AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

Use of *Streptomyces fradiae* and *Bacillus megaterium* as probiotics in the experimental culture of tiger shrimp *Penaeus monodon* (Crustacea, Penaeidae)

[1]Sheikh Aftabuddin, [2]M. Abul Kashem, [1]M. Abdul Kader, [1]M. Nurul Azim Sikder, and [3]M. Abdul Hakim

[1]Institute of Marine Sciences and Fisheries, University of Chittagong, Bangladesh; [2] Prime Shrimp Hatchery Limited, Kolatoly, Cox's Bazar, Bangladesh; [3]Department of Microbiology, University of Chittagong, Bangladesh. Corresponding author: S. Aftabuddin, email: aftabimsf@yahoo.com

Abstract. Shrimp hatcheries often beset with diseases, mainly the bacterial infection and antibiotics are widely used for prevention of disease. Presently, beneficial bacteria (probiotics) are used to prevent diseases instead of antibiotics and increasing the production. In the present study, the two new microbial strains *Bacillus megaterium* and *Streptomyces fradiae* isolated from mangrove sediments were applied (experimental culture) for the post larval rearing of *Penaeus monodon* which is compared with control culture tanks (without probiotics). The water quality condition such as temperature (27-29°C), salinity (26-28‰) and dissolved oxygen (4.7-5.0 mg L^{-1}) of both control and experimental culture were more or less similar. Concentration of ammonia and pH were significantly different ($p<0.05$) between the control and experimental culture during the study period. The feed assimilation efficiency is higher (above 80%) in experimental culture tank when compared to control tank (74.76%). The growth rate was higher - 1.70 and 1.67 in *S. fradiae*, 1.66 and 1.63 in *B. megaterium* - through feed and water, respectively, while in control tank it was 1.4. The FCR values were 2.06 and 2.12 in *S. fradiae* treated tanks through feed and water, respectively, while 2.51 and 2.55 were observed in *B. megaterium* treated through feed and water respectively. The FCR value was found higher (4.02) in the control tank. The average total heterotrophic bacteria (THB) and total presumptive *Vibrio* bacteria both in culture water and post larvae were lower during experimental culture in compared to control culture. The present study indicates that the probiotic treatment using two new microbial strains such as *B. megaterium* and *S. fradiae* would help in better aquaculture production.
Key Words: *Penaeus monodon*, post larvae, *Streptomyces fradiae*, *Bacillus megaterium*, probiotics.

Introduction. Shrimp farms are growing throughout the world to meet the demand for shrimp which is one of the most popular types of seafood in the world and Bangladesh is no exception. The tiger shrimp, *Penaeus monodon* is one of the largest shrimp among penaeids and it is most suitable for aquaculture in Bangladesh (Aftabuddin et al 2005). Shrimp cultivation and export in Bangladesh have undergone rapid expansion over the last two decades. During 2008-2009 fiscal year, export of shrimp and fish products was 117.31 million Lbs earning 454.53 million US dollar. Shrimp alone contributed about 78% of total export earning from fishery product (BFFEA 2009). With the rapid expansion of shrimp growout farm, the hatchery industry has progressed rapidly during the last ten years. There are 50 shrimp hatcheries (at various stages of operation and management) in Bangladesh and they produced nearly 5 billion post larvae in 2004 (Aftabuddin & Kader 2006) which was sufficient to supply the entire shrimp culture industry in Bangladesh. However, many of these hatcheries had to stop production in the last three years due to invasion of different pathogens in their system (Aftabuddin et al 2008).

Bacteria are the most common biological agents in the aquaculture sector and it is known that marine crustaceans can be infected by one or more type of bacteria. In many shrimp producing countries, *Vibrio* and *Aeromonas* are considered as the most common

and significant infectious pathogens (Lightner 1996; Moriarty 1997; Vaseeharan et al 2005; Aftabuddin et al 2008). Bacterial diseases are the major problem affecting the shrimp hatcheries and most of the mass mortalities reported in shrimp (*P. monodon*) hatcheries are associated with luminous bacteria (Lavilla-Pitogo et al 1990; Jiravanichpaisal et al 1994; Karunasagar et al 1994). *Vibrio* spp. are by far the major bacterial pathogens and can cause severe mortalities, particularly in hatcheries. Vibriosis is caused by a number of *Vibrio* species including: *V. harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. penaeicida* (Brock & Lightner 1990; Ishimaru et al 1995). Different antibiotics *i.e.* Oxytetracycline, Chloramphenicol, Erythromycin, Furazolidone, Neomycin sulphate, Kenamycin sulphate, Ciprocin and others are used in *P. monodon* shrimp hatcheries in Bangladesh (Aftabuddin & Kader 2006). Prolonged exposure of a bacterial population to a given antibiotic can result in development of pathogens that are resistant to those antibiotics. This resistance can occur either by mutation within the target bacterial population or by inadvertent selection of a minor percentage of the original population that is innately resistant (Chamberlain 1988). On the other hand, the use of beneficial bacteria (probiotics) to displace pathogens by competitive processes is being used in the animal industry as a better remedy than administering antibiotics and is now gaining acceptance for the control of pathogens in aquaculture (Havenaar et al 1992). The probiotics are live microbial feed supplements, used for environment-friendly aquaculture. The probiotics are usually applied through water or feed, they act beneficially in improving the water quality, increasing growth of the shrimp and making the animals active and healthy (Gatesoupe 1999). The probiotics includes Vibrios, Pseudomonades, lactic acid bacteria, bacilli and yeasts. Among them *Bacillus* spp. and yeasts are commonly studied probiotics (Cherif et al 2001; Vaseeharan & Ramasamy 2003; Dahan et al 2003; Duc et al 2004; Soundarapandian & Babu 2010).

Marine environment contains a wide range of distinct microorganisms that are not present in the terrestrial environment (Ramesh & Mathivanan 2009). Many promising bioactive compounds including antimicrobial, antitumour, immunosuppressive agents and enzymes are being discovered from marine actinomycetes (Tanaka & Omura 1990). Marine mangrove ecosystems are a great source for isolation of new microbes that are potentially rich to produce the important bioactive metabolites (Mangamuri et al 2012).

The environment of the mangrove ecosystem is saline, and highly rich in organic matter due to its various microbial enzymatic and metabolic activities (Kizhekkedathu & Parukuttyamma 2005). Approximately 70% of thousands of naturally occurring antibiotics have been isolated from marine actinomycetes (Takizawa et al 1993) and the majorities were isolated from the genus *Streptomyces* (Zheng et al 2000; Atta & Ahmad 2009; Reddy et al 2011). Marine actinomycetes, especially *Streptomyces*, have been considered in antibiotic production for chemical factories (Okazaki & Okami 1972; Ellaiah & Reddy 1987). However, in addition, little information was found on the application of *Streptomyces* as probiotics in aquaculture (Das et al 2006).

On the other hand, *Bacillus megaterium* is a gram positive, spore producing bacteria. It is found in diverse environments from rice paddies to dried food, seawater, sediments, fish, normal flora, and even in bee honey (Vary 1994). Taxonomically, *B. megaterium* is positioned into the *B. subtilis* group of Bacilli (Priest 1993; Vary 1994).

In contrast to other bacilli strains *B. megaterium* has an advantage, that no alkaline protease is present (Vary et al 2007). This fact enables excellent production and secretion of foreign proteins without degradation (Meinhardt et al 1989; Rygus & Hillen 1991). In compare to gram negative organisms like *Escherichia coli*, it does not produce endotoxins associated with the outer membrane (Vary et al 2007). These features make *B. megaterium* well applicable in food and even in pharmaceutical industry. *B. megaterium* has economic importance because of its commercially important enzymes such as penicillin amidase and steroid hydrolases (Vary et al 2007).

Presently, probiotics used in shrimp culture industry in Bangladesh is entirely import oriented. It is also not clear about the source of beneficial microorganisms used in the preparation of imported probiotics. In recent years, the increasing consumer concern about the residues of antibiotics and the danger of development of antibiotic resistant

bacterial strains have led to the use of probiotic feed additives in the aquaculture industry.

Therefore, the present study was undertaken to examine the effects of *B. megaterium* and *S. fradiae* as probiotics when treated through feed and culture medium of *P. monodon.* Water quality parameters of cultured environment, net growth efficiency, assimilation, food conversion ratio (FCR), growth rate, total heterotrophic bacteria (THB) and total *Vibrio* bacteria were also studied, with a view to develop a potent microbial supplement to ensure better aquaculture production maintaining the environment friendly.

Material and Method. In the present study, strains of *B. megaterium* and *S. fradiae* were isolated from mangrove sediments of Chokaria mangrove area, Cox's Bazar, Bangladesh (Figure 1). This study was carried out from January 2011 to December 2011 in Prime Shrimp Hatchery Limited, Cox's Bazar, where adequate research facilities for the study of hatchery operation and management were readily available and Shrimp & Fish Disease Diagnosis Laboratory, Institute of Marine Sciences and Fisheries, University of Chittagong, Bangladesh.

Figure 1. Sediment sampling area of the present investigation.

Isolation and identification of Bacillus megaterium. The sediment samples were collected by inserting a grab sampler at 1-2 meter depth. The sampler was sterilized with 70% alcohol before sampling. The sample was then transferred to a sterilized polyethylene bag. Sediment was dried overnight in a laminar flow hood and when clumping occurred, was grinded with mortar and pestle. Ten gram of sediment sample was suspended in 100 ml distilled water in a 200 ml conical flask and serial dilutions were made. From the serial dilutions 100 µl of soil suspension at different dilutions were drawn and poured onto the Blood Agar medium and incubated at 35 to 37°C for 24-48 hours (HPA 2007). Bacterial cells from typical colonies were observed with a light microscope (400X) and purified on nutrient agar medium. Colonies were white, round smooth and shiny. During the microscopic examination all the isolates were found gram positive rods. The isolated colonies were identified as *B. megaterium* using standard morphological, physiological and biochemical plate and tube tests (Holt et al 1994). All strains were stored in Luria-Bertani (LB) broth cultures with sterile glycerol (15% v/v).

Isolation and identification of Streptomyces fradiae. The collected sediment samples were air-dried aseptically. All the marine sediment samples were subjected to pre-heat treatment prior to serial dilution. Pre-heat treatment was performed by incubating the sediment samples in a water bath at 50° C for 60 minutes (Takizawa et al 1993). Ten grams of sediment samples were suspended in 95 ml of sterile aged seawater (7 day old seawater) and these suspensions were considered as 10^{-1} dilution. Starch casein agar (SCA) medium was used for isolation of *Streptomyces* spp. Serial dilutions were done and overlaid on the surface of SCA. The medium consisted of 10 g soluble starch, 0.3g casein, 2g KNO_3, 2g KH_2PO_4, 0.05g $MgSO_4.7H_2O$, 2g NaCl, 0.02g $CaCO_3$, 0.01g $FeSO_4.7H_2O$, 20g Agar and natural aged sea water 1000ml. The final pH of the medium was adjusted to 7.0 ±2 with 0.1 N NaOH before sterilization (Ramesh & Mathivanan 2009). The medium was supplemented with 50 mg L^{-1} and 20 mg L^{-1} of tetracycline and nystatin respectively as antibacterial and antifungal agents to inhibit the bacterial and fungal contamination (Reddy et al 2011). The plates were incubated at room temperature 28±2°C for one week.

Purification. The appearance and growth of marine actinomycetes were observed everyday on SCA plates. Again, the colonies have been observed using a light microscope for their filamentous nature, spores, width of hyphae and spiral sporophores. Individual colonies were picked up, and subcultured on SCA and on International Streptomyces Project medium 2 (ISP2) i.e. Yeast extract-malt extract agar (Pridham et al 1956) by repeated streaking to ascertain their purity. The medium consisted of Bacto Yeast Extract (Difco, USA) 4g, Bacto Malt Extract (Difco, USA) 10g, Bacto Dextrose (Difco, USA) 4g, distilled water 1 liter, Bacto agar 20g and adjusted pH 7.3. The pure cultures of marine actinomycetes were sub-cultured in SCA slants, incubated at room temperature for 5–7 days to achieve good sporulation, and then preserved in 20% glycerol vials at -80°C (Williams & Cross 1971). The inocula used in all the experiments were seven-day-old cultures, unless otherwise stated.

Characterization of the Streptomyces isolates. The microorganisms were characterized by acid-fast staining and Gram's staining techniques. The morphological, physiological and biochemical characterization of the isolated was performed according to the method of International Streptomyces Project (ISP) (Shirling & Gottlieb 1966; Kokare et al 2004). Hydrolysis of starch was performed by using the media of Gordon et al (1974) Liquefaction of gelatine was done by the method of Waksman (1961). Growth characteristic was also observed using tyrosine agar medium (ISP-7) (Shinobu 1958). Carbon source utilization (CSU) tests have been used for the classification and identification of bacteria for many years in the form of simple tests such as citrate utilization, D-glucose, sucrose, inositol etc. Biochemical tests and carbon and nitrogen utilization were performed according to standard methods described by Shirling & Gottlieb (1966) and Tresner et al (1968).

Experimental set up. Healthy and active post larvae (PLs_{20-22}) were acclimatized to the laboratory conditions for 5 days. One hundred PL were used for each treatment tank of 100 liters capacity. The tank was filled with 90 liters of filtered seawater and was aerated using air-stones. Salinity was maintained between 22 and 25 ppt. The experimental animals were fed with formulated feed (Brine Shrimp flakes, Higashimaru 3, Singapore) at 10% body weight of the PL.

The experiment was done in two ways using the cultures of *B. megaterium* and *S. fradiae*. One way was that the cultures with a density of 10^9 cells per ml and total 10ml were inoculated into the tank having the 90 liters of seawater considering that this small amount of culture media would not be affected the experiment. Another way was that the cultures were mixed thoroughly with feed at 10^9 cells per gram of the feed. The treated feed was given two times a day at 7.00 a.m. and 7.00 p.m. All the treatments (water and feed) were done at an interval of every 5 days of the total 60 days of culture. In control tanks, no microbial treatment either through water or through feed was made. Water exchange was made for 35% of the culture water every morning. Ten tanks were used (five for *Bacillus* spp. and five for *Streptomyces* spp.) for conducting the experiment. ANOVA test was done to see the treatment effects of the new probiotics used.

Water parameters analysis. The water quality parameters of the probiotics (*B. megaterium* and *S. fradiae*) treated and control tanks were regularly monitored. Water quality parameters such as salinity, temperature, pH, dissolved oxygen, and ammonia were estimated daily in the morning hours. The water salinity was measured by using a hand refrectometer (ATAGO, Japan). The pH of the water was measured by using electronic pen pH meter manufactured by Hanna Instrumental Company (Singapore). Water temperature was measured by using a digital thermometer (DT801, China). Dissolved oxygen (mg L^{-1}), and ammonia (mg L^{-1}) were measured by HANNA instruments products, Singapore, Model no. HI 3810 and HI 3826, respectively.

Microbial evaluation. For enumeration of total heterotrophic bacteria (THB) in water and post larvae the pour plate method was used with sterile Zobell's marine agar medium (Hi-media, Mumbai, India). For presumptive *Vibrio* count TCBS and TSA medium (Oxoid, England) were used. This work was carried out for 10 days interval i.e. 10th, 20th, 30th, 40th, 50th and 60th days of culture period.

Analysis of shrimp growth. The shrimp growth in terms of length and weight was measured after 60 days of culture. Total length was measured with the help of a plastic graduated scale. Fresh weight was taken by weighing the live shrimps on an electronic balance. From the data collected, the food consumption, mean production weight, assimilation, metabolism, growth rate, relative growth rate, digestibility, gross conversion efficiency, net conversion efficiency, food conversion ratio (FCR) and food-coefficient were calculated by Crisp (1971), Qasim & Easterson (1974) and Easterson (1987). Before feeding and water exchange, unutilized feed and faecal matter were collected separately by siphoning out the water using a 2 cm (dia) plastic hosepipe to determine the feed assimilation rate. The collected unutilized feed and faecal matter were placed separately on a pre-weighted filter paper (WHATMAN™ No. 1), cleaned with distilled water, dried in an oven at 60°C for 24-h and then weighted.

Results and Discussion. The results shown in Table 1 demonstrate the physiological and biochemical characteristics of *B. megaterium* and *S. fradiae*. The enumerated total heterotrophic bacterial (THB) population was higher in treated tanks than in the control tank. In control tank, the total heterotrophic bacterial populations in water were recorded $63.80 \pm 1.1 \times 10^3$ to $15.10 \pm 0.72 \times 10^4$ cfu ml^{-1} while in post larvae, the recorded THB was $5.4 \pm 1.70 \times 10^2$ to $2.38 \pm 0.78 \times 10^4$ cfu g^{-1}. In the *S. fradiae* treated tank, the value ranged from $65.07 \pm 1.0 \times 10^3$ to $44.30 \pm 0.5 \times 10^5$ cfu ml^{-1} for feed experiment and from $69.56 \pm 1.2 \times 10^3$ to $43.10 \pm 0.60 \times 10^6$ cfu ml^{-1} for water experiment. In the *B. megaterium* treated tank, the recorded values varied from 62.21×10^3 to 42.90×10^5 cfu ml^{-1} and $8.4 \pm 0.58 \times 10^3$ to $2.18 \pm 0.72 \times 10^4$ cfu g^{-1} in water and post larvae respectively while

treated with food and from 59.83 x 10^3 to 39.22x10^6 cfu ml^{-1} and 6.1±1.1x10^2 to 1.99±0.35x10^4 cfu g^{-1} in water and post larvae respectively while treated with water. In the *S. fradiae* treated tank's post larvae, the THB ranged from 3.48±1.24x10^3 to 3.45±0.82x10^4 in feed experiment and from 8.8±2.3x10^2 to 6.1±1.2x10^3 cfu g^{-1} in water experiment. The THB values are much different between the control and treated tanks (Figure 2). The observed total *Vibrio* bacteria were lower in *S. fradiae* and *B. megaterium* treated tank compared to control tank (Figure 3) and the values were significantly different ($p<0.05$). The average THB and *Vibrio* bacteria in water and post larvae are shown in Table 2.

Table 1

Different physiological and biochemical characteristics of *B. megaterium* and *S. fradiae*

Tests	*B. megaterium (n=10)*	*S. fradiae (n=10)*
Gram staining	+	+
Motility	+/-	-
Catalase	+	+
Reduction of nitrate	-(d)	ND
Liquefaction of Gelatin	+	+
Starch hydrolysis	+	-
Citrate test	+	+
Egg yolk reaction	-	ND
Anaerobic utilization of glucose	-	ND
Voges proskauer reaction	-	-
Growth at 45°C	-	+
Growth at NaCl 7% (w/v)	-	+
Melanin Pigment on Peptone yeast extract –iron agar medium (ISP-6)	ND	+
Tyrosin decomposed (ISP-7)	+/-	+
Lysozyme-resistant	-	-
Acid produced from		
Manintol	+	-
Glucose	+	+
Acetylglucosamine	+	ND
Arbutin	+	ND
D-fructose	+	+/-
D-trehalose	+	+/-
Ribose	+	ND
Rhamose	ND	+
Carbon utilization		
D-glucose	+	+
L-arabinose	ND	+
Sucrose	ND	-
Inositol	ND	-
Rhamnose	ND	+

"+" = 90-100% strains positive; "-" = 90-100% strains are negative; ND = Not done; "+/-" = 50-50% strains are positive; "–(d)" = most strains are negative.

Microbes are unavoidable in the culture system and they have beneficial as well as detrimental effects (Das et al 2006). In aquaculture systems, total heterotrophic bacteria play a significant role through mineralization and decomposition of wastes and provide supplementary feed for shrimp larvae (Sunilkumar 1996) whereas *Vibrio* can cause diseases and mass mortality (Karunasagar et al 1994; Lightner 1993). In the present study, total heterotrophic bacteria increased in the experimental tanks due to the addition of *S. fradiae* and *B. megaterium* cells but decreased in the control tank. In contrast, the *Vibrio* count decreased with the introduction of probiotics and increased in

the control tank. This finding coincide with the works of Moriarty (1998), Prabhu et al (1999), Dalmin et al (2001) and Das et al (2006).

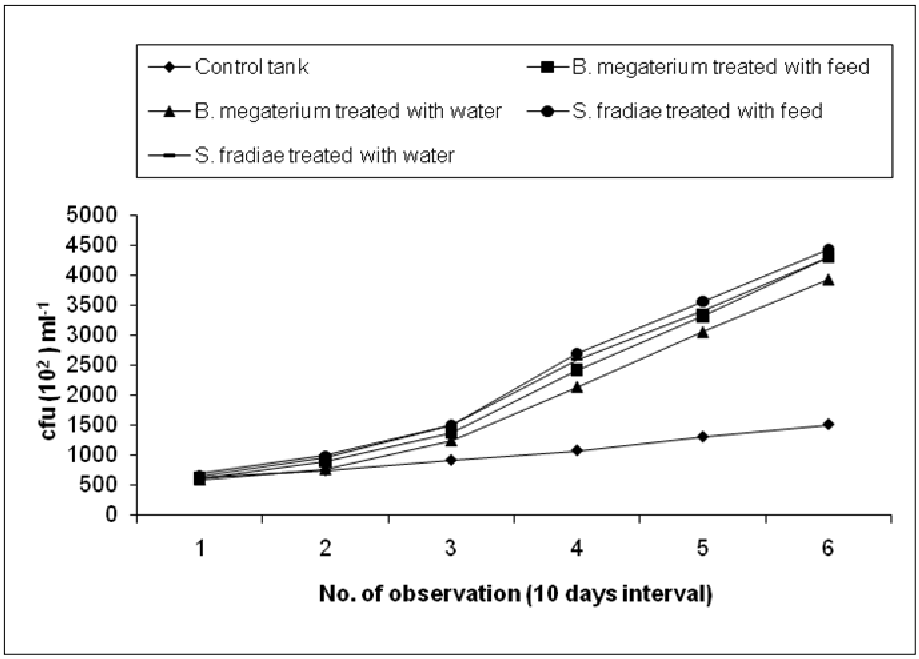

Figure 2. Total heterotrophic bacteria (cfu ml^{-1}) observed in control and treated tanks water.

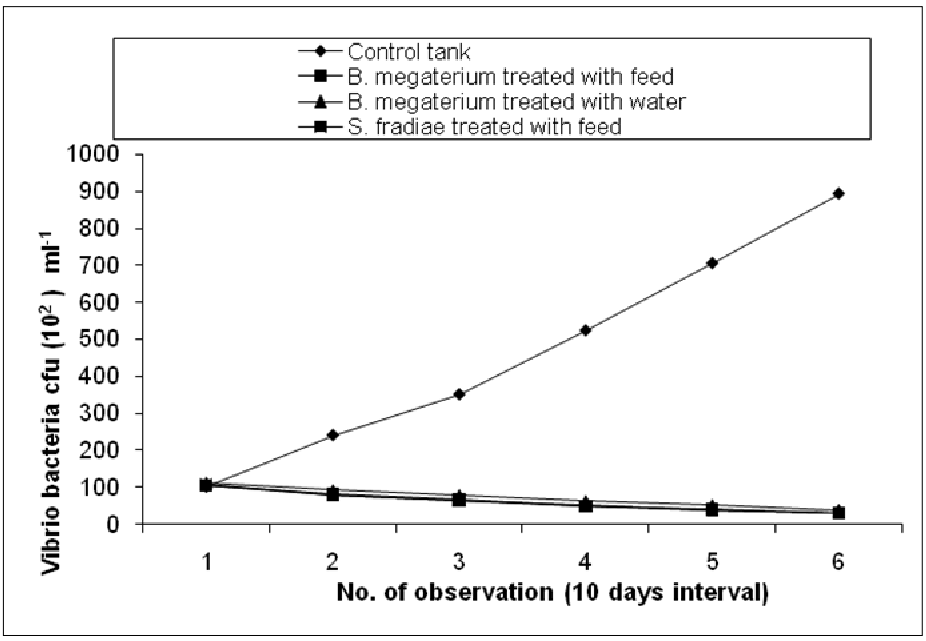

Figure 3. No. of *Vibrio* bacteria cfu ml^{-1} (10^2) in treated and control tanks water.

Water quality plays an important role in *P. monodon* post larvae production. Managing of water quality during the larval rearing phase is very important due to the sensitivity of larvae to the fluctuation of water parameters (Aftabuddin et al 2009). The quality of water during the production period will deteriorate mainly due to the accumulation of metabolic wastes of living organisms, decomposition of unutilized feed and decay of biotic materials. Efficient removal of imbalances, which cause impairment in water quality, is difficult. However, addition of some commercial preparations such as chemicals, antibiotics and probiotics are reported to effectively deal with these substances and in this way they are helpful in maintaining water quality parameters and improving growth

rate and survival rate (Soundarapandian & Babu 2010). The average water quality parameters of experimental and control tanks were shown in Table 2.

Table 2

Water quality parameters and total heterotrophic bacteria (THB) and *Vibrio* (TVC) counts in *P. monodon* culture tanks treated with *S. fradiae* and *B. megaterium*

Variables	*Average value*				
	Control	*B. megaterium treated with feed*	*S. fradiae treated with feed*	*B. megaterium treated with water*	*S. fradiae treated with water*
Temperature (^oC)	29	27.6	27.2	27.4	27
Salinity (ppt)	28	27	27	26	26
Dissolved oxygen (mg L^{-1})	4.8	4.7	5.0	4.8	4.9
pH	6.8	7.5	7.7	7.6	7.5
Ammonia (NH_3 – N) (mg L^{-1})	0.45	0.025	0.018	0.022	0.020
THB (cfu ml^{-1}) in water	$10.33\pm0.5x10^4$	$21.54\pm1.2x10^4$	$22.98\pm0.8x10^4$	$19.56\pm0.6x10^4$	$22.52\pm1.1x10^4$
THB (cfu g^{-1}) in post larvae	$2.3\pm0.3x10^3$	$11.2\pm1.5x10^3$	$18.5\pm0.9x10^3$	$4.14\pm0.7x\ 10^3$	$6.42\pm0.9x10^3$
Vibrios (cfu ml^{-1}) in water	$469.66\pm1.2x10^2$	$63.33\pm0.90x10^2$	$60.66\pm1.0x10^2$	$72.83\pm0.72\ x10^2$	$62.33\pm1.3x10^2$
Vibrios (cfu ml^{-1}) in post larvae	$233.11\pm2.3x10^1$	$10.12\pm0.5x10^1$	$9.36\pm0.3x10^1$	$13.38\pm0.45x10^1$	$14.12\pm0.65x10^1$

NB: Values are average of 60 days of analysis for temperature, salinity, DO and pH; THB (total heterotrophic bacteria) and Vibrios are averages of 6 days of analysis (10th, 20th, 30th, 40th, 50th and 60th days of culture time).

The optimum range of temperature for the black tiger shrimp larval rearing is between 28 to 32^oC (Kannupandi et al 2002). The temperature varied from 27 to 29^oC in both control and experimental tanks during the study period. There were no marked differences in temperature between control and experimental tanks of the present study.

P. monodon can tolerate a wide range of salinity. The range of salinity with which the normal growth of *P. monodon* can be maintained is between 15 and 30 ppt (Chen 1976; Chen 1985). In *P. monodon*, the optimum salinity range for growth may change during their life history with 10 ppt for early post larvae (Valencia 1976), 15-20 ppt for late post larvae (Chen 1976) and 15-25 ppt for juvenile (Chen 1984). In the present study, the salinity ranged between 26 and 28 ppt on all the tanks. There were slight differences between control and treated tanks (*S. fradiae* and *B. megaterium*). Salinity was slightly higher in *S. fradiae* and *B. megaterium* treated tanks mixed with feed than mixed in water. For a shrimp hatchery, the recommended salinity range is 28-35 ppt (Kannupandi et al 2002).

The average DO level ranged between 4.7 and 5.0 mg L^{-1} in the experimental and control tanks during the study and there was no significant difference ($p>0.05$) noticed between the control and treated tanks. Liao & Murai (1986) reported that the respiration rate of *P. monodon* remained constant at dissolved oxygen (DO) concentrations above 3.0 to 4.0 ml L^{-1} at salinities 4-45 ppt and temperatures of 20-30^oC.

The average pH was observed from 6.8 to 7.7 in both control and treated tanks. There was a significant difference ($p<0.05$) between the control and treated tanks. Law (1988) reported that *P. monodon* could tolerate pH down to 6.0 to 6.5. High mortality was observed at pH below 6.0. In the present study the pH level was less in the control tank (6.8) and considerably high in experimental tank (7.7). The results pointed that probiotics (*B. megaterium* and *S. fradiae*) present in the experimental tanks were helpful in maintaining the pH at desired level.

Ammonia exists in water in both ionized (NH_4+) and unionized (NH_3) forms. Unionized ammonia is considered the more toxic form of ammonia due to its ability to

diffuse readily across cell membrane (Fromm & Gillette 1968; Emerson et al 1975). The reaction of NH_3 depends on pH, temperature and to a lesser extent on salinity (Bower & Bidwell 1978). As pH or temperature rises, NH_3 increases relative to NH_4+, and the toxicity of ammonia to animals increases (Chien 1992). The average ammonia level ranged between 0.018 to 0.45 mg L^{-1} in the experimental and control tanks during the study. There was a significant difference ($p<0.05$) between the control and treated tanks. Chien (1992) reported that the safe levels of ammonia for various stages of *P. monodon* are 0.15, 0.1 and 0.08 mg L^{-1} for PL_{30-50}, juvenile and adolescent respectively.

Shrimp growth. The results obtained from the experiment are presented in Table 3a. Growth (in terms of wet weight gain) was significantly higher ($p<0.05$) in the treated than in the control tanks. The percent of increment was 100.10 and 98.09 in *B. megaterium* treated through feed and water respectively. The percent increment was 102.33 and 100.44 in *S. fradiae* treated through feed and water respectively while in the control group it was 89.32 percent (Table 3b).

Table 3a

Weight gain of *P. monodon* post larvae after 60 days culture treated with new probiotics *S. fradiae* and *B. megaterium* (mixed with feed and water)

Treatment	*Initial weight w1*	*Final weight w2*	*Weighed means (W) = w1 + w2/2*	*Feed consumed (C) = feed given-unutilized feed*	*Faecal output (F)*	*Assimilation (A) = Feed consumed – faecal matter (C-F)*	*Production Growth (P) = (w2-w1)*	*Metabolism (R) = C-(P+F)*
Control tank	0.34	2.32	1.33	5.35	1.35	4.00	1.98	2.02
S. fradiae (mixed with feed)	0.34	4.28	2.31	4.78	0.75	4.03	3.94	0.09
S. fradiae (mixed with water)	0.35	3.90	2.12	4.50	0.85	3.65	3.55	0.10
B. megaterium (mixed with feed)	0.33	3.60	1.96	4.92	0.90	4.02	3.27	0.75
B. megaterium (mixed with water)	0.35	3.44	1.89	4.85	0.88	3.97	3.09	0.88

The relative growth rate in shrimp was higher in treated tanks than in control. The best relative growth rates 1.70 and 1.67 were observed in the tank inoculated with *S. fradiae* respectively through feed and water. The growth rates were 1.66 and 1.63 respectively in the *B. megaterium* treated through feed and water. However, the lowest growth rate 1.48 was recorded in the control tank (Table 3b).

The shrimp showed high assimilation efficiency (digestibility) of 84.30 and 81.85% in *S. fradiae* and *B. megaterium* treated through feed and water respectively. Assimilation efficiency was also observed at 81.70 and 81.11% in *B. megaterium and S. fradiae* treated through feed and water respectively (Table 3b). Qasim & Easterson (1974) observed the assimilation efficiency ranged from 89 to 97% in the shrimp, *Metapenaeus monoceros* in India.

Table 3b

FCR and relative growth of *P. monodon* post larvae after 60 days culture treated with new probiotics *S. fradiae* and *B. megaterium* (mixed with feed and water)

Treatment	*Assimilation efficiency or digestibility = A/C (%)*	*Gross growth (conversion) efficiency (k_1) = P/C (%)*	*Net growth (conversion) efficiency (k_2) = P/A (%)*	*Food Conversion Ratio = Total food consumed/Total live weight increased*	*Food co-efficient C/P*	*Relative Growth Rate = P/W x 1*	*Growth Rate = Fw- Iw/W x 60*
Control tank	74.76	37.00	49.50	4.02	2.70	1.49	89.32
S. fradiae (mixed with feed)	84.30	82.42	97.76	2.06	1.21	1.70	102.33
S. fradiae (mixed with water)	81.11	78.88	97.26	2.12	1.26	1.67	100.44
B. megaterium (mixed with feed)	81.70	66.46	81.34	2.51	1.50	1.66	100.10
B. megaterium (mixed with water)	81.85	63.71	77.83	2.55	1.56	1.63	98.09

The gross growth (conversion) efficiency in the shrimps was 82.42 and 78.88% through feed and water respectively in *S. fradiae* treated tank. Gross growth (conversion) efficiency were 66.46 and 63.71% in *B. megaterium* treated through feed and water respectively (Table 3b). The gross conversion efficiency was found very high in *S. fradiae* treated tanks in comparison to the control and *B. megaterium* treated tanks. In crustaceans, the reported gross growth (conversion) efficiency was 4-68% (Qasim & Easterson 1974).

The highest value of net conversion efficiency in shrimps was recorded as 97.76 and 97.26% in *S. fradiae* treated through feed and water respectively. The net conversation efficiency was 81.34 and 77.83 in *B. megaterium* treated through feed and water respectively. Net conversion efficiency in the control tank was 49.50 (Table 3b). Qasim & Easterson (1974) reported that net conversion efficiency was 24 to 71% in crustaceans. The maximum food coefficient value was 2.70 recorded in animals of the control tank, followed by the values of 1.56 and 1.50 in *B. megaterium* and 1.26 and 1.21 in *S. fradiae* treated tanks through feed and water, respectively.

The food conversion ratio (FCR) was lower in treated tanks than in control tank. The FCR values were 2.06 and 2.12 in *S. fradiae* treated tank through feed and water respectively while 2.51 and 2.55 were observed in *B. megaterium* treated through feed and water, respectively. The FCR value is higher 4.02 in the control tank.

The present study has demonstrated the positive effect of beneficial microorganisms such as *S. fradiae* and *B. megaterium* on the production of the *P. monodon* shrimp post larvae. The microbial supplements improve the growth and survival of the tiger shrimp, besides biomass production, food conversion efficiency, food conversion ratio and assimilation efficiency (Tables 3a and 3b). This can be attributed to the better water quality ensured by the microbial supplements. This may also be due to activation of immune system induced by the microbes through the production of enzymes and antibiotics produced by the treated microbes that activate the defense system of the shrimps (Itami et al 1998). However, the precise mechanism of action of microbial supplements in improving the disease resistance of shrimp is not known. This is perhaps because shrimps possess a less developed immune system and are relatively more dependant on the non-specific immune processes. Porubcan (1991) has recorded that the microbial strain isolated from

the tiger shrimp pond improves water quality growth and productions of *P. monodon*. The beneficial effect is attributed to the efficiency of bacteria in antagonisms to pathogen, gut colonization with possible adhesion to intestinal mucus (Evelyn 1996) and to increase resistance of the host to pathogens.

The actinomycetes i.e. *Steptomyces* sp. are well known for their production of antibiotics, growth-promoting substances. Therefore, it is probably that the *S. fradiae* used in the present study has improved the growth of shrimps through growth promoting substances produced and in making the shrimps healthy by reducing the load of pathogenic vibrios in the culture medium through antibiotics formed. It is therefore, necessary to characterize the growth promoting substances and antibiotics produced by the actinomycetes to develop them as microbial production for better aquaculture practices.

Moriarty (1998) has noted an increase of prawn survival in the pond water inoculated with bacillus species. This is attributed to the water quality improved by the bacterial inoculations because of degradation of organic matter. This treatment also has decreased the pathogenic lumionous vibrio species and other bacteria (Maeda 1994). The antagonistic activities of *Bacillus* species may be mediated by many inhibitory substances, such as organic acids, hydrogen peroxide (Ringø & Gatesoupe 1998). The strains of bacillus are also known to compete for nutrients and for space for pathogenic bacteria. These mechanisms of bacillus may even prevent the emergence of resistant strains of pathogenic bacteria, a well-known risk of antibiotics treatments (Moriarty 1998).

Conclusion. The two microbial species namely *B. megaterium* and *S. fradiae* have been proved to have potential for their use as probiotics in shrimp aquaculture to improve water quality, balance microbial population and to reduce pathogenic bacteria. This practice is a new approach, deserving more attention. There is a great scope for application of marine actinomycetes as probiotics for a glorious future of aquaculture.

Acknowledgement. The authors are thankful to Professor Dr. Md. Abul Kalam Azad, Dean, Faculty of Science, University of Chittagong and Professor Dr. Md. Ashraful Azam Khan, Director, Institute of Marine Sciences and Fisheries, University of Chittagong, Bangladesh for providing the fund through special allocation no. 350/P&D/7-28/2012 (Part-3).

References

Aftabuddin S., Das N. G., Kamal A. H. M., 2005 Maturation and spawning of penaeid shrimp *Penaeus monodon* Fabricius collected from off shore water of the Bay of Bengal. Int J Agric Biol 7(6):925-927.

Aftabuddin S., Kader M. A., 2006 The use of antibiotics in shrimp hatcheries in Bangladesh. J Fish Aquat Sci 1(1):64-67.

Aftabuddin S., Zafar M., Noman A. S. M., Sharmin A., 2008 Isolation and identification of *Vibrio* spp. and *Aeromonas* spp. in wild and hatchery reared *Penaeus monodon* post larvae from Cox's Bazar, Bangladesh. Pak J Mar Sci 17(Special Issue):161-167.

Aftabuddin S., Kamal A. H. M., Kader M. A., Akhtar A., Hossain M. S., 2009 Assessment of water quality at Kalatoly coast and a shrimp hatchery, Cox's Bazar. Curr World Environm 4(2):123-130.

Atta M. A., Ahmad M. S., 2009 Actinomycin-A antibiotic biosynthesis produced by *Streptomyces* sp. AZ-AR 262: taxonomy, fermentation, purification and biological activities. Aust J Basic Sci 3:126-135.

BFFEA, 2009 Bangladesh Frozen Foods Exporters Association, Dhaka. Annual Report, pp. 55.

Bower C. E., Bidwell J. P., 1978 Ionization of ammonia in seawater: effects of temperature, pH and salinity. J Fish Res Board Canada 35(7):1012-1016.

Brock J. A., Lightner D. V., 1990 Diseases of crustaceans. In: Diseases of marine animals. Kinne O. (ed), Biologische Anstalt Helgoland, Hamburg, pp. 245-424.

Chamberlain G., 1988 Rethinking shrimp pond management. Coastal aquaculture. Texas Agricultural Extension Service. Sea Grant College Program 5(2), pp. 1-19.
Chen J. Y., 1984 The effect of salinity on the growth and survival rate of four *Penaeus* juvenile. Masters thesis. Institute of Oceanography, National Taiwan University, Taipei, Taiwan, 175 pp. [in Chinese].
Chen T. P., 1976 Aquaculture practices in Taiwan. Fishing News Books Ltd. Farnham, Surrey, England. 162 p.
Chen T. P., 1985 Water quality criteria for farming the grass shrimp, *Penaeus monodon*. In: Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps. Taki Y., Primavera J. H., Llobrera J. A. (eds), Aquaculture Department, Southeast Asian Fisheries Development Center, Iloilo, Philippines, 165 pp.
Cherif A., Ouzari H., Daffonchio D., Cherif H., Ben Slama K., Hassen A., Jaoua S., Boudabous A., 2001 Thuricin 7: a novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7, a new strain isolated from soil. Letters in Appl Microbiol 32:43–247.
Chien Y. H., 1992 Water quality management for marine shrimp culture. Water quality management review. In: Proceedings of the special session on shrimp farming. Wyban J. (ed), World Aquaculture Society, Baton Rouge, LA, USA, pp. 144-156.
Crisp D. J., 1971 Methods for the study of marine benthos. In: International Biological Programme (IBP) Handbook No. 16. Holme N. A., McIntyre A. D. (eds), Blackwell, Oxford, 197 pp.
Dahan S., Dalmasso G., Imbert V., Peyron J. F., Rampal P., Czerucka D., 2003 *Saccharomyces boulardii* interferes with enterohemorrhagic *Escherichia coli*-induced signaling pathways in T84 cells. Infect Immun 71(2):766–773.
Dalmin G., Kathiresan K., Purushothaman A., 2001 Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem. Indian J Exp Biol 39:939-942.
Das S., Lyla P. S., Ajmal Khan S., 2006 Application of *Streptomyces* as a probiotic in the laboratory culture of *Penaeus monodon* (Fabricius). Israeli J Aquacult Bamidgeh 58(3):198-204.
Duc L. H., Hong H. A., Barbosa T. M., Henriques A. O., Cutting S. M., 2004 Characterization of *Bacillus* probiotics available for human use. Appl Environ Microbiol 70(4):2161–2171.
Easterson D. C. V., 1987 Nutritional bioenergetics in fishes and shellfishes. In: Proceedings of the summer institute in recent advances in finfishes and shellfish nutrition. Technical Paper II, Central marine fisheries research institute, Cochin, India, 25 pp.
Ellaiah P., Reddy A. P. C., 1987 Isolation of actinomycetes from marine sediments off Visakhapatnam, east coast of India. Indian J Mar Sci 16:134-135.
Emerson K., Russo R. C., Thurston R. V., 1975 Aqueous ammonia equilibrium calculations: effect of pH and temperature. J Fish Res Board Canada 33(2):379-388.
Evelyn T. P. T., 1996 Infection and disease. The fish immune systems: organism, pathogen and environment. In: Hoar W. S., David J. R., Farrell A. P. (eds), Fish Physiology series, 15, pp. 339.
Fromm P. O., Gillette J. R., 1968 Effect of ammonia on blood and nitrogen excretion of rainbow trout (*Salmo gairdneri*). Comp Biochem Physiol 26:887-896.
Gatesoupe F. J., 1999 The use of probiotics in aquaculture. Aquaculture 180:147–165.
Gordon R. E., Barnett D. A., Handerhan J. E., Pang C. H. N., 1974 *Nocardia coeliaca, Nocardia autotrophica* and the nocardin strain. Int J Syst Bacteriol 24:54-63.
Havenaar R., Ten Brink B., Huis in't Veld J. H. J., 1992 Selection of strains for probiotic use. In: Probiotics: the scientific basis. Fuller R. (ed), Chapman and Hall, London, pp. 209-224.
Health Protection Agency (HPA), 2007 Identification of *Bacillus* species. National Standard Method BSOP ID 9 Issue 2.1. http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

Holt J. C., Krieg N. R., Sneath P. H. A., Staley J. T., 1994 Bergey's manual of determinative bacteriology. 9th ed. Baltimore, MD: Williams and Wilkins Company, pp. 559.

Ishimaru K., Akarawa-Matsushita M., Muroga K., 1995 *Vibrio penaeicida* sp., nov., a pathogen of kuruma prawns (*Penaeus japonicus*). Int J Syst Bacter 45:134-138.

Itami T., Asano M., Tokushige K., Kubono K., Nakagawa A., Takeno N., Nishimura H., Maeda M., Kondo M., Takahashi Y., 1998 Enhancement of disease resistance of Kuruma shrimp *P. japonicus* after oral administration of peptidoglycon derived from *Bifidobacterium thermophilum*. Aquaculture 164:277-288.

Jiravanichpaisal P., Miyazaki T., Limsuwan C., 1994 Histopathology, biochemistry and pathogenicity of *Vibrio harveyi* infecting black tiger prawn *Penaeus monodon*. J Aquat Anim Health 6:27-35.

Kannupandi T., Soundarapandian P., Anantharaman P., 2002 Hatchery operation manual for *Penaeus monodon* Fabricius. Annamalai University, CAS in Marine Biology, pp. 1-99.

Karunasagar I., Pai R., Malathi G. R., Karunasagar I., 1994 Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture 128:203–209.

Kizhekkedathu N. N., Parukuttyamma P., 2005 Mangrove Actinomycetes as the source of lignolytic enzymes. Actinomycetologica 19:40-47.

Kokare C. R., Mahadik K. R., Kadam S. S., Chopade B. A., 2004 Isolation of bioactive marine actinomycetes from sediments isolated from Goa and Maharashtra coastlines (west coast of India). Ind J Mar Sci 33:248-256.

Lavilla-Pitogo C. R., Baticados M. C. L., Cruz-Lacierda E. R., De La Pena L. D., 1990 Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture 91:1–13.

Law A. T., 1988 Water quality requirements for *Penaeus monodon* culture. Proceedings of the Seminar on Marine Prawn Farming in Malaysia. Serdang, Malaysia, Malaysia Fisheries Society, pp. 53-65.

Liao I. C., Murai T., 1986 Effects of dissolved oxygen, temperature and salinity on the oxygen consumption of grass shrimp, *Penaeus monodon*. In: The First Asian Fisheries Forum. Maclean J. L., Dizon L. B., Hosillos L. V. (eds), Asian Fisheries Society, Manila, Philippines, pp. 641-646.

Lightner D. V., 1993 Diseases of cultured penaeid shrimp. In: CRC Handbook of Mariculture, 2nd ed., vol. 1. Crustacean Aquaculture. McVey J. P. (ed), CRC Press, Boca Raton, FL, pp. 393-486.

Lightner D. V., 1996 A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, LA, USA. pp. 305.

Maeda M., 1994 Biocontrol of the larvae rearing biotope in aquaculture. Bull Nat Res (Institute of Aquaculture) 1:71-74.

Mangamuri U. K., Vijayalakshmi M., Sudhakar P., Sreenivasulu K., 2012 Isolation, identification and molecular characterization of rare Actinomycetes from mangrove ecosystem of Nizampatnam. Malaysian Journal of Microbiology 8(2):83-91.

Meinhardt F., Stahl U., Ebeling W., 1989 Highly efficient expression of homologous and heterologous genes in *Bacillus megaterium*. Appl Microbiol Biotechnol 30:343-350.

Moriarty D. J. W., 1997 The role of microorganisms in aquaculture ponds. Aquaculture 151:333-349

Moriarty D. J. W., 1998 Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164:351–358.

Okazaki T., Okami Y., 1972 Studies on marine microorganisms. II. Actinomycetes in Sagami Bay and their antibiotic substances. J Antibiotic 25:461-466.

Prabhu M., Nazar A. R., Rajagopal S., Ajmal Khan S., 1999 Use of probiotics in water quality management during shrimp culture. J Aquacult Trop 14:227-236.

Pridham T. G., Anderson P. C., Foley C., Lindenfelsr L. A., Hesseltine C. W., Benedict R. G., 1956 A selection of media for maintenance and taxonomic study of Streptomyces. Antibiotics Annual pp. 947-953.

Priest F. G., 1993 Systematics and ecology of *Bacillus*. In: *Bacillus subtilis* and other Grampositive Bacteria. Sonenshein A. L., Hoch J. A., Losick R. (eds), Washington DC: American Society for Microbiology, pp. 3-16.
Porubcan R. S., 1991 Reduction in chemical oxygen demand and improvement in *Penaeus monodon* yield in ponds inoculated with aerobic *Bacillus* bacteria. Program and Abstract of the 22nd Annual Conference and exposition 16-20 June 1991 San Juan, Puerto Rico, World Aquaculture Society, 70 pp.
Qasim S. Z., Easterson D. C. V., 1974 Energy conversion in the shrimp, *Metapenaeus monoceros* (Fabricius) fed on detritus. Indian J Mar Sci 3:131-134.
Ramesh S., Mathivanan N., 2009 Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. World J Microbiol Biotechnol 25:2103–2111.
Reddy N. G., Ramakrishna D. P. N., Raja Gopal S. V., 2011 A morphological, physiological and biochemical studies of marine *Streptomyces rochei* (MTCC 10109) showing antagonistic activity against selective human pathogenic microorganisms. Asian J Biol Sci 4(1):1-14.
Ringø E., Gatesoupe F. J., 1998 Lactic acid bacteria in fish: a review. Aquaculture 160:177–203.
Rygus T., Hillen W., 1991 Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. Appl Microbiol Biotechnol 35:594-599.
Shinobu R., 1958 Physiological and cultural study for the identification of soil actinomycete species. Mem Osaka Univ B Nat Sci 7:1-76.
Shirling E. B., Gottlieb D., 1966 Methods for characterization of *Streptomyces* species. Int J Sys Bact 16(3):313-340.
Soundarapandian P., Babu R., 2010 Effect of probiotics on the hatchery seed production of black tiger shrimp, *Penaeus monodon* (Fabricius). Int J Anim Vet Advan 2(1):9-15.
Sunilkumar M. K., 1996 Heterotrophic marine bacteria as supplementary feed for larval *Penaeus monodon*. NAGA, ICLARM 19(1):23-26.
Takizawa M., Colwell R. R., Hill R. T., 1993 Isolation and diversity of actinomycetes in the Chesapeake Bay. Appl Environm Microbiol 59:997–1002.
Tanaka Y., Omura O., 1990 Metabolisms and products of actinomycetes - an introduction. Actinomycetologica 4:13-14.
Tresner H. D., Hayes J. A., Backns E. J., 1968 Differential tolerance of *Streptomyces* to sodium chloride as a taxonomic aid. Appl Microbiol 16:1134-1136.
Valencia M. C., 1976 The effect of salinity and temperature of the growth and survival of penaeid post larvae. Philippine J Fisheries 14:1-22.
Vary P. S., 1994 Prime time for *Bacillus megaterium*. Microbiology 140:1001-1013.
Vary P. S., Biedendieck R., Fuerch T., Meinhardt F., Rohde M., Deckwer W. D., Jahn D., 2007 *Bacillus megaterium* - from simple soil bacterium to industrial protein production host. Appl Microbiol Biotechnol 76:957-967.
Vaseeharan B., Ramasamy P., 2003 Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Letters in Appl Microbiol 36:83–87.
Vaseeharan B., Ramasamy P., Murugan T., Chen J. C., 2005 In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. Int J Antimicrobial Agents 26:285-291.
Waksman S. A., 1961 The actinomycetes classification, identification and description of genera and species, 2. Baltimore, USA. Williams and Wilkins Company pp. 327.
Williams S. T., Cross T., 1971 Actinomycetes isolation from soil. In: Methods in microbiology. Booth C. (ed), Vol 4. Academic Press, New York, pp. 295-334.
Zheng Z., Zeng W., Huang Y., Yang Z., Li J., Cai H., Su W., 2000 Detection of antitumor and antimicrobial activities in marine organism associated actinomycetes isolated from the Taiwan Strait, China. FEMS Microbiol Lett 188:87-91.

Received: 27 January 2013. Accepted: 07 February 2013. Published online: 04 March 2013.
Authors:
Sheikh Aftabuddin, Institute of Marine Sciences and Fisheries, University of Chittagong, Chittagong-4331, Bangladesh, e-mail: aftabimsf@yahoo.com
Abul Kashem, Prime Shrimp Hatchery Limited, Hatchery Zone, Kolatoly, Cox's Bazar, Bangladesh, e-mail: md.kashem@gmail.com
Abdul Kader, Institute of Marine Sciences and Fisheries, Chittagong University, Chittagong-4331, Bangladesh, e-mail: makaderims@yahoo.com
Nurul Azim Sikder, Institute of Marine Sciences and Fisheries, Chittagong University,Chittagong-4331, Bangladesh, e-mail: sikderims@gmail.com
Abdul Hakim, Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh, e-mail: hakimcu@yahoo.com

How to cite this article:
Aftabuddin S., Kashem M. A., Kader M. A., Sikder M. N. A., Hakim M. A., 2013 Use of *Streptomyces fradiae* and *Bacillus megaterium* as probiotics in the experimental culture of tiger shrimp *Penaeus monodon* (Crustacea, Penaeidae). AACL Bioflux 6(3):253-267.