International Journal of Animal and Veterinary Advances 2(1): 9-15, 2010
ISSN: 2041-2908

Submitted Date: September 10, 2009 Accepted Date: January 02, 2010 Published Date: February 10, 2010

Effect of Probiotics on the Hatchery Seed Production of Black Tiger Shrimp, *Penaeus monodon* (Fabricius)

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Abstract: In recent years, the diseases of shrimps hindered the development of shrimp culture. Hence, the use of probiotic bacteria in aquaculture has tremendous scope and the study of the application of probiotics in aquaculture has a glorious future. In the present study, the probiotics was applied (experimental) for the larval rearing of *P. monodon* which is compared with control tanks (without probiotics). The temperature and alkalinity of both control and experimental tanks were more of less same. The pH of the control tank was 8.4 and the experimental tank was 8.2.The dissolved oxygen was higher in experimental tank (6.12ml/litre) and lower in control tanks (5.75ml/litre). Likewise, the ammonia was higher in control tanks (0.19 mg/litre) rather than experimental tanks (0.15mg/litre). The survival rate of different larval stages (nauplii, zoea and mysis) were maximum in the present study than that of control tanks. The final survival rate of the post larvae from the control and experimental tank was 70 and 30% respectively. The average length of all post larvae was maximum when reared in experimental tank than control tanks. The general conclusion obtained from the present study is that the probiotics plays a vital role in maintaining water quality parameters throughout the larval cycle. It is clear from the microbial load data that *vibrio* sp. is dominant in the control tanks than in experimental tanks.

Key words: Mysis, *Penaeus monodon*, post larvae, seed production, *vibrio* and zoea

INTRODUCTION

Though the shrimp hatchery technology has advanced over the decades, the hatchery production is more often hampered by severe mortalities caused mostly by bacteria. Increasing *Vibrio* population in larvae and rearing tank water has been reported to reduce the survival rate of larvae and post larvae (Singh, 1986; Hameed, 1993). Luminous species of *V. harveyi* have been associated with causing mass mortalities in shrimp hatcheries (Sunaryanto and Mariam, 1986; Baticados *et al*., 1990; Karunasagar *et al*., 1994a, b). Ways of *Vibrio* entry into hatchery includes seawater, faecal matter and exoskeleton of spawners and feeds (Lavilla-Pitogo *et al*., 1990). So far, conventional approaches, such as the use of disinfectants and antimicrobial drugs, have had limited success in the prevention or cure of aquatic disease. The role of probiotics bacteria in farming of shrimp was attempted but such studies in hatchery are not that much reported especially in giant tiger shrimp, *P. monodon*. Hence, the beneficial effect of probiotics on the commercial seed production of Indian major candidate shrimp, *P. monotone* is very much need of the hour. Therefore, the present study, was aimed to examine the effect of probiotics on the hatchery seed production of black tiger shrimp, *P. monodon* was studied.

MATERIALS AND METHODS

The present study was carried out in CP (Charion Pockphand, Aquaculture India Pvt. Ltd.,) hatchery located at Chettikuppam village near Marakkanam. The hatchery is situated about 24km north of Puducherry. This hatchery is well designed, equipped and maintained for commercial production of *P. monodon* seeds for the past ten years. There are four production units operating simultaneously and the annual production is around 200 - 300 million seeds.

The seawater for the hatchery was pumped from the sea directly using a 7 HP motor. The suction point is located about 20m from the shoreline. The water was initially pumped into a sand-gravel filter. From the sand filter, water was lifted into chlorination tank using a 5 HP motor. Chlorination was done with 20ppm chlorine. After 24hrs, the chlorinated water was stored in overhead tank after passing through activated carbon filter. Subsequently the filtered water was passed through cartridge filter (0.5 – 1.0μm mesh size) and UV filter before filling into tanks. The residual chlorine available in the treated seawater was determined with chlorine test kits by using O-tolidine. After knowing the availability of excess chlorine in treated sea water sodium thiosulphate (hypo) was used to

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neutralize the residual chlorine. The chelating agent, EDTA (10ppm) was added in treated seawater to ensure clear seawater. A 15HP air blower and a 7.5HP standby provided continuous supply of air. The air generated by the blower was supplied to individual tanks through PVC pipes.

The brooders were collected from the wild and transported to the hatchery in oxygenated polythene bags. Initially the brooders were quarantined and maintained in a receiving section. Subsequently they were bathed in 10ppm $KMnO_4$, washed in seawater and maintained in separate thermocol boxes with aeration. Samples were experienced to PCR test to detect the presence of WSSV and MBV. Only negative brooders were stocked in brooder maintenance tanks and fed with polychaete worms and squid at twice in a day. Fifty percent of water was exchanged daily. The brooders were then transferred to the maturation tanks and treated with water probiotics (Super Biotic) of 20ppm to control the luminous bacteria.

To enhance artificial spawning, eyestalk ablation was performed. A pre-heated forceps was used to remove the eye and subsequently transferred into broodstock tank. Males and females were stocked in 2:1 ratio in 50 t rectangular tanks. Ripe females were transferred to 0.5 t capacity FRP tanks for spawning. After spawning, females were removed and eggs were filtered and transferred to hatching tanks after treating with 20 ppm Iodine solution.

Before stocking of nauplii, the larval rearing tanks were first filled with 2 t of chlorinated seawater (30ppt) and all the water quality parameters were checked. The larval rearing tanks are divided into two for our convenience. The probiotics treated tanks are considered as experimental and without probiotics treated tanks are considered as control. The water probiotics namely Super Biotic and Biodream (this probiotics contains *Bacillus* sp. and *Streptococcus* sp.) was added in the experimental tanks alone @ 5ppm each. In addition to probiotics 0.05 ppm of Treflon was also added both experimental and control tanks to prevent the fungal disease. The newly hatched nauplii from hatching tank was harvested and transferred in polythene bags and stocked in control and experimental tanks @ 2 lakhs per tank.

Twenty four hours after stocking, the nauplii converted into zoea – I in experimental tanks. Where as in the control tanks, the conversion time was extended to 24 to 30hrs. First feeding was started when the zoea I appeared. The zoeal stages (I to III) were fed with *Chaetoceros* sp. @ $1x10^6$ cells /ml in twice daily both control and experimental tanks. The mysis stages (I to III) were fed with algae @ a density of 5×10^4 cells/ml in both control and experimental tanks. In addition to algae, mysis stages were fed with knock *Artemia* (*Artemia* killed by hot water) @ 5 to 10g/tanks. Post larval stages were fed exclusively on freshly hatched live *Artemia* nauplii @ 5 nos per PL per feeding. In general, the animals in experimental tanks consumed more than control tanks. The water probiotics, Super PS (CP Aquaculture Pvt. Ltd.) was added daily @ 5-10ppm from zoeal stage onwards and probiotics Zymatin @ 5ppm was added after the appearance of post larval stage in experimental tanks alone.

Stock cultures of *Chaetoceros* sp was maintained in temperature controlled room (20-24ºC) with 2000 – 5000 lux light intensity. Conway Walney's Medium or Guillard (F2) Medium was used for indoor culture. Culturing Chaetoceros sp. in 2litre glass bottles, 25 litre plastic bags, 500litre FRP tanks and 20 – 30t cement outdoor tanks did scaling up of culture. For outdoor culture, TMRL and Skelon media were used. Once the algal culture reached into exponential phase and sufficient cell concentration were pumped into larval rearing tanks.

The commercial *Artemia* cysts were aerated for half an hour prior to decapsulation. Two litres of liquid chlorine and 120 ml of Sodium hydroxide solution were mixed well. Cysts were transferred to this solution with constant stirring below 40ºC. The colour changed from dark brown to orange indicates the cyst underwent decapsulation. At this stage, cysts were transferred to *Artemia* hatching tanks after thorough washing in freshwater until chlorine smell disappears. Continuous aeration and illumination with a 60V lamp was provided to accelerate the hatching process. After 24hrs, using phototactic behaviour of the Atemia nauplii collected hatched nauplii.

Water exchange was done from the mysis III stage onwards. Using mesh size of 0.5 mm reduced around 50% of the water. Once post larvae appeared, the salinity was reduced slowly and maintained to 20ppt especially in experimental tanks, because, the effect of probiotics was good in low salinity.

The water quality parameters of the probiotics treated and control tanks were regularly monitored. Water quality parameters such as salinity, temperature, pH, dissolved oxygen, ammonia and alkalinity were estimated daily in the morning hours. The water salinity was measured by using a hand refrectometer (Erma- Japan). The pH of the water was measured by using electronic pH pen manufactured by Hanna Instrumental Company, Japan. Water temperature was measured by using a standard centigrade thermometer. Dissolved oxygen meter estimated the dissolved oxygen. First using sodium bicarbonate after standardized the sulphuric acid then the samples were titrated with the standardized sulphuric acid by using Methy red indicator. Ammonia level was monitored regularly by adopting the method of Solorzano (1969) and Koroleff (1969).

Water samples were taken from the rearing tanks (both control and experimental tanks) and various larval stages (i.e. nauplii, zoea, mysis and post larvae). Total plate count (TPC) was performed by plating serial ten-fold dilutions in Tryptic Soy Agar containing 1% NaCl (TSAS) by the spread plate method. Each sample was plated in duplicate. The plates were incubated at 29.1ºC and observed after 24 hrs. To determine luminous bacterial counts, plates were observed in a dark room.

After reaching PL15, then it was ready to sale. Before the dispatch of post larvae, the healthiness was assessed by PCR test, MBV, and Quality control. To maintain the reputation of the hatchery only the negative seeds were sold to the farmers.

RESULTS

The salinity was maintained as 30 ppt for both control and experimental tanks. The pH of the control tank was 8.4 and the experimental tank was 8.2.The temperature (31ºC) of both control and experimental tanks were more of less it. The dissolved oxygen was higher in experimental tank (6.12ml/litre) and lower in control tank (5.75ml/li. The alkalinity (150 ppm) of both control and experimental tanks are more or less similar. The ammonia was higher in control tanks (0.19 mg/litre) rather than experimental tanks (0.15mg/litre) (Table 1).

The survival rate of the nauplii of both control and experimental tanks were more or less same. The survival rate of the zoea reared in the experimental tanks was higher (90%) than that of control tanks (75%). The survival rate of the mysis was maximum (85%) in experimental tanks and minimum was in control tanks (60%). The survival rate of all the post larval stages (PL1-PL15) was higher in experimental tanks than that of control tanks (Table 2).

The average length of all post larvae (PL1-PL15) was maximum when reared in experimental tanks than control tanks (Table 3).

Green colonies were comparatively more in control tanks than experimental tanks (both water and larval stages). And bacterial count was maximum in larval stages than water (Table 4).

Table 1: Water quality parameters of both control and experimental larval rearing tanks of *P. monodon*.

Water Quality parameters	Control	Experimental
Salinity (ppt)	30±1	30±1
pH	8.4±0.17	8.2±0.12
Water Temperature (º C)	31±0.5	31 ±- 0.5
Dissolved Oxygen(ml / litre)	5.75±0.18	6.12±0.13
Alkalinity (ppm)	150±0.5	150±0.5
Ammonia (mg/litre)	0.19±0.00	0.15±0.01

Table 2: Survival rate of different larval stages of *P. monodon* reared in both control and experimental tanks.

Stages	Survival rate (%)	
	Control	Experimental
Nauplii	99±1.2	99±0.2
Zoea	75±0.1	90±0.3
Mysis	60±1.4	85±1.5
PL1	45±2.1	85±1.8
PL5	35±2.2	80±1.5
PL10	30±0.1	75±0.8
PL15	30±0.4	75±0.9

Table 3: The average length of *P. monodon* post larvae reared in both control and experimental tanks.

Stages	Length (mm)	
	Control	Experimental
PL1	4.78±1.2	5.38±0.1
PL5	5.68±0.9	7.83±0.4
PL10	7.98±0.4	9.84±0.1
PL15	9.84±0.8	11.81±0.4

Table 4: Bacterial loads (Vibriosis) in the rearing water and *P.monodon* larval stages of both control and experimental tanks.

Stages	Control (Green colony)		Experimental (Green colony)	
	Water (CFU/ml)	larvae (CFU/ml)	Water (CFU/ml)	larvae (CFU/ml)
Zoea	1.60 X 102	2.00 X 103	0.70 X 102	1.50 X 103
Mysis	1.30 X 103	2.90X 103	0.60 X 102	1.30 X 103
PL 1	2 X 103	9.00 X 103	0.50 X 102	1.00 X 103
PL 5	2.50 X 103	1.00 X 104	0.70 X 102	0.80 X 103
PL 10	>3 X 103	>1.50 X 104	0.80 X 102	0.60 X 103
PL 15	>3 X 103	>1.50 X 104	0.65 X 102	0.60 X 103

DISCUSSION

There has been a considerable increase in the culture of brackish water shrimp, *P. monotone* due to its taste, market demand for both national and international markets. Traditional and non-scientific farms depended only on shrimp seeds caught from the wild or those entered with the tides for stocking. However, in scientific shrimp farming a steady and heavy demand for healthy and quality seeds throughout the year. Thus successful shrimp culture is reliant in stocking disease free, healthy seeds raised in the hatcheries. The present study was undertaken to ascertain the efficiency of probiotics on the survival of the most important cultivable shrimp larval farms, *P. monodon* in addition to its influence on important water quality parameters. Important water quality parameters monitored during the present study were, salinity, pH, temperature, dissolved oxygen, alkalinity and ammonia.

Water quality plays an important role in aquaculture production. A complete understanding of the relationship between water quality and aquatic productivity is absolutely essential for optimum growth and production. The quality of water during the culture period will deteriorate mainly due to the accumulation of metabolic wastes of living organisms, decomposition of unutilized feed and decay of biotic materials. Generally organisms are in a state of balance between potential disease causing microorganisms and their environment. Change in this equilibrium through the way of impairment in water

quality parameters can influence survival of organisms as they become vulnerable to disease due to stress, so also growth. Efficient removal of imbalances, which cause impairment in water quality, is difficult. However addition of some commercial preparations such as probiotics is reported to effectively deal with these substances and that way helpful in maintaining water quality parameters thereby improving growth rate and survival rate.

In the present study, the water quality parameters of hatchery, which is applied with microbial supplement through probiotics, was good because of the various roles played by the microbes. Improved water quality has especially been associated with *Bacillus* sp. The rationale is that gram-positive bacteria are better converters of organic matter back to Co_2 that gram-negative bacteria. During the production cycle, high levels of gram-positive bacteria can minimize the buildup of dissolved and particulate organic carbon. A similar observation was found in the present study. The tank that was treated with probiotics (Super Biotic, Biodream, Super PC and Zymetin) was abundant with *Bacillus* sp. was showing a low level of ammonia, which was converted into nitrate through nitrite.

Water temperature is probably the most important environmental variables for larval rearing, because it directly affects metabolism, oxygen consumption, growth, moulting and survival. In general, a sudden change of temperature affects the larval immmune system. The optimum range of temperature for the black tiger shrimp larval rearing is between 28 to 32ºC (Kannupandi *et al*., 2002). The temperature in the present study was 31ºC. There was no marked difference in temperature between control and experimental tanks of the present study.

Salinity is the most important factor influencing many functional responses of the organisms as metabolism, growth, migration, osmotic behavior, reproduction etc. Marine organisms maintain their internal salt concentration (salt concentration of blood and body fluids) by osmoregulation. They need considerable energy for osmoregulation to maintain their internal salt balance in relation to the external medium in which they are living. When nutrient energy is used for osmoregulation, the growth may be reduced. For a shrimp hatchery the recommended salinity range is 28-35ppt (Kannupandi *et al*., 2002.) In the present study, filtered seawater was used and the salinity was falls in the desired range of 30 ppt for control and experimental tanks. Krishnaprakash (2007) also reported almost similar salinity (31ppt) for the larval rearing of *P. monodon*.

pH of the culture is having an important say on the metabolism and other physiological processes of an organisms. It changes with accumulation of residual feed, dead algae and excreta. In the optimum range of pH, ammonia will not cause much problem. Toxicity of nitrite and hydrogen sulphide is increased in low pH. The required range of pH for shrimp larval culture is 8.2-8.5 (Kannupandi *et al*., 2002). In the present study the pH level was more in the control tank (8.4) and considerably less in experimental tank (8.2) but anyway falls on optimum range. The results attribute that probiotics present in the experimental tanks was helpful in maintaining the pH in desired level. Krishnaprakash (2007) also maintained almost similar pH (8.27- 8.96) for the rearing of *P. monodon* larvae.

Oxygen dissolved in the rearing medium is an important factor not only for the respiration of aquatic organisms but also to maintain favourable chemical and hygienic environment of the water body. It controls many of the oxidation reactions and maintains aerobic conditions in water. When oxygen level is very low and anaerobic conditions exist, nitrate is reduced into ammonia, which will be toxic. This also increases the *p*H. Low-level of oxygen tension hampers metabolic performances in shrimp larvae and can reduce growth and moulting and cause mortality (Gilles, 2001). Oxygen level in the culture medium was maintained in the desired range by aeration. Continuous aeration was done during the present study and therefore the oxygen level did not vary significantly between control and experimental tanks and was in the range of 5.75-6.12ml/litre.

The alkalinity of both control and experimental tanks are more or less similar (150 ppm) in the present study. Alkalinity values were found to be in the range of from 140 to 160 ppm in the larval rearing of *P. monodon* (Krishnaprakash, 2007). In the present study both control and experimental tanks the levels of ammonia were 0.19 mg/litre and 0.15 mg/litre respectively. The less amount of ammonia in the experimental tank indicates mainly due to the microorganisms present in the probiotics, which initiate nitrification. The concentration of ammonia was found to be between 0 and 2.1ppm in the larval rearing of *P. monodon* (Krishnaprakash, 2007).

In the present study both *Chaetoceros* sp. and *Artemia* nauplii were used as a feed for all the larval stages of *P. monodon*. Hanson and Haraold (1977) used *C. gracilis* as a feed in desirable concentrations to shrimp larval farms to prevent mortality. Crocos and Coman (1997) concluded that micro algal species plays a vital role on larval growth and survival of *Penaeus* sp. Devi *et al*. (2004) described the influence of live and supplementary feed on the growth of post larval stages of *P. monodon* (Fab.). Krishnaprakash (2002) also used similar feeds as in the present study for the larval rearing of *P. monodon* to get better survival.

The commercial production of penaeid shrimp seeds has been hampered by the occurrence of infectious and non-infectious diseases. A number of microbial agents are involved in causing mortalities. Bacterial diseases are

considered to be a major cause of mortality in shrimp hatcheries (Wyban and Sweeney, 1991). They are also a constraint in consistent larval production (Grisez and Ollevier, 1995). Bacteria, particularly *Vibrio* spp have been reported to cause larval mortalities in southern and southeastern Asia. *V. harveyi*, a luminous species, has been implicated in a number of cases causing mass mortalities (Sunaryanto and Mariam, 1986; Karunasagar *et al*., 1994a, b). Even though disease-spreading organisms are always present in the water, they attack larvae only when the larvae are weak due to environmental stress or nutritional deficiency.

A number of factors influence the microflora in shrimp hatcheries. The natural flora present in raw seawater may be altered by filtration, chlorination and other treatment methods adopted in the hatcheries. Microflora present in the chlorinated water represents those, which survived the treatment, and those derived from biofilms formed in the water pipes and tank surfaces (Otta *et al*., 2001). During larval rearing different microflora may enter the hatchery system through the eggs and live feeds such as algae and *Atremia*. Otta *et al*. (2001) observed a qualitative change in the microflora between intake seawater and hatchery water, with a clear dominance of *Vibrio* sp. in the hatchery water.

To avoid bacteriological problems, commercial shrimp hatcheries adopt extensive water treatments, which include filtration, chlorination, and ultraviolet treatment. Traditionally, the control of bacterial problems in penaeid shrimp hatcheries has relied on the chemical compounds. The abuse of antimicrobials can result in the development of resistant strains of bacteria (Weston, 1996). More recently probiotic organisms are being used. Probiotics, which is generally used in grow-out systems, finds use in hatcheries also. A commercial probiotic product with *Lactobacillus*, *Bacillus* and *Streptococcus* was used in the present study. Water quality and microbial load was within the permissible limits. However luminous bacteria were observed in the hatchery systems in lower counts.

The microbial community inside the gut of some animals confers some degree of resistance or protection against diseases (Fox, 1988). In natural populations of aquatic animals, the microflora of the gut might reflect that of the aquatic environment. But in artificial larval rearing systems, the balance is altered by the use of disinfected water, microalgae, *Artemia* nauplii, rotifers and antibacterials. As a result, the protective microbial community may not develop either in the environment or the digestive system of the larvae. The post larvae reared in relatively sterile environment of a hatchery do not grow well and show poor survival when exposed to complex microbial populations which makes them susceptible to environmental stress and potential pathogenic bacteria.

Regular health checks and examination of animals were carried out in the hatchery at various stages of rearing. Post larval stages were examined regularly for necrosis, luminous bacteria, WSSV, MBV, endoparasites etc. Stress tests were also conducted. Only healthy larvae, which passed the health limits, were sold to the farmers. The survival rate in the present study is 30 and 75% for control and experimental tanks respectively. This clearly shows that probiotics used in the experimental tanks check the water quality and disease causing organisms than control tanks. The average length of PL also much higher in the animals exposed to probiotics treated tanks than control tanks. Krishnaprakash (2007) also obtained 43.07% for the seed production of *P.monodon*.

In aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty, 1999). Probiotics in aquaculture have been shown to have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality; enhancement of immune response of host species; and enhancement of nutrition of host species through the production of supplemental digestive enzymes (Verschuere *et al*., 2000). Because *Bacillus* bacteria secrete many exoenzymes (Moriarty, 1996; 1999), these bacteria have been used widely as putative probiotics. Studies have shown that when these bacteria were administered as probiotics in the shrimp larval rearing tanks, growth and survival were improved and immunity was enhanced (Rengpipat *et al*., 1998; 2000).

Some bacteria used as candidate probiotics have antiviral effects. Although the exact mechanism by which these bacterial action is unknown. Direkbusarakom *et al*. (1997) isolated two strains of *Vibrio* spp. NICA 1030 and NICA 1031 from a black tiger shrimp hatchery. These isolates displayed antiviral activities against IHNV and *Oncorhynchus Mosaic virus* (OMV). Some researches have suggested that microorganisms have a beneficial effect in the digestive processes of aquatic animals. Balca´zar (2003) demonstrated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrios* sp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against the pathogens *V. harveyi* and white spot syndrome virus. This protection was due to a stimulation of the immune system, by increasing phagocytosis and antibacterial activity.

Administration of the *Bacillus* bacteria to shrimp larval rearing tanks resulted in an increase in the specific activity of lipase, protease and amylase in the larval digestive tract. Because gram-positive bacteria, particularly members of the genus *Bacillus*, do secrete a wide range of exoenzymes (Moriarty, 1996; 1999), we cannot distinguish between activity due to enzyme synthesized by the larvae and activity due to enzyme synthesized by the bacteria. They observed increases in specific activities of digestive enzymes in probiotic

treatments might have led to enhanced digestion and increased absorption of food, which in turn contributed to the improved survival and growth in *P. indicus*. In the present study also the feed consumption was maximum in probiotics treated tanks than control tanks. In contrast, Shariff *et al.* (2001) found that treatment of *P. monodon* and *Litopenaeus vannamei* with a commercial *Bacillus* probiotics did not significantly increase (P N0.05) either survival or growth. For shrimp receiving probiotic in both the hatchery and the farming stages, all of the growth parameters except total length and carapace length were significantly higher in treatments than in controls. The correlation of higher bacterial counts with higher digestive enzyme activity and improved survival and growth parameters in treatment PP over controls strongly suggests that adding the probiotics during the hatchery stages and continuing its administration throughout the farming stages is necessary to maximize survival and growth in the shrimp. The general conclusion obtained from the present study is that the probiotics plays a vital role in survival and disease resistance of the larval farms by maintaining good water quality parameters through out the cycle. It is clear from the microbial load data that *Vibrio* sp. is dominant only in the control tanks not in experimental tanks.

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