

Study of Water Quality and Biochemical Characterization of Bacterial Isolates from Water Samples of Ponnagi Area in Krishna District

Jyothi Kshatri^{1*}, C.V. Rao¹ and Vijaya Saradhi Settaluri²

¹Sneha Biotech, Chukkapalli Vari Street, Labbipet, Vijayawada-520010, Andhra Pradesh, India.

²Associate Professor, Department of Biotechnology, K L University, Vaddeswaram-522502, Andhra Pradesh, India.

<http://dx.doi.org/10.13005/bbra/2551>

(Received: 31 July 2017; accepted: 09 August 2017)

Aquaculture also known as fish farming is one of the methods for breeding rearing and harvesting of freshwater and marine species of fish and shellfish, in ponds, rivers, lakes and oceans. In this article an attempt has been put forth to study and apply techniques that could help in improving the water quality in aquaculture ponds and to further understand the role played by enzymes and probiotics by means of bio remedial procedures and protocols. Aquaculture productivity needs to be improved to cater the ever-increasing demand, no doubt, but simultaneously a proactive role for environment protection is required. The gathering of organic wastes worsens the water quality and reduces the level of dissolved oxygen in the fish aquaculture ponds. This further increases the formation of toxic metabolites such as ammonia (NH₃), nitrite (NO₂⁻) and hydrogen sulphide (H₂S). Hence, NH₄⁺ or NO₃⁻ removal processes (nitrification and denitrification) become essential for the pond water quality. This can be carried out by applying different types of nitrifying and denitrifying bacteria such as Nitrosomonas, Nitrobacter and Alcaligenes. In the present investigation, 2 earthen ponds were selected from Ponnagi near Eluru in Krishna district, Andhra Pradesh, India. One pond (Pond A) was treated with probiotics having Nitrosomonas, Nitrobacter and Alcaligenes sps. And the other pond (Pond B) was kept as control. During the culture period, the water samples were collected from probiotics treated and control ponds for analysis of water quality parameters. The current study is aimed to focus on the changes in water quality and biochemical characterization of bacterial isolates from water samples of Ponnagi area in probiotic treated ponds and to compare the results with ponds not treated with probiotics.

Keywords: Probiotics, Microorganisms, quality, characterization, ponds, Aquaculture.

At present the fastest growing food production industry in the world is Aquaculture (Josupieh.H; *et al*, 2000). The aquaculture market has been growing at impressive rates in recent years, which is expected with our growing population. Supplying fish sustainably is a challenge as ponds and hatcheries are easily contaminated with biomass, shell, faecal matter

and other pollutants. These contaminants reduce the levels of O₂ and produce ammonia and other gases that are harmful to species and lead to disease. Excessive and irresponsible harvesting can create large scale disease outbreaks, such as early mortality syndrome seen in recent years in some parts of the world.

The culture environment becomes unstable, with the increase in fish biomass, and is highly susceptible to changes in water quality conditions. Due to such changes there may be a direct effect on the health of the fish, and for

* To whom all correspondence should be addressed.
E-mail: snehabiotech@yahoo.com

this purpose the water quality has to be regularly monitored on a daily basis and managed effectively. When the fish is exposed to toxic gases, it suffers from severe stress and this stress finally terminates in a disease (Irianto *et al* 2002). The occurrence of disease primarily depends on the existing soil conditions and the deteriorating water quality. The present study revealed that the probiotic bacteria in aquaculture to improve water quality (Padmavathi *et al*, 2012). In this context wide use and application of probiotics would be the most sought after technology for bioremediation in fish aquaculture ponds. Application of probiotics and/or enzymes to ponds is a new and emerging eco-friendly technology which is being used for improving the water quality in aquaculture ponds. This could be effectively achieved through “Bioremediation”, which involves proper use and application of microorganisms in fish ponds to supplement mineralization of organic matter and at the same time get rid of disagreeable waste compounds. When microorganisms and/or the products obtained from them are used as accessories to improve the water quality, these enhancers of water quality are referred as “Bioremediators” or “Bioremediating agents” (Moriarty, 1998). The application of Bioremediation to improve water quality in aquaculture can be enhanced by performance of probiotics. Probiotics as an efficient biological treatment method (Akpor O. B. & Muchie M, 2010), which is considered as biocontrol agent is related to the elimination of waste like parasites or specific pathogens (Moriarty 1998).

Moriarty (1998) proposed that the microbial probiotics can effectively act as water additives (Burford *et al*, 2003; Devaraja *et al*, 2002; Vezzulli *et al*, 2004). A few bioremediators which have occupied potential place in the market include *Bacillus* species, Nitrifiers, pseudomonas and some sulphur containing bacteria.

A number of waste products are released during aquaculture, different forms of these wastes include, residual food and faecal matter, residues related to biocides and biostats, metabolic by products, wastes derived from fertilizers, wastes produced during the processes of moulting and also wastes produced due to collapsing algal blooms. (Sharma And Scheeno, 1999). Ammonia is considered as the major excretory product in most of the aquatic species. Accumulation of ammonia in

ponds is a critical water quality actor as it is terribly toxic to fishes and other invertebrates. Ammonia is excreted as waste product from gills, kidneys and also during normal respiration by fishes, prawns and shrimps. . Due to an increased microbial activity in ponds and rivers Ammonia production is also seen from unconsumed feeds, shell moults of prawn and shrimp, dead algae, zooplankton etc. At optimum levels the concentration of ammonia should be below 0.1mg/L (total ammonia). The best way to achieve these levels is to use bioremediators, which can initiate and effectively carry out the oxidation of ammonia to nitrate and utilize other bacterial species, which could effectively convert nitrite to nitrate. Nitrite is formed either by the reduction of nitrate (denitrification) or by the oxidation of ammonia (nitrification). The presence of nitrite in ponds is deemed to be toxic to many fish and a few invertebrates and hence its levels should be significantly maintained below 0.1mg/L. In general, nitrate should be maintained below 50mg/L (measured as NO_3^-) but it is a very crucial factor in maintaining the water quality. The most common method to reduce nitrate is to regularly change the water and grow fresh plants. A denitrifying bioremediator generates an anaerobic atmosphere where anaerobic bacteria can effectively grow and reduce nitrate to nitrogen gas. (Rao *et al*, 2002), (Kurosu O *et al*, 2001).

A few corrective measures like optimising the nitrification rates to lower the concentration of ammonia; optimising denitrification process to eliminate excess oxygen from ponds in the form of H_2 gas; maintaining oxidation of sulphide to reduce accumulation of H_2S ; make best use of carbon mineralization to CO_2 ; minimizing sludge accumulation; increasing the primary productivity that stimulates shrimp or fish production and secondary crops; and finally maintaining a diverse and stable pond community where undesirable species do not become dominant are a few steps which help in successful bioremediation (Bratvold *et al*, 1999). In the current study, the authors have used probiotic bacteria in fish culture ponds of ponnagi near Eluru, to improve water quality by removing unwanted gases toxic to the fish ponds using probiotic untreated ponds as control. In addition to this, the identification of the probiotic bacteria is carried out using morphological, cultural and biochemical tests.

MATERIALS AND METHODS

Fish ponds

The study was carried out in Ponnagi near Eluru town of Krishna district in the state of Andhra Pradesh, India. Two earthen ponds were selected and designated as Pond A and Pond B. Pond A which measures about 5 acres was stocked with the fish variety Catla-catla (2500 numbers) and Selavathi (10,000 numbers) in the ratio of 1:4 where as Pond B was maintained as control. 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 during the period from August 2012 to July 2013 in the culture ponds.

Probiotics containing, *Nitrobacter* species and *Nitrosomonas* species, and *Alcaligenes* manufactured at Sneha Biotech, Vijayawada, Krishna district, Andhra Pradesh, India were used for the present investigation.

Water samples

In the present investigation, physico-chemical parameters such as temperature, transparency, dissolved oxygen, pH, nitrate, ammonia present in water samples were estimated using standard and suggested protocols of Golterman and Clymo (1969); (Wetzel and Likens (1979); APHA (1999); (Claude E. Boyd *et al.*, 2011). 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 (Ahmed *et al.*, 2006).

Biochemical characterization

The isolates 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, ammonia utilisation test) according to the method described by Cappuccino and Sherman (1996) and Bergey's manual (Holt *et al.*, 1994).

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 decolorization, 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°C for 48h. 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 hr. After incubation few drops of **Baritt'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°C for 24 hours. 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°C for 48hr. 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°C for 48 hours. 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°C for 48 hours. 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.

Table 1. Physico-chemical characteristics of pond before and after treatment with probiotics.

Parameters	Pond A (Before and After treatment with probiotics)		Pond B
	Before	After	Control
P _H	8.9	7.2	8.1
Salinity(ppt)	30	20	28
Alkalinity(ppm)	132	110	130
Total hardness(ppm)	172	163	170
Ammonia nitrogen(ppm)	6	4	5
Unionized ammonia(ppm)	2	1	3
Dissolved oxygen(ppm)	8	3	7

Table 2. Biochemical tests conducted for identification of bacteria from pond water

Biochemical tests	Nitrosomonas	Nitrobacter	Alcaligenes
Indole production Test	Negative	Negative	Negative
Methyl red	Negative	Negative	Negative
Voges proskauer	Negative	Negative	Negative
Citrate utilisation	Negative	Negative	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
Ammonia utilization	Positive (Blue Black)	Negative	Negative
Nitrite	Positive	Positive	Positive
Nitrate	Negative	Negative	Positive (red color)
H ₂ S production	Positive(Black)	Negative	Negative

Oxidase test**Wet filter paper method**

A 1% solution of the reagent (tetra methyl-p- phenylene-diamine dihydrochloride) 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 to 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.

Ammonia utilization test

A drop of bacterial culture from the ammonium sulphate medium was transferred to the 3 drops of Trommsdorf's reagent with one drop of dilute sulphuric acid on a spot plate. Blue-black colour indicates the presence of nitrite.

RESULTS

After carrying out the above tests the results of analysis were tabulated. Table 1 below shows the results of water analysis in ponds A and B which were treated prior to and after addition of probiotics. The results showed the presence of toxic gases in pond A, but after probiotic treatment for 3 to 5 days, there was significant decrease in the levels of toxic gases. For effective comparison of results pond B was kept as control. Pond B was maintained as a control. A series of Biochemical characterization tests were carried out to identify different types of bacteria from pond water and the results are shown in Table-2.

DISCUSSION

Earlier investigations have been carried out by scientists and researchers to improve the water quality of ponds and rivers to make more viable for aquaculture. In an earlier study carried out in by Ahmed *et al* in 2006, in an article Seasonal Changes in Bacterial Flora of Fish Pond Sediments in Saudi Arabia, characteristic studies pertaining to motility, cell shape, flagellation, spore formation, encapsulation mechanisms and staining procedures using gram staining methods were studied. It was also observed that to improve the water quality denitrification could be a better option and in this direction studies were carried out by (Rao *et*

al, in 2002) where they employed a denitrifying bioremediator (or filter) which creates an anaerobic region where anaerobic bacteria can grow and reduce nitrate to nitrogen gas. 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 of ponnagi areas of Eluru in Krishna District Andhra Pradesh, India. In order to achieve the above said objectives, Nitrosomonas, Nitrobacter and Alcaligenes 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: Nitrosomonas sp., Nitrobacter and Alcaligenes were all found to be gram negative and having a characteristic rod shaped morphology. Nitrosomonas showed positive biochemical reactions with methyl red test, oxidase test and H₂S tests and showed negative results with indole test. Similar tests were also conducted for Nitrobacter and Alcaligenes species and the positive and negative results have been duly tabulated in Table-2. From the above tests the probable identification of isolates were found to be Nitrosomonas sp., Nitrobacter sp. and Alcaligenes denitrificans with reference to Bergey's manual of Determinative Bacteriology. When compared to earlier studies carried elsewhere it was observed that Bacteriological nitrification is the most practical method for the removal of ammonia from closed aquaculture systems. Nitrobacter converts nitrite to nitrate. In a study carried out earlier it was observed that Nitrification not only produces nitrate but also alters the pH slightly towards the acidic range, facilitating the availability of soluble materials (Ayyappan and Mishra 2003). In this paper also certain nitrifying bacteria have been put to utmost use for improving the quality and a quantity of ponds for sustained aquacultural practices. When compared to earlier studies it is quite evident that probiotics are effective in bioremediation of fish aquaculture ponds. Earlier studies have shown the effect of bioremediation to improve the health of aquaculture (Venkateswara AR, 2007). In the current study bioremediation has been effectively carried out using probiotics and hence the study is more viable. Usage of probiotics reduced and prevented the accumulation of organic sludge at bottom of the pond as well as formation

of toxic gases like NH₃, NO₂, H₂S and thus helped to improve water quality 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 industry.

ACKNOWLEDGEMENTS

The authors are thankful to the management and staff of SNEHA BIOTECH for providing the financial assistance and lab facilities to carry out the research work.

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