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Population of *Vibrio Parahaemolyticus* (Pathogen) and *Bacillus* (Beneficial Bacteria) in *Penaeus Monodon* (Fabricus, 1798) Culture

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Abstract: Problem statement: The present work was attempted to evaluate the probiotic activity of *Bacillus* spp. in shrimp health management. **Approach:** Penaeid culture ecosystem as a confined environment with huge inputs always encourages the proliferation of pathogenic bacteria like Vibrios. **Results:** From the present study and earlier reports it is understood that these pathogens alone or combine with virus especially White Spot Syndrome Virus (WSSV) lead to sudden disease outbreaks and mortality. However the unwarranted and unwanted usage of antibiotics causes negative impacts rather than controlling diseases. **Conclusion:** Based on this, it is concluded that application of suitable and effective probiotics (Bacillus) will certainly reduce population of pathogens and provide a congenial pond environment for shrimp culture. However, this could be achieved by the effective usage of commercially available probiotics for maintaining good pond environment.

Keywords: Available probiotics, *Bacillus* sp., congenial pond environment, phytoplankton density, Triple Sugar Iron (TSI), food and drug administration, pathogenic reduction, commercial shrimp

INTRODUCTION

Over exploitation of seafoods has decreased the supply of seafoods in general and shrimps (living dollars) in particular. Hence, it necessities to increase their production through other sources like aquaculture. Shrimp culture is grown as a million dollar industry and rearing of shrimps in culture system becomes popularized throughout the world especially in Southeast Asia. In India, bestowed with an extensive coastal area, roughly 8,154 km long coastline, which includes estuaries, backwaters, creeks, mud flats and lagoons, has made remarkable progress in shrimp culture. Shrimp culture has become one of the major industries earning more foreign exchange (Sugumar *et al*., 2001). The total area utilized for shrimp farming is around 95,000 sq ha. This reflects the rapid development of shrimp culture in India for the past 10 years.

Unfortunately the uncontrolled development of commercial shrimp farming has led to the outbreak of infectious diseases by various microorganisms (Lightner *et al*., 1992). Among microorganisms, bacteria and virus play a major role in shrimp culture ecosystem. Particularly bacteria have dual role as they can act as pathogenic and also as beneficial microflora (Moriarty, 1997).

The relative abundance of penaeid diseases is depending upon the type of culture system employed. The intensive culture of shrimps results in serious diseases and mass mortality (Chen *et al*., 1992). High stocking density per unit volume of water encourages the transmission of many diseases (Lightner *et al*., 1992). When intensive methods are adapted, concomitant appearance of diseases are known to occur. This might be due to accumulation of organic matter, unconsumed feed, faecal wastes, increased of phytoplankton density, high stoking density, NH_3, H_2S and human interference (Sharmila *et al*., 1996). All these negative parameters combine together and ultimately lead to poor pond environment which leads to stress and animals becomes susceptible to disease and eventually cause mass mortality and huge economic losses. Beneficial bacteria are helpful in organic matter degradation, nutrients recycling and pathogenic reduction (Moriarty, 1984).

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Pathogenic bacteria are responsible for various infectious disease outbreaks as they can act as both primary and secondary pathogens (Nash *et al*., 1992; Karunasagar *et al*., 1992).

In shrimp pond ecosystem, *Bacillus* sp. was reported as the most abundant beneficial bacteria are *Bacillus* spp. (Moriarty, 1997). The species of Nitrosomonas and Nitrobactor are also present. However, *Bacillus* degrades organic matter, facilitates nutrients recycling, competes with the pathogenic bacteria like vibrios for food and substrates and secretes enzymes to contain gram negative pathogenic bacteria such as the species of Vibrio (Rengpipat *et al*., 2003). The beneficial microflora involved conversion of anaerobic pond bottom environment into an aerobic one. *Bacillus* has got antagonistic activity against vibrios. Also they are helpful in maintaining the healthy balance of intestinal microflora of the cultured shrimps.

In coastal ecosystem the most important beneficial bacteria are *Bacillus* (Vaseeharan and Ramasamy, 2003) as they degrade complex organic matter, facilitate nutrients recycling, compete with the pathogenic bacteria for food and substrate and secrete enzymes to kill/inactivate the gram-negative pathogenic bacteria. *Bacillus* is gram positive and spore forming bacteria. The species of *Bacillus* will minimize the build up of dissolved and particulate organic carbon during the culture cycle while promoting more stable phytoplankton blooms through the increased production of CO_2. They are also prevailing in coastal ecosystems like shrimp culture pond. Thus, the indigenous flora can balance the microbial composition in shrimp culture ponds by reducing the pathogens.

Pathogenic and beneficial bacterial population in shrimp culture pond, as a confined environment, is influenced by many factors. Factors such as the accumulation of phytoplankton at the water/sediment interphase (Sugita *et al*., 1998), high stocking density, excess feed (Sharmila *et al*., 1996), faecal matter, human interference and other inputs (Vaseeharan, 2001) play a major role in the distributing pattern of microbial flora in shrimp culture ecosystem. In addition environmental parameters such as temperature, salinity, pH and dissolved oxygen have a role in the microbial population. Therefore, the present work is designed to find out the distribution of pathogenic *Vibrio parahaemolyticus* and beneficial *Bacillus* spp. in water, sediment and cultured shrimps in relation to physic-chemical parameters and days of culture.

Beneficial bacteria as probiotic to displace pathogens by competitive processes can be used effectively in the shrimp culture industry as a better remedy than administrating antibiotics. Thus, the baseline data on bacteriological aspects both of beneficial and pathogenic bacteria in shrimp culture ponds are necessary for the successful farming without any disease outbreak and mortality. The most required information as on today is the comprehensive knowledge on the distribution of bacteria, water quality and immune system of the cultured species. This in turn help to overcome the disease problem through rapid identification and screening of the pathogenic and beneficial bacteria, commercial scale development of beneficial bacteria (probiotics) and usage of effective immunomodulator. Hence, the present work was carried out to study the above factors in a modified extensive shrimp (Penaeus monodon) culture farm located at the Marakanam, Tamil Nadu India with the following objectives. To find out the population density of pathogenic bacteria (Vibrio *parahaemolyticus*) and beneficial bacteria (*Bacillus* spp.) in the water, sediment samples and cultured shrimps in relation with the physico-chemical parameters and days of culture in two ponds of a modified extensive farms and to ascertain the immunomoduling effects of vitamins C and E and β-1,3 glucan on the health status of cultured shrimps.

MATERIALS AND METHODS

Sample collection: The present work was carried out for one culture operation (July-Dec 2007) in a modified extensive shrimp culture farm located at Marakkanam region. Samples were collected from two ponds having 0.6 ha water spread area each. One pond (Pond-A) was operated without any probiotic application. Another pond (Pond-B) was treated with *Bacillus* based probiotics. Water and sediment samples were collected once in 30 days from each pond that covered pond preparation and post-harvest. Sampling was made during early morning. Collected samples were transferred to the laboratory within 3 h in potable icebox. Bacteriological analysis was carried out immediately to avoid any further bacterial growth.

Estimation of physico-chemical parameters: Temperature, salinity, pH and water transparency were measured in the pond site itself by using thermometer, salinometer, pH pen and sechi disc respectively. Dissolved oxygen fixed on the spot and the bottles were taken to the laboratory to perform titration by following the methods of Strickland and Parsons (1976). Sediment was collected in new polythene bags and transferred to the laboratory for the estimation of total organic carbon by following the method of Walkley and Black (1934).

Estimation of Vibrio *parahaemolyticus*: In the case of water, 0.1 ml of the sample was directly taken. For

sediment samples, one gm was added to 9 mL^{-1} of sterile water blank and mixed thoroughly. From this, 0.1 mL was taken. In the case of animal samples, 10 gm was added to 90 mL of sterile water blank and homogenized uniformly in an alcohol rinsed pestle and mortar. From this mixture, 0.1 mL was used to isolate V. *parahaemolyticus*.

Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar medium was used for enumeration of V. *parahaemolyticus*. After enrichment of samples in alkaline peptone water, they were transferred to TCBS agar medium bymeans of spread plate methods of sterile 'L' shaped glass rod. Then the sample added plates in duplicate were kept in bacteriological incubator in an inverted position for 20-24 h. at 35-37°C.

Suspected colonies of V. *parahaemolyticus* (green, large and smooth) were picked and streaked on to Brain heart infusion agar (BHIA) for further characterization. Preliminary screening tests for the isolates were the Triple Sugar Iron (TSI) agar reaction, oxidase reaction, growth in trypton water with 0, 3 and 9% sodium chloride and sensitivity to 2, 2, diamino-6, 7, -disopropyl-pteridine phosphate (0/129) phosphate, sigma, St Louis, (Mo., USA) with 10 and 150 µg discs. Further biochemical tests were made by following methods outlined in U.S. Food and Drug Administration (FDA) Bacteriological manual (1984). The results were expressed as Colony Forming Units (CFU) per ml for water and per gram for sediment and animal samples.

Estimation of bacillus: Sample preparation was same as mentioned in Chapter 4.2.3. Zobell Marine agar medium was used. One ml of sample was added by pour plating technique. After complete mixing of sample and medium, plates in duplicate were kept in bacteriological incubator in an inverted position at room temperature (28±2°C) for three days. Then, the pure cultures in the nutrient broth were identified up to generic level by following the scheme of Oliver (1982). The results were expressed as CFU mL^{-1} for water and CFU g^{-1} for sediment and animal samples.

RESULTS

Physico-chemical parameters:

Temperature: In the pond A, the temperature ranged from 26°C (December 2007) to 30°C (July 2007) where as in the Pond B, it varied between 26°C (December 2007) and 29°C (July 2007) (Fig. 1).

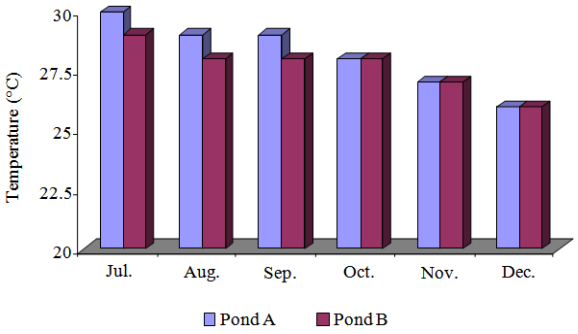

Fig. 1: Temperature recorded in pond A and pond B during the culture period

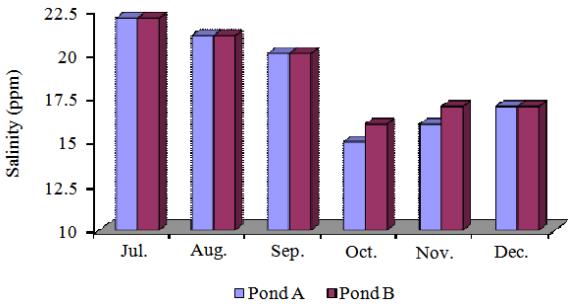

Fig. 2: Salinity recorded in pond A and pond B during the culture period

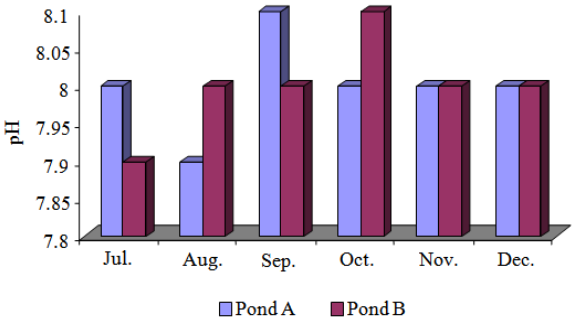

Fig. 3: pH recorded in pond A and pond B during the culture period

Salinity: The maximum salinity recorded at the pond A in July 007 (22 ppt) and the minimum (15 ppt) recorded in October 2007. In pond B, the maximum (22 ppt) observed in July 2007 and the minimum (16 ppt) was observed in October 2007 (Fig. 2).

pH: In the pond A, pH varied between 7.9 (August 2007) and 8.1 (September 2007) whereas in the pond B, it ranged from 7.9 (August 2007) to 8.1 (October 2007) (Fig. 3).

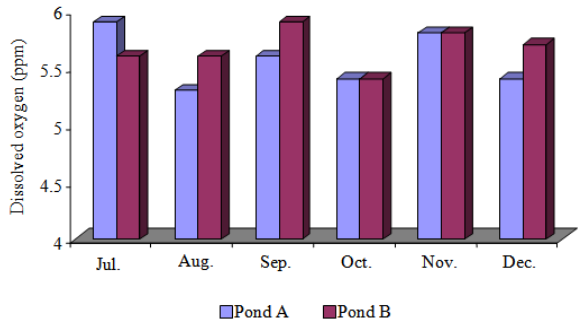

Fig. 4: Dissolved oxygen recorded in pond A and pond B during the culture period

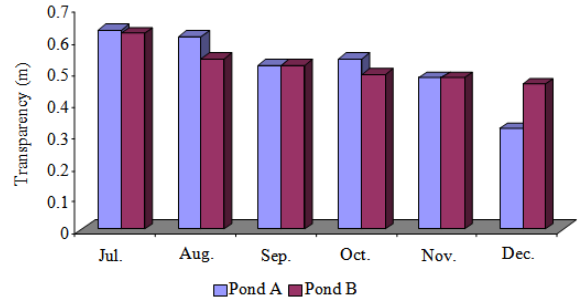

Fig. 5: Transparency recorded in pond A and pond B during the culture period

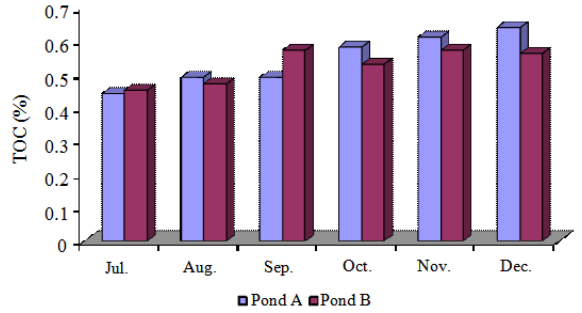

Fig. 6: Total Organic carbon recorded in pond A and pond B during the culture period

Dissolved oxygen: The level of dissolved oxygen ranged from 5.3 ppm (August 2007) to 5.9 ppm (July 2007) at the pond A and 5.4 ppm (November 2007) to 5.8 ppm (November 2007) in the pond B (Fig. 4).

Transparency: In the pond A, the minimum transparency was observed in December 2007 (0.32m) and the maximum was observed in July 2007 (0.63m). It varied between 0.46 m (December 2007) and 0.62 m (July 2007) in the Pond B (Fig. 5).

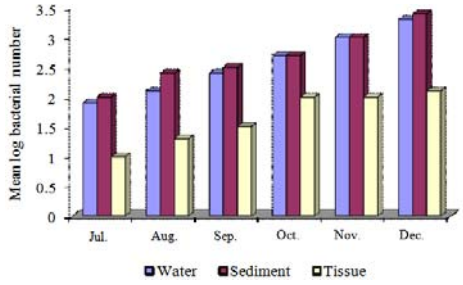

Fig. 7: Population density of *Vibrio paraheamolyticus* recorded in water, sediment and animal samples of Pond-A

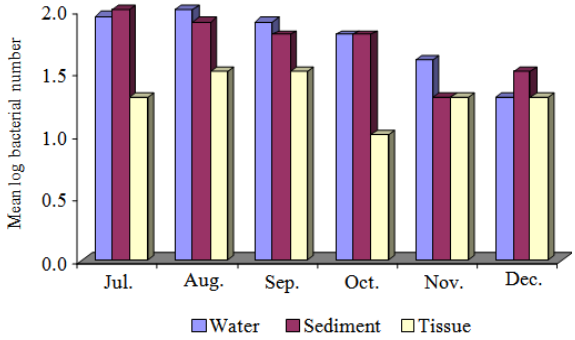

Fig. 8: Population density of *Vibrio paraheamolyticus* in water, sediment and animal samples of Pond-B

Total organic Carbon (TOC): In pond A, maximum percentage of Total Organic Carbon (TOC) (0.64%) was reported in December 2007 and the minimum was in July 2007 (0.44%). In pond B, it ranged from 0.45% (July 2007) to 0.56% (November 2007) (Fig. 6).

Population of *Vibrio parahaemolyticus* water: In the pond A, it varied between $8.0x10^{1}$ CFU mL^{-1} (July 2007) to 1.9×10^{3} CFU mL^{-1} (December 2007) whereas in the Pond B, it ranged from $9.0x10^{1}$ CFU mL^{-1} (July 2007) to 1.0×10^{2} CFU mL^{-1} (August 2007) (Fig. 7, 8).

Sediment: The maximum number of *Vibrio parahaemolyticus* was recorded in December 2007 (2.7×10^{3}CFU g^{-1}) and the minimum was observed in Jul- 2007 in pond A. In pond B it varied between 2.0×10^{1} CFU g^{-1} (November 2007) and 9.0×10^{1} CFU g^{-1} (Jul-2007) (Fig. 7 and 8).

Animal's muscle: In the pond A, it ranged from 1.0×10^{1} CFU g^{-1} (July 2007) to $1.2x10^{2}$ CFU g^{-1} (December 2007) and in the Pond B, the maximum load was found as 3.0×10^{1}CFU g^{-1} in September 2007 and the minimum was recorded as $1.0^{-1}10^{1}$ CFU g^{-1} in October 2007 (Fig. 7 and 8).

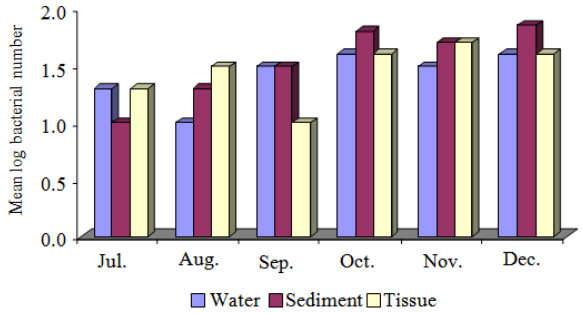

Fig. 9: Population density of *Bacillus* recorded in water, sediment and animal samples of Pond-A

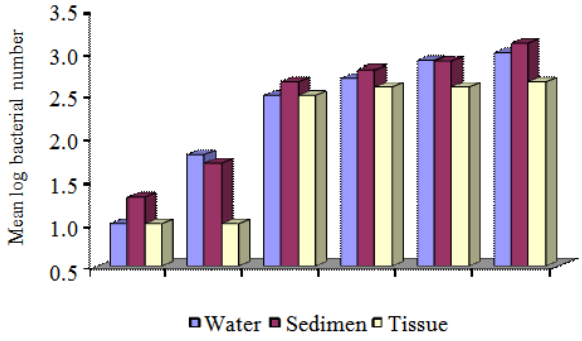

Fig. 10: Population density of *Bacillus* in water, sediment and Animal samples of Pond-B

***Bacillus* spp.:**

Water: The maximum population of *Bacillus* was noted in December 2007 (3.9×10^{1}CFU mL^{-1}) and the minimum number was reported in August 2007 (2.0×10^{1} CFU mL^{-1}) in the Pond A, where as in the Pond B, it ranged from 1.0×10^{1} CFU mL^{-1} (Jul- 2007) to 9.8×10^{2} CFU mL^{-1} (December 2007) (Fig. 9 and 10).

Sediment: In the pond A, the maximum load was observed in December 2007 (7.0×10^{1} CFU g^{-1}) and the minimum was recorded in July 2007 (1.0×10^{1}CFU g^{-1}). In the pond B, it was in the range of $2.0x10^{1}$ CFU g^{-1} (July 2007) and 1.3×10^{3} CFU g^{-1} (December 2007) (Fig. 9 and 10).

Animal's muscle: The population of *Bacillus* spp. ranged from 1.0×10^{1} CFU g^{-1} (September 2007)to 5.0×10^{1} CFU g^{-1} (November 2007) in the pond A. In the Pond B, it ranged from 1.0×10^{1} CFU g^{-1} (July 2007) to 4.6×10^{2} CFU g^{-1} (December 2007) (Fig. 9 and 10).

DISCUSSION

The present study showed an increasing trend of *Vibrio parahaemolyticus* population towards days of culture in the control pond in which no probiotic was added (Fig. 7). The same trend was observed in the water, sediment and animal samples also. However, sediment samples had slightly more number of vibrios than water and the animal samples. Samples collected from the pond that was treated with probiotic had comparatively less V. *parahaemolyticus*. In contrast to the pond A, here decreasing trend of vibrios was noticed towards days of culture. The result on the presence of *Bacillus* in the pond B clearly indicates the increasing trend of this bacterium towards days of culture. However, in the pond A, there was not any specific pattern of *Bacillus* distribution. The result of the present study reconciles with previous reports of Maclean *et al*. (1994) and Sharmila *et al*. (1996) as they also experienced the increasing trend of pathogenic vibrios towards the culture operation. This might be due to the steady increase in the accumulation of organic matter in pond bottom (Moriarty, 1984; Sujatha, 2007). Large organic matter in shrimp culture pond is possible due to high stocking density, over feeding, uneaten feed, faecal matter, fertilizers and over blooming (Lloberra *et al*., 1990; Chen *et al*., 1992; Kautshy *et al*., 2000).

Present finding also supported this view as decreasing trend of transparency (0.63-0.32 m) and increasing trend of total organic carbon (0.44-0.64%) were observed, especially in the pond A. However, other environment parameters such as temperature, salinity, pH and dissolved oxygen did not show any specific trend, as they did not vary much. From the results, it is inferred that transparency and total organic carbon could influence the distribution of bacteria in shrimp culture system. However other environmental factors did not have any role on the bacteriology of shrimp culture environment. It stands with the results of the work done by Sharmila *et al*. (1996) who stated that, pond as a confined environment is easy to be maintained by optimum physico-chemical parameters through proper water exchange, aeration and lime application. The results of the present study are concordance with this as it was possible to maintain the optimum level of temperature, salinity and pH and dissolved oxygen (26-30°C, 15-22, 7.9-8.1 and 5.3-5.9 ppm respectively). However, it is slightly contrast with some other researchers, who pointed out that environmental parameters such as temperature, salinity, pH and dissolved oxygen play a major role in the distribution of bacteria in any aquatic system.

In general, sediments had more bacterial load than the water. This might be due to the facts such as bacteria are not free floating forms and they need to attach any substrates. Also sediments provide some sort

of protection to bacteria to tide over the unfavourable environmental conditions. Continuous availability of substrates and nutrients in the form of uneaten feed, faecal matter and plankton has led to the increased population of bacteria in sediment. This is in agreement with the earlier findings of Sharmila *et al*. (1996). Animal samples had more beneficial bacterial population (around 10^1-10^2 CFU g^{-1}) than water and sediment. However, the size of the animal played a major role in the abundance of bacteria as large sized animals had more number bacteria than small sized animals. This coincides with the report of Cobb *et al*. (1973), as they mentioned that the bacterial populations reach maximum diversity during rapidly growing seasons. In addition, abuse of prawn feed may also increase the bacterial population in cultured shrimps. Also the distribution of bacteria depends upon the moulting of the exoskeleton and the shedding of the chitinous lining (Dempsey *et al*., 1989).

The result of the present study clearly indicates the presence of V. *parahaemolyticus* in all samplings. However the number of vibrios was reported as 1.0×10^2 CFU mL^{-1} in water during the early days of culture and minimum population as 1.0×10^1 CFU g^{-1} was observed in animal samples during the end of culture. By contrast, in pond A, which was not treated with probiotic, the maximum as 2.7×10^3CFU g^{-1} in sediment was noticed at the end of culture and the minimum as 1.0×10^1CFU g^{-1} in animal was found at the initial stage of culture. Low population obtained in the present study reconciles with early findings released by Velammal (1993) and Ruangpan and Tubakaew (1991). Likewise the maximum population observed in the present study is supported by Kumazawa and Kato (1985) who were able to observe the population of vibrios as 10^4 CFU g^{-1} in brackish water area used for shrimp culture. As already discussed, environmental parameters did not play any role in the distribution of V. *parahaemolyticus*, which stands with the early reports made by Baross and Liston (1970). From the above-mentioned works and the present findings, it could be concluded that loading of nutrients and organic matter increases the load of vibrios.

Vibrios are the predominant flora of the shrimps (Yasuda and Kitao, 1980; Karunasagar *et al*., 1984; Velammal, 1993; Sharmila *et al*., 1996). This statement is supported by the present investigation also as all samplings showed the presence of V. *parahaemolyticus* in muscle of P. monodon. Chitinous exoskeleton of shrimps gives a better substrate for the proliferation of Chitinoclastic vibrios like V. *parahaemolyticus* (Kaneko and Colwell, 1975). Besides, feeding on the remains of tiny animals and large proportions of unrecognizable materials also lead to the proliferation of V. *parahaemolyticus* in shrimps (Dalmin, 1998). From the present investigation and the reports of various researchers (Natarajan *et al*., 1980; Ruanpan and Kitao, 1991; Lavilla-Pitogo and Pena, 1998; Goarant *et al*., 2000; Lee *et al*., 1996; Sujatha, 2007), it is understood that vibrios are the autochthonous flora of coastal and marine ecosystem. Hence it is obvious that V. *parahaemolyticus* was isolated from all the pond samples. It is again apparent that later stages of culture operation had more organic matter, which in turn led to more V. *parahaemolyticus* population.

The pond (A), which was not treated with probiotic, faced serious white spot disease problem at 140th day of culture. Within a week mass mortality of the cultured shrimps has happened. At this period more population of V.*parahaemolyticus* (10^3CFU mL^{-1} and g) was recorded in all samples. The high abundance of V.*parahaemolyticus* coincided with White Spot Syndrome Virus (WSSV). Vibrios seem to be the secondary pathogens in the viral disease. Karunasagar *et al*. (1992) recognize that mortalities in viral diseases are generally due to secondary bacterial infections like vibriosis.

The second pond (pond-B) treated with probiotic did not face any disease problem. Here the maximum number of Vibrio was as 1.0×10^2 CFU m^{-1} in water that too in earlier stage of culture. A decreasing trend of V. paraheamolyticus was reported. Also growth and survival rates were more in the pond, which eventually led to good production and more profit. This desirable quality might be due to the probiotic application, which apparently increased the beneficial bacteria (*Bacillus* spp.) population. The result clearly illustrates the increasing trend of *Bacillus* load towards days of culture in all samples. The maximum number of *Bacillus* was observed in sediment sample of the pond as 1.0×10^3 CFU g^{-1} during the early stage of culture. The minimum of 1.0×10^1 CFU mL^{-1} in water was recorded in both the ponds. Unlike the pond B, the pond A untreated with probiotic did not have more *Bacillus* spp and has not shown any specific pattern of their distribution. It agrees with the earlier findings of Sharmila *et al*. (1996). Naturally *Bacillus* spp. are prevailing in the coastal ecosystem with minimum number as they contribute only 15-20% of the total bacteria present in coastal ecosystem where as vibrios contribute above 40%. The trend is supported by the present observation.

However, in the pond B, the load of beneficial bacteria like *Bacillus* was artificially increased by probiotic, hence this pond showed the presence of more *Bacillus* and escaped from disease outbreak. The results

of the present study demonstrate that the beneficial bacterial load was keep on increasing. As the number of probiotic application increases, the load of *Bacillus* also increases. Simultaneously the load of V.paraheamolyticus was coming down. This was totally contrast to the results obtained in the Pond A that was not treated with probiotic. It reflects that more population of beneficial bacteria can significantly reduce the pathogenic bacteria in shrimp culture ecosystem. Since both of them are indigenous flora of the coastal ecosystem (Jayanth *et al*., 2002; Matias *et al*., 2002), they have the tendency to fight each other for survival. If beneficial bacteria like *Bacillus* is more in shrimp culture ecosystem, it inhibits the proliferation of pathogenic bacteria like vibrios by competing for nutrients, substrate and damaging the slimy layers of vibrios through secreting enzymes that degrade slimes (Moriarty, 1998; Torrento and Torres, 2005; Verschuere *et al*., 2000; Chythanya *et al*., 2002; Devaraj *et al*., 2002; Wang *et al*., 2005).

The bacteria could also secrete enzymes that will breakdown the organic flocks and excess nutrients in pond ecosystem, which was noticed in the present study by recording optimum transparency and low organic matter in the pond B. Based on the present study and earlier reports, it is apparent that high load of pathogenic bacteria like V.*parahaemolyticus* can cause diseases directly through vibriosis and indirectly leading to white spot disease. Besides, more pathogenic bacteria cause stress to the culture species. Hence, it is essential to keep the level of V. *parahaemolyticus* within a limit. This can be achieved by increasing beneficial bacterial population in the same ecosystem. Present study and previous findings reveal that higher population of beneficial bacteria leads to good pond water quality, reduction of pathogenic vibrios, stress and disease free culture, enhanced growth and survival rates. This eventually makes the culture a successful and profitable one.

CONCLUSION

Based on the present study, it is concluded that application of suitable and effective probiotics (Bacillus) will certainly reduce population of pathogens and provide a congenial pond environment for shrimp culture. However, this could be achieved by the effective usage of commercially available probiotics for maintaining good pond environment.

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