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NEUTRALIZATION OF TOXINS IN AQUA CULTURE USING PROBIOTICS

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ABSTRACT: Bioremediation is a waste management method where we can employ different types of microorganism's to nullify the effect of pollutants from a contaminated site. In the current study the authors have carried out a three months study to find out how different bacterial strains of *Bacillus licheniformis, B. substilis* and pseudomonas species can be used to assess the water quality in terms of parameters like pH, dissolved oxygen, free ammonia, total ammonia and nitrite and turbidity from the discharge of common ponds. The study was carried to evaluate the physico-chemical parameters of the catla and selavathi culture ponds by applying probiotics. During the process of investigation one pond was treated with probiotics having *Bacillus licheniformis, B. subtilis* and *Pseudomonas* species and to study the efficacy of results the pond to which probiotics were not added was kept as control. The current study is aimed not only to focus on the changes in water quality but also biochemical characterization of bacterial species and to understand what concentration of the bacterial species is more effective in bioremediation of probiotic treated ponds and ponds not treated with probiotics.

INTRODUCTION: The beneficial microbial consortium plays an important role in natural balances in the pond, in cultivate farming, culture growth in pond, maintaining high fish or prawn densities, high feeding rate (Ecologically because fish feed have high protein content mainly from fish meal), faecal matter, blood vessels, along with (moulting) shells and left over food. The term solid is very broad and is found to be taken in relation to plankton, fish wastes, clay particles and uneaten fish feeds that are usually suspended in the water.

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The large particles which commonly settle down to the pond bottom are referred as suspended solids. During the process of fish production in aquaculture [12] ponds huge amounts of settled solids and suspended particles are obtained. The waste materials produced by fish are a major source for the water to get contaminated and hence can lead to very poor water quality [4, 9, 14, 21] as they are very likely to contain up to 70 percent of the nitrogen load in the system.

Most of the turbidity issues in pond water is often associated with the presence of phytoplankton (microscopic plants) and zooplankton (microscopic animals) and is not directly harmful to the fish. Phytoplankton (green algae) not only provides a food source for zooplankton feeding fish/shellfish, but also produces oxygen. Ammonia [14] produced by the fishes is considered as an essential nutrient

source for Phytoplankton's. Zooplankton is also considered an important food source for fishes. Owing to their ability to consume extra oxygen algae [14] can lead to increased rates of respiration during the night. Varying oxygen levels can be a major concern for low oxygen concentrations. Temperature also determines the amount of dissolved gases (oxygen, carbon dioxide, nitrogen, *etc*.) in the water. In the process called thermal stratification the physical parameter temperature plays a major role. Fish wastes, nutrients (solids) and dissolved gases are evenly mixed throughout the pond.

In the bottom layer the dissolved oxygen levels decrease due to photosynthesis and as a result the contact with air is reduced. The existing low oxygen levels show further decline through decomposition of waste products, which settle to the pond bottom. The primary metabolic waste of fish is ammonia and is primarily excreted through the gills. Ammonia occurs in two forms namely the ionized (NH_4^{+}) and un-ionized (NH_3). Among the two forms of ammonia the unionized form is deemed to be more toxic when compared to the ionized form. Both forms are collectively referred as total ammonia nitrogen. Toxic ammonia present in the ponds can be converted to non-toxic nitrates using standard biological procedures

The levels of ammonia are found to increase as and when there is an increase in temperature and hydrogen ion concentration. In terms of the hardness of water, when the hardness is found equal to the combined concentrations of carbonate and bicarbonate alkalinity, it is called as carbonate hardness. When the hardness values are comparatively greater than the sum of the carbonate and bicarbonate alkalinity, then such a condition is termed as non-carbonated hardness. There is a variation in toxicity levels for ammonia and this invariably depends on the nature of the species involved. When the levels are around 0.02 ppm, it is considered safe for the pond and the species living there. Through the process of nitrification ammonia is initially oxidized to nitrite and then terminally to nitrate. Ammonia and nitrite are by far the most toxic metabolites to the fish and other aqua species. Denitrification also results in the formation of nitrite. Ammonia concentrations have been reported as toxic to fish.

When the fishes are exposed to ammonia, there have been observed many epithelial changes in the form of epithelial liftings on the gill filaments resulting in increased risk of respiratory tract infections and mortality. Except when there is an imbalance the concentration of nitrite is generally present at low concentrations in defined natural systems, because nitrite is considered to occur as a common intermediate in nitrification [16] and denitrification, catabolic ammonification and nitrate incorporation. As a result of the denitrification process, nitrite is obtained as an intermediate in the conversion of nitrate to nitric oxide, nitrous oxide and nitrogen gas. Where water is continually recycled there is dangerously high ammonia concentrations and are usually limited to water reuse systems.

However, the intermediate form of ammonium nitrate - has been known to occur at toxic levels in fish ponds. pH, alkalinity, and hardness. The acceptable pH range for fish culture is normally between pH 6.5 and 9.0. In spite of the classical belief that this process is predominated by autotrophic bacteria, certain bacterial species like the Bacillus sp. [11, 19] also is considered for nitrogen removal. Certain species of the Bacillus group, especially one members of this group, such as *B. substilis* [19, 21, 22], are able to grow under facultative aerobic, aerobic, and anaerobic conditions, allowing for shifts in nitrogen uptake that facilitate both nitrification and denitrification.

The pattern of nitrite metabolism by *B. substilis* [21, 22] of nitrite and nitrate metabolism Nitrite oxidation is common in heterotrophic bacteria such as Bacillus species. The reduction of phosphate concentration in culture systems has also been demonstrated through addition of Bacillus species. The improvement in solubilisation of phosphate is also thought to facilitate removal of phosphate and reduction of algal blooms [14].

MATERIALS AND METHODS:

Fish Ponds: The study was carried out in Eluru town in the state of Andhra Pradesh, India. Two ponds (one as control and one treated with probiotics were selected which had two fish varieties Catla-catla (2, 500 numbers) and Selavathi (10,000 numbers) in the ratio of 1:4. The supplementary feed supplied to these ponds having

crude protein including, ground nut oil cake, rice bran, coconut oil cake, dry fish, Vitamin and minerals premix. In addition to the supplementary feed supplied certain inorganic fertilizers such as superphosphate, poultry manure, cattle dung, were applied before and after the release of fish. For the present study, the investigations were carried out for a fortnight during the period from in the culture ponds.

Probiotics: Probiotics containing, *Bacillus licheniformis, B. substili* and pseudomonas sp. manufactured at Sneha Biotech, Vijayawada, Krishna district, Andhra Pradesh, India were used for the present investigation,.

Water Samples: In the present investigation, physicochemical parameters such as temperature, transparency, dissolved oxygen, pH, nitrate, ammonia present in water samples were estimated using standard and suggested protocols. All these parameters were studied at fortnight intervals by collecting water samples in between 6 am and 11 am.

Identification of the Probiotics: The isolated probiotic bacteria were identified by morphological, cultural and biochemical characterization methods.

Morphological and Culture Characterization: Morphology and cultural characteristic studies were carried out to find out essential characteristics like the shape, colour, size, edge elevation, transparency and surface texture. One of the major concerns was to find out the genus or species of the selected bacterial isolates. Once purified the isolates were subjected to determine the motility, cell shape, flagellation, spore formation, encapsulation mechanisms and staining procedures using gram staining methods.

Biochemical Characterization: The different bacteria were then subjected to biochemical tests (Indole, methyl red, voges-proskauer, citrate utilization, starch hydrolysis, urease test, caseinolytic activity and catalase test, oxidase test, nitrate reduction) according to the method described by Bergey's manual.

Simple Staining: The bacterial smears were treated with crystal violet (60 seconds), rinsed with distilled water, air dried and observed under microscope.

Gram Staining: A thin bacterial smear was made on a clean glass slide and heat fixed. Then the smear was stained with crystal violet for 1 minute and then washed with water. Gram's iodine was added for 1 minute and decolorized with alcohol. After decolourization, the smear was counter stained with saffranin for 1 minute. Finally the smear was washed, air dried and observed under the microscope.

Indole Production Test: Peptone broth (1 %) was prepared, sterilized and incubated with the bacterial suspension and incubated at 37 ^{o}C for 48 h. The incubated sample was treated with 1ml of Kovac's reagent and shaken gently. After allowing the tubes to stand at room temperature, the results were observed. Formation of a cherry red precipitate showed positive results.

Methyl Red Test: The sterilized MR-VP broth was incubated with the bacterial cultures, 5 drops of methyl red indicator was added and the tubes were observed for a colour change to red that indicates a positive reaction.

Voges-Proskauer Test: The sterilized MR - VP broth was incubated with the bacterial cultures at 37 °C for 48 h. After incubation few drops of Barritt's reagent A and B were added and the result noted. Development of crimson to pink colour indicates a positive reaction.

Citrate Utilisation Test: Simmon's citrate agar slants were prepared and sterilized. Bacterial cultures were streaked on the surface of the slant and incubated at 37 ºC for 24 h. A change in green colour to Prussian blue indicates the positive results.

Starch Hydrolysis: Starch agar medium plates were prepared, inoculated (streaked) with the bacterial culture and incubated at 37 ^{o}C for 48 h. After incubation, the plates were flooded with Gram's Iodine. Amylase production was indicated by colourless zone and rest of the plate appeared purple.

Catalase Test: A clean glass slide was taken and a drop of culture suspension was placed on the slide. To this, few drops of hydrogen peroxide was added. A positive reaction indicates the release of air bubbles from the suspension.

Casein Hydrolysis: Skim milk agar medium plate were prepared and inoculated with the bacterial culture by streaking and incubated at 37 ºC for 48h. The opaque zones surrounding the microbial growth consist of casein milk powder, indicating protease activity.

Nitrate Reduction Test: Nitrate broth was prepared, sterilized and incubated with the bacterial culture and then incubated at 37 ºC for 48 h. Presence or absence of nitrate was identified by adding a few drops of sulfanilic acid and naphthylamine reagent to each of the tubes. Results were observed without shaking the tubes. A distinct red colour that may slowly convert into brown indicates that nitrate has been reduced.

Oxidase Test:

Wet Filter Paper Method: A 1% solution of the reagent (tetra methyl-p-phenylene-diamine di-hydrochloride was prepared and strip of filter paper was soaked. A small amount of the culture was rubbed on it with the help of a platinum loop. A positive reaction is indicated by the formation of a deep purple colour within 5 - 10 seconds, a delayed positive reaction by the presence of blue colouration in 10- 60 sec, and even after 60 seconds if no colour is formed it indicates a negative reaction.

RESULTS: After carrying out the above tests the results of analysis were tabulated. **Table 1** below shows the results of water analysis in ponds over a period of three months, with day 1 as control , for different parameters like pH, total ammonia, free ammonia, dissolved oxygen and nitirite levels which were treated prior to (day 1) and after addition of probiotics (day 2 - day 90). A series of Biochemical characterization tests were carried out to identify *B. Substilis*, *Bacillus licheniformis* and Pseudomonas species bacteria from pond water and the results are shown in **Table 2**. Variation of physicochemical parameters day wise before and after treatment of probiotics is depicted in **Fig. 1**. The first day values are treated as control.

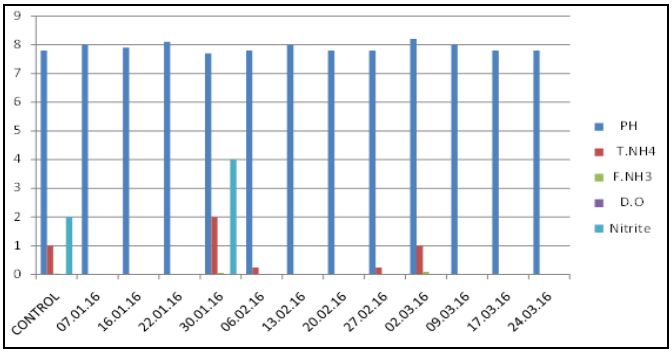

FIG. 1: VARIATION OF PHYSICOCHEMICAL PARAMETERS DAY WISE BEFORE AND AFTER TREATMENT OF PROTBIOTICS (DAY 1 IS CONTROL)

TABLE 1: BIOCHEMICAL TESTS CONDUCTED FOR IDENTIFICATION OF BACTERIA FROM POND WATER

Biochemical tests	*Bacillus substilis*	*Bacillus licheniformis*	Pseudomonas sp.
Indole Test	Negative	Negative	Negative
Methyl red	Negative	Negative	Negative
Voges-proskauer	Positive	Positive	Negative
Citrate utilisation	Positive(blue)	Positive (blue)	Positive (blue)
Catalase	Positive (release of air bubbles)	Positive	Positive
Oxidase	Positive (formation of opaque zones)	Positive	Positive
Motility	Motile	Motile	Motile
Urease	Negative	Negative	Negative
Nitrate	Positive (red colour)	Positive (red colour)	Positive
H_2S production	Negative	Negative	Negative

TABLE 2: PHYSICOCHEMICAL CHARACTERISTICS OF POND BEFORE AND AFTER TREATMENT WITH PROBIOTICS

Test	pH	T. NH_4	F. NH_3	D. O	Nitrite
Control	7.8	1	0.038	0	2
07.01.16	8	0	0	0	0
16.01.16	7.9	0	0	0	0
22.01.16	8.1	0	0	0	0
30.01.16	7.7	2	0.06	0	4
06.02.16	7.8	0.25	0.01	0	0
13.02.16	8	0	0	0	0
20.02.16	7.8	0	0	0	0
27.02.16	7.8	0.25	0.01	0	0
02.03.16	8.2	1	0.098	0	0
09.03.16	8	0	0	0	0
17.03.16	7.8	0	0	0	0
24.03.16	7.8	0	0	0	0

DISCUSSION: In the current study, after reviewing the literature it was proposed to introduce probiotics prepared in our laboratory by including a number of bacterial species, to study the water quality in ponds. In order to achieve the above said objectives, *Bacillus substilis* [21], *Bacillus licheniformis* [19] and Pseudomonas species [19] bacterial isolates from ponds were identified and a series of biochemical tests were carried out.

For the isolates, the probable identity of each genera include: *Bacillus substilis* [11, 21, 22] *Bacillus licheniformis* [19] and Pseudomonas species [19] were all found to be gram positive and having a characteristic rod shaped morphology. *Bacillus substilis* [11, 21, 22] showed negative biochemical reactions with methyl red test, indole test and H_2S tests and showed positive results with oxidase, catalase and nitrate test.

Similar tests were also conducted for *Bacillus licheniformis* [19] and Pseudomonas species [19] the positive and negative results have been duly tabulated in **Table 1**. From the above tests the probable identification of isolates were found to be with reference to: *Bacillus substilis* [11, 21, 22], *Bacilus licheniformis* [23] and Pseudomonas species [19]. When compared to earlier studies carried elsewhere it was observed that Bacteriological nitrification [16, 17] is the most practical method for the removal of ammonia from closed aquaculture systems. According to earlier studies of *B. substilis* [21, 22], are able to grow under aerobic, facultative aerobic and anaerobic conditions, allowing for switches in nitrogen metabolism that facilitate both nitrification [16, 17] and denitrification.

The pattern of nitrite metabolism by *B. substilis* [21, 22] of nitrite and nitrate metabolism nitrite oxidation [16] common in heterotrophic bacteria such as Bacillus sp. The reduction of phosphate concentration in culture systems has also been demonstrated through addition of Bacillus species. The improvement in of phosphate, through solubilisation, is also thought to facilitate removal of phosphate and reduce of algal blooms [14]. Certain Bacillus species also alters the pH slightly towards the acidic range, When compared to earlier studies it is quite evident that probiotics are effective in bioremediation of fish aquaculture [12, 20, 21] ponds. Earlier studies have shown the effect of bioremediation to improve the health of aquaculture. In the current study bioremediation has been effectively carried out using probiotics and hence the study is more viable. Usage of probiotics showed stable pH and other physicochemical constituents and at the same time prevented the accumulation of organic sludge [15, 17] at bottom of the pond as well as formation of toxic gases [15] like NH_3, NO_2, H_2S and thus helped to improve water quality [1, 21] and subsequently fish health.

CONCLUSION: The management of pond microbial ecology is an area where applied research can lead to important findings for improving the productivity and environmental friendliness of the fish farming industry worldwide. The use of bioremediators will gradually increase and the success of aquaculture in future may be synonymous with the success of bioremediators that, if validated through rigorous scientific investigation and used wisely, may prove to be a boon for the aquaculture [12, 20, 21] industry.

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CONFLICT OF INTEREST: Nil

REFERENCES:

1. Swann LD: A Fish Farmer's Guide to Understanding Water Quality, Department of Animal Sciences, Ill inois-Indiana Sea Grant Program, Purdue University.
2. Samani MN, Jafaryan H, Gholipour H, Harsij M and Farhangi M: Effect of different concentration of profitable Bacillus on bioremediation of common carp (crprinu scarpio) pond discharge, P. Iranian Journal of Aquatic Animal Health, 2016; 2(2): 44-54.
3. Akpor OB and Muchie M: Bioremediation of polluted wastewater influent: Phosphorus and nitrogen removal. Scientific Research and Essays, 2010; 5: 3222-3230.
4. Bhatnagar A and Devi P: Water quality guidelines for the management of pond fish culture. International Journal of Environmental Sciences 2013; 3(6).
5. Claude E, Boyd, Craig S. Tucker and Viriyatum R: Interpretion of pH, acidity and alkalinity in aqua culture and fisheries; North American Journal of Aquaculture. 2011; 73(4).
6. Http:/Thefishsite.com/Article/Bioremediation-An-Advanced-Strategy-To-Restore-The–Health-Of-AquaculturePond-Ecosystems.
7. Lakshmanan R and Soundarapandian P: Effect of commercial probiotics on large scale culture of black tiger shrimp *Penaeus mondon* (Fabricius), Research Journal of Microbiological 2008; 3(3): 198.
8. Mohammed Ghouse S: Use of probiotics as biological

control agents in aquaculture for sustainable development. International Journal of Food, Agriculture, Veterinary Sciences, 2015; 5(1): 112-119.
9. Padmavathi P, Sunitha K and Veeraiah K: Efficacy of probiotics in improving water quality and bacterial flora is fish ponds, African Journal of Microbiology Research 2012; 6(49): 7471-7478.
10. Devi RPI and Gowri VS: Biological treatment of a culture discharge waters by seaweeds, J. of Industrial Pollution Control, 2007; 23(1): 135-140.
11. Raja S, Ganesan S, Sivakumar K and Thangaradjou T: Screening of marine actinobacteria for amylase enzymes inhibitors. Indian J Microbiol, 2010; 50: 233-37.
12. Barad MM, Bhatt AJ, Singh V and Sarman V: Live feed importance, constrains and future prospects in Aquaculture production system, International Journal of Multi disciplinary Educational Research, 2017; 6 (8.2).
13. Akter MN, Parvez I and Patwary ZP: Beneficial effects of Probiotics in aquaculture, International Journal of Fisheries and Aquatic studies, 2016; 4(5): 494-499.
14. Sergeant C: The management of ammonia levels in an aquaculture environment, Pollution News Articles-Pollution Solutions Online, 2014.
15. George EGJ, Jeyaraj GP and Balaraman D: Bacillus probiotic strains of ecotoxnil as exo-friendly and efficient Bio-decomposing agent in curbing sludge and toxic gases from litopenaeus vannamei (Boone, 1931) Shrimp Culture Ponds, International Journal of Fisheries and Aquatic Studies 2017; 5(5): 283-291.
16. Daims H, Lebedeva EV, Pjevac P, Han P, Herbold C, Albertsen M, Jehmlich N, Palatinszky M, Juliavierheilig, Bulaev A, Kirkegaard RH, von Bergen M, Rattei T, Bendinger B, Nielsen PH and Wagner M: Complete nitrification by Nitrospira Bacteria/Nature, Biogeo chemistry Environmental Microbiology, 2015; 528: 504 509.
17. Narihiro T: Microbes in the water Infrastructure: Underpinning Our Society, Microbes and Environments, 2016; 31(2): 89-92.
18. Bezuneh TT: The role of microorganisms in distillery wastewater treatment: A Review/ Open Access Journals, 2016.
19. Sankaran K, Pisharody L, Narayanan GS and Premalatha M: Bacterial assisted treatment of anaerobically digested distillery wastewater, RSC Advances, 2015; 87.
20. Nemutanzhela ME, Roets Y, Gardiner N and Lalloo R: The use and benefits of Bacillus based biological agents in aquaculture. Intech, 2014.
21. Gazi M. Uddin N, Larsen MH, Christensen H, Aarestrup FM, Phu TM and Dalsgaard A: Identification and antimicrobial resistance of bacteria isolated from probiotic products used in shrimp culture, Journals. Plosone. 2015.
22. Olmos J and Paniagua-Michel J: *Bacillus subtilis*: A potential probiotic bacterium to formulate functional feeds for aquaculture, Journal of Microbial & Biochemical Technology. 2014.
23. Swapna B, Venkatrayulu C and Swathi AV: Effect of Probiotic Bacteria *Bacillus licheniformis* and *Lactobacillus rhamnosus* on growth of the Pacific white shrimp Litopenaeus Vannamei (Boone, 1931), Pelagia Research Library, European Journal of Experimental Biology, 2015; 5(11): 31-36.

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