and D respectively.

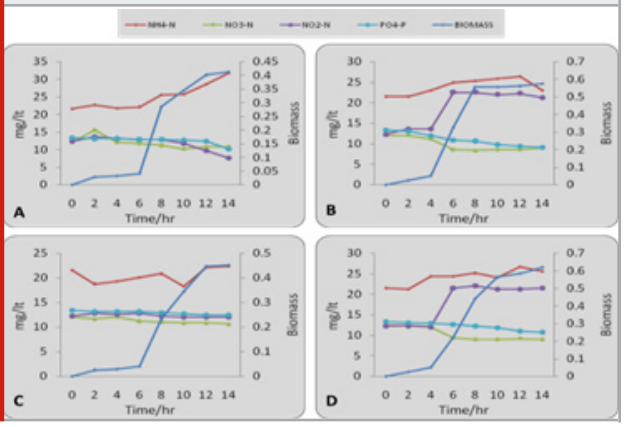
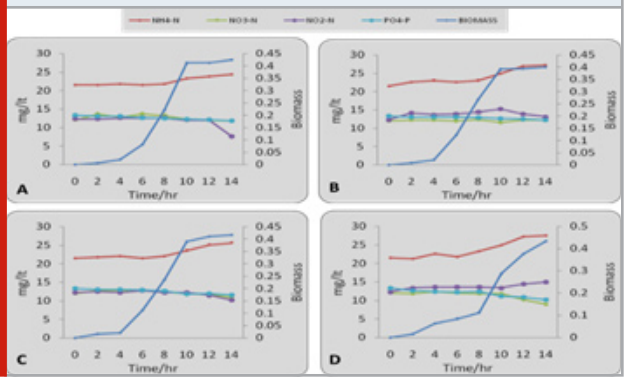


Figure 3: Nutrient utilization pattern for ammonia, nitrate, nitrite and phosphorous of the isolates RJ5, RJ6, RJ7 and RJ8 in synthetic pond water depicted in A, B, C and D respectively.



Although we have incorporated all the nutrients so for nitrification and denitrification the directly the NO3-N, NO2-N were consumed not the NH4-N. Thurlow et al. (2019), observed reduced nitrate-nitrogen (75%) and total nitrogen (43%) in catfish pond water treated with *Bacillus* velezensis AP193. A long-term incubation may result to

further demineralization as observed in our study. But mostly *Bacillus* spp. are recommended as ammonifiers by its taxonomic classification and also there may be a strain variation as the reported experiments are not for the same bacterial species used as in our study.

Figure 4: Nutrient utilization pattern for ammonia, nitrate, nitrite and phosphorous of the isolates RJ9, RJ10, RJ11 and RJ12 in synthetic pond water depicted in A, B, C and D respectively.

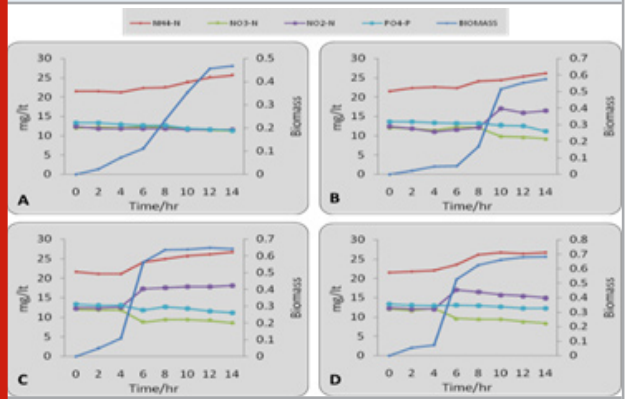
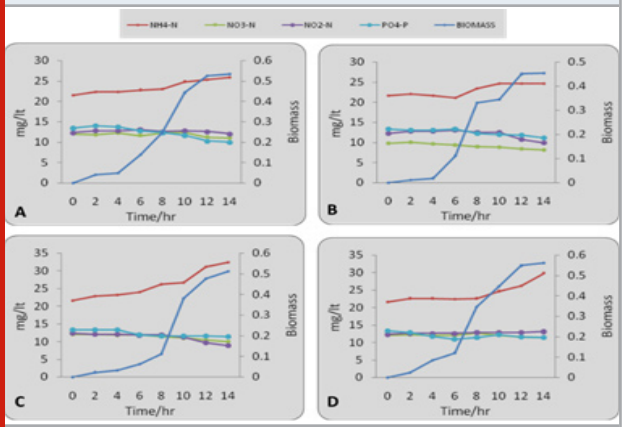


Figure 5: Nutrient utilization pattern for ammonia, nitrate, nitrite and phosphorous of the isolates RJ13, RJ14, RJ15 and RJ16 in synthetic pond water depicted in A, B, C and D respectively.



In phosphorus metabolism RJ2 was found to be utilize highest phosphorus in synthetic pond water with 32% decrease in phosphorus concentration whereas most of the strains showed no change in the activity. Like nitrate accumulation phosphate also leads to algal bloom in fish culture systems (Lalloo et al., 2007) as required for biological processes. The increase in phosphate concentration leads to eutrophic water (Luo et al., 2016; Reddy et al., 2018) and Bacillus spp. have been observed as potential phosphate solubilizers. In many studies it has been observed that *Bacillus* spp. can reduce phosphate ions and 81% reduction was reported (Reddy et al., 2018) in a study having equal proportions of *B. subtilis, B. mojavensis* and, *B. cereus*.

Also, it was evidenced in the presence of pathogens also (Lalloo et al., 2007) In a pond treated with *Bacillus* spp. decreased phosphorus levels was found as compared to control of shrimp culture ponds (Wang et al., 2005). Total phosphorus reduction was also documented in catfish ponds treated with B. velezensis (Thurlow et al., 2019). The finding of this study proved that *Bacillus* spp. are ammonifiers, denitrifies and have the ability to mineralize phosphorous, however *in vivo* study in aquaculture ponds are necessary to draw final conclusion.

Conflict of Interests: We declare that no competing interests exist among the authors of this article.

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