

Full Length Research Paper

Screening, identification and antagonistic activity of halo stable *Bacillus* sp. Mk22 used as probiotic in *Penaeus monodon* Fabricius, 1798

Sekar Ashokkumar^{1*} and Packyam Mayavu²

¹International Maritime Academy, Chennai, India.

²Faculty of Marine Sciences, Annamalai University, Parangipettai, India.

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The research of probiotics for aquatic animals is increasing with the demand for environmental friendly aquaculture. Most attempts to propose probiotic have been undertaken by isolating and selecting strains from saltpan environment. 16S rRNA gene sequencing showed that strain *Bacillus* sp. Mk22 (accession number: JF794553) was gram positive, had rod shape and was 937 nm in size. The isolated strain that was applied to *Penaeus monodon* culture under laboratory condition revealed that maximum survival rate was $92.7 \pm 1.53\%$, wet weight was 6.9 ± 0.15 g, production was 378 ± 1.0 g and feed conversion ratio was 0.9 ± 0.03 in experiment-III compared to those of control experiment. The present study also showed that the halophilic (*Bacillus* sp.) bacterium was able to colonize both the culture in water and shrimp digestive tract. The minimum total bacterial counts ($6.0 \pm 1.0 \times 10^5$ Cfu ml⁻¹) and maximum *Bacillus* counts ($5.6 \pm 0.75 \times 10^4$ Cfu g⁻¹) were recorded in experiment-III and not in the control.

Key words: *Bacillus* sp., polymerase chain reaction, *Penaeus monodon*, probiotic, Saltpan.

INTRODUCTION

The UN FAO estimates that half of the world's seafood demand will be met by aquaculture in 2020, as wild capture fisheries are overexploited and are in decline. Shrimp (or prawn) culture is wide spread throughout the tropical world. It is in an industry set for a period with strong growing demand and is currently worth around US\$10 billion. In most of the world, aquaculture industry is beset by disease outbreak, caused mostly by bacteria (especially for genome *Vibrio*) and viruses. The high density of animals in hatchery tanks and ponds is conducive for the spread of pathogens; and the aquatic environment as well as regular applications of protein-rich feed is ideal for culturing bacteria. Mostly, *Vibrio* spp. (*V. parahaemolyticus*, *V. harveii*, *V. alginolyticus*) cause major problems in aquaculture, resulting in reduced growth rate, poor feed consumption, loss of body weight and ultimately mass mortality.

A number of alternative strategies for the prevention and control of diseases have been proposed and have already been applied successfully in aquaculture, such as antibiotics, vaccines and immunostimulants. Antibiotics, one of the feed additives, were commonly used in the early 1950s (Ahilan et al., 2004). The problem of antibiotic contamination of aquaculture facilities and livestock and the indiscriminate worldwide use of antibiotics in aquaculture have led to the development of drug-resistant bacteria which are becoming increasingly difficult to control and eradicate (Hayashi et al., 1993; DePaola, 1995; Bruun et al., 2000; Sahul and Balasubramanian, 2000; Van der Waaij and Nord, 2000; Miranda and Zemelman, 2002). Subsequently, certain antibiotics such as chloramphenicol have been banned in many countries (Robert et al., 1995; FAO website – URL in reference list). As a result of resistant bacterial strains becoming

*Corresponding author. E-mail: kumarebt@gmail.com.

more prevalent and difficult to treat, alternative methods of controlling the microbial environment are being investigated. One of the methods gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria (Verschuere et al., 2000; Irianto and Austin, 2002a).

Some halophilic bacteria against aquaculture pathogens are used in aquaculture. These bacteria both live in high saline and medium saline environment. Halophiles can be classified as slight, moderate and extreme microorganisms depending on their NaCl requirement. Slight halophiles require 0.2 – 0.85 M (2-5%) NaCl, moderate halophiles require 0.85 – 3.4 M (5-20%) NaCl and extreme halophilic microorganisms grow optimally above 3.4 – 5.1 M (20-30%) NaCl concentrations. Hence, in the present study, probiotic microorganisms were isolated from the saltpan environment and applied.

MATERIALS AND METHODS

Sediment samples were collected monthly from Marakkanam saltpan environment (Lat. 12° 14' 29N; Long. 79° 56' 28E) at Tamilnadu, India. Isolation of probiotic bacteria was done using well plate method in antagonistic activity. Physico-chemical character was not included in the results.

Isolation of halophilic bacteria

Isolation of halophilic bacterial was done using the halophilic agar (Hi-Media, Mumbai) (Mellado et al., 1998). Totally, 25 strains were isolated from the saltpan environment and kept at 4°C for further analysis.

Identification

16S rDNA sequencing was used to identify the beneficial bacteria. Once the DNA sequence was edited and assembled, identification was done using Microseq Analysis Software and Sequence Database and universal primers. Identification was based on the pairwise alignment algorithms and phylogenetic tree. The two shrimp pathogenic *Vibrio* strains namely, *V. parahaemolyticus* and *V. harveyi* were used to screen the antagonistic activity of the isolated halophilic bacteria. The beneficial bacteria were sequenced using the forward and reverse primer; 16S rRNA forward primer: AGA GTT TGA TCC TGG CTC AG and reverse primer: ACG GCT ACC TTG TTA CGA CTT. The sequences were assembled using Clustal W software version 1.82 (Thompson et al., 1994) available at <http://www.ebi.ac.uk>. Strains' morphology and their surface were observed under the scanning electron microscope (Hitachi-s-1500X-SEM).

Rearing of shrimp

Separate experiment was conducted to examine the effect of probiotic administrated to black tiger shrimp (*P. monodon*). The stocking (*P. monodon*), post larval-15 were purchased from commercial hatcheries and screened for WSSV using 2 step PCR test based on the methods of Lo et al. (1996). WSSV-negative PL was brought to the laboratory and stocked in 50 L capacity culture tanks having 35 L chlorinated filtered estuarine water. The animals were provided with proper aeration and feeding was given at 7.5% of the body weight initially; subsequent feeding was adjusted to 5 to 3.5% of the body weight per day according to the left-out, unutilized

feed and increasing body weight of animals. The feed was given twice a day; 60% at dawn (6.00 a.m.) and 40% at dusk (6.00 p.m.). In the experimental tanks, the water quality parameters were maintained at the optimum range; the bottom water in the tank along with excess feed and fecal matter was siphoned out using 2 cm dia plastic hose to enhance the survival of the animals.

Experiment I

The effect of without probiotic on juveniles (*P. monodon*) was examined in experiment I. Shrimp was stocked in 50 L plastic tanks at density of 20 per tank in triplicate. The commercial pellet feed was used as a control. Water was exchanged weekly and animal behavior was observed every day.

Experiment II

The effect of commercial probiotic on juveniles (*P. monodon*) was examined in experiment II. Shrimps were stocked in 50 L plastic tank at density of 20 per tank in triplicate. The commercial pellet feed mixed with commercial probiotic contained spore of two species of *Bacillus* sp. at a concentration of 1.2×10^5 CFU/ml. Water was exchanged weekly and animal behavior was observed every day.

Experiment III

The effect of halophilic bacteria (*Bacillus* sp.) on juveniles (*P. monodon*) was examined in experiment III. Shrimps were stocked in 50 L plastic tanks at a density of 20 per tank in triplicate. The commercial pellet feed was mixed with halophilic bacteria (*Bacillus* sp.) at a concentration of 1.2×10^6 CFU/ml. Water was exchanged weekly and animal behavior was observed every day.

Survival and growth rate

At the end of each experiment, the percentage survival was determined and 10 shrimps were sampled randomly from each tank to determine wet weight, feed conversion ratio (FCR), production and survival rate with the following equation.

Production

The production rate was calculated by the following formula:

$$\text{Production} = \text{Initial weight (g)} / \text{Final weight (g)}$$

Feed Conversion Ratio (FCR)

The feed conversion was calculated using the formula:

$$\text{FCR} = \text{Feed taken in (dry weight in g)} / \text{Weight gain (wet weight g)}$$

Monitoring of bacteria

Water samples and digestive tract samples from shrimp were used to determine the counts of total bacteria and counts of the halophilic probiotic bacteria (*Bacillus*). Prior to dissection or homogenation, the shrimps were rinsed with sterilized distilled water, washed with 0.1% benzalkonium chloride according to the method of Gatesoupe (1999) and then rinsed again with sterilized distilled water to remove all external bacteria. In all the experiment, the digestive tract was dissected out using sterile technique and then was homogenized. All samples were diluted serially with sterilized normal saline solution (0.85% w/v NaCl). Total counts of bacteria were determined by plating on zobal marine agar (with 1% w/v NaCl). *Bacillus* bacteria in water and digestive tract samples were cultured

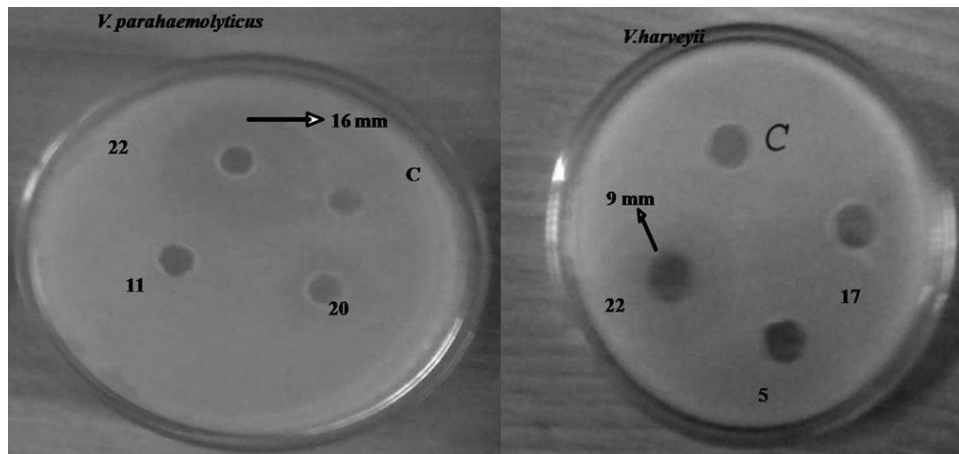


Figure 1. Experimental studies showed antagonistic activities of halophilic strain (*Bacillus* sp.)

according to the method recommended by Probiotics International Ltd. (Protexin Aquatech, Registration Dossier, unpublished pamphlet). The number of colonies on each plate was counted after incubation for 68 h at 25°C for water samples and for 72 h at 37°C for digestive tract samples. Most of the *Bacillus* spp. (*Bacillus* sp. MK22) cultured in fresh or marine water grow well in high and moderate salt concentration. *Bacillus* sp. had maximum cell count inside the digestive tract of shrimp for 54 h. All statistical analysis was significance; the level of $P < 0.05$ was used for all tests. Data are reported as means \pm standard deviation.

RESULTS

The present study showed that every month, the total bacterial population increased between 12×10^6 CFUg⁻¹ to 45×10^6 in saltpan environment.

A total of 25 halophilic bacterial strains were preliminary screen based on the zone of inhibition; five strains showed acceptable activities. Among these, a strain, *Bacillus* sp. Mk22 showed large clear zones around the bacterial colonies and it was selected for further studies.

The initial screening is shown in Figure 1. The strain showed maximum zone of inhibition against *V. parahaemolyticus* (16 mm) and the minimum was observed against *V. harveyi* (9 mm) (Figure 1).

The SEM images were taken to verify the size and morphology of bacterial cells. Figure 2 shows the SEM image of *Bacillus* sp: rod shape, smooth and had approximate size of 3.0 μ m. The isolate, *Bacillus* sp. Mk22 was subjected to molecular level identifications.

In the Phylogenetic analysis based on a comparison of the 16S rDNA sequence, data showed that the genus *Bacillus* is phylogenetically homogeneous, forming a distinct lineage within the radiation of the bacteria. The dendrogram placed the strain *Bacillus* sp. Mk22 in a separate line of descent within the genus *Bacillus*, representing a distinct phylogenetic lineage (Figure 3).

Further, representatives of other species like *Bacillus baekryungensis*, *B. marisflavi*, *Cloacibacterium normanense*, *B. ferrariarum*, *B. aquimaris*, *Bacillus* sp.,

Bacillus sp., and *Bacillus* sp. Mk22 (JF794553.1) with 99% similarity in the genus levels formed a distinct cluster.

The maximum survival ($92.7 \pm 1.53\%$) was recorded in experiment-III and the minimum ($60 \pm 2.53\%$) was observed in experiment I (Table 1). Administration of the probiotic significantly increased survival in all treatments (generally by 20–30%) over the controls, except in experiment III, where survival was significantly ($P < 0.05$) different from that in the controls (Table 2). The maximum wet weight (6.9 ± 0.15 g) was recorded in experiment III and minimum (3.5 ± 0.10 g) was observed in experiment I (Table 1). Wet weight was significantly ($P < 0.05$) greater in treatments than in the control. The production rate was found to be higher (378 ± 1.0 g) in experiment III followed by experiment II (301 ± 1.0 g); in the control experiment, it was low (159.3 ± 1.53 g) (Table 1). Production was more ($P < 0.05$) significant in experiment III than in the control. The maximum (1.2 ± 0.28) FCR was recorded in experiment I and minimum (0.9 ± 0.03) FCR was recorded in experiment III (Table 1). There was 5% level of significance in experiment-III compared to the control.

Monitoring of bacteria

In all the experiments, Halophilic (*Bacillus* sp.) bacteria successfully colonized both the culture water and the digestive tract of the shrimp (Table 2). The maximum total count ($24 \pm 0.79 \times 10^5$ Cfu ml l⁻¹) was recorded in experiment I and minimum ($6.0 \pm 1.0 \times 10^5$ Cfu ml l⁻¹) was observed in experiment III; and the maximum *Bacillus* count ($3.7 \pm 0.58 \times 10^4$ Cfu g⁻¹) was recorded in experiment III and minimum (0×10^4 Cfu g⁻¹) was observed in experiment I in water (Table 2).

The maximum total *Bacillus* count ($35.4 \pm 0.59 \times 10^5$ Cfu ml l⁻¹) was recorded in experiment I and minimum ($4.1 \pm 0.47 \times 10^5$ Cfu ml l⁻¹) was observed in experiment III; and the maximum *Bacillus* count ($5.6 \pm 0.75 \times 10^4$ Cfu g⁻¹) was recorded in experiment III and minimum (0×10^4 Cfu g⁻¹) was observed in experiment I in the digestive tract (Table

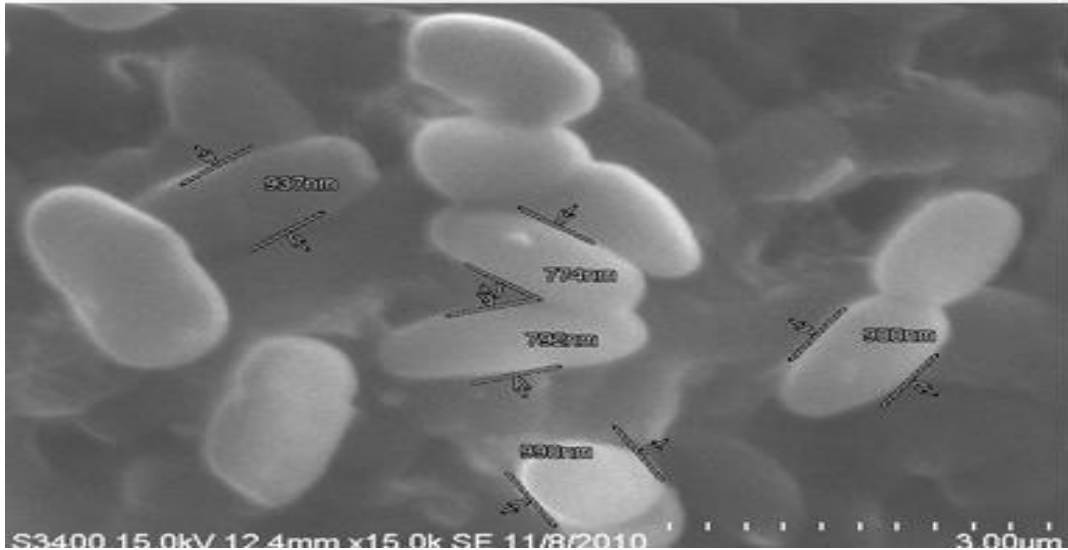


Figure 2. Scanning electron microscopy (SEM) image of *Bacillus* sp. Mk22.

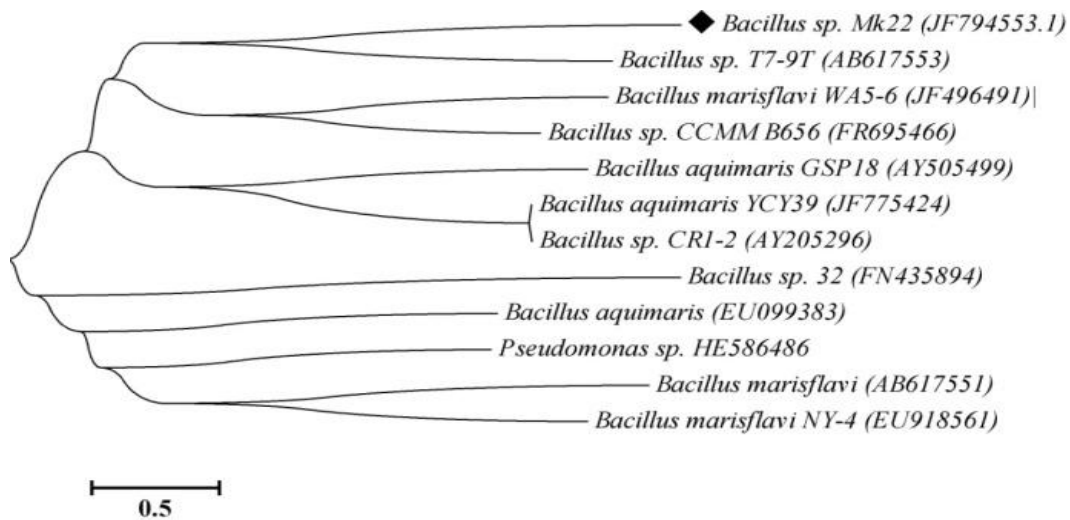


Figure 3. 16S rRNA gene sequence-based phylogenetic relationships of *Bacillus* sp. Mk22 (830 nucleotides) and closely related members of the genus *Bacillus*. The tree was constructed using the neighbourjoining algorithm. GenBank accession numbers are given in parentheses. Only bootstrap values above 50% are shown (1000 replications). Bar 0.5 substitutions per 100 nucleotides. Triangle indicates the present study strain.

Table 1. Growth and survival parameters of *P. monodon* reared with and without *Bacillus* probiotic added to water.

Experiment	Stocking density (<i>P. monodon</i>)	Survival (%)	Wet weight (g)	Final production(g)	FCR
I	60	60 ± 2.52	3.5 ± 0.10	159.3 ± 1.53	1.2 ± 0.28
II	60	83 ± 2.0	5.5 ± 0.36	301 ± 1.0	1.0 ± 0.04
III	60	92.7 ± 1.53	6.9 ± 0.15	378 ± 1.0	0.9 ± 0.03

Mean ± S.D. Exp-I (Control no probiotic provided); Exp-II- Commercial probiotic (Superbiotic) provided; Exp- III *Bacillus* sp. Mk22 provided.

Table 2. Total bacterial count and *Bacillus* count in water and in digestive tracts of *P. monodon* reared with and without *Bacillus* probiotic added to water.

Experiment	Water		Digestive tract	
	Total counts (10 ⁵ CFU ml ⁻¹)	<i>Bacillus</i> count (10 ⁴ CFU ml ⁻¹)	Total counts (10 ⁵ CFU g ⁻¹)	<i>Bacillus</i> count (10 ⁴ CFU g ⁻¹)
I	24 ± 0.79	0	35.4 ± 0.59	0
II	8 ± 1.00	5 ± 1.00	8.7 ± 0.85	4.4 ± 0.42
III	6 ± 1.00	3.7 ± 0.58	4.1 ± 0.47	5.6 ± 0.75

Mean ± S.D. Exp-I (Control no probiotic provided); Exp-II- Commercial probiotic (Superbiotic) provided; Exp- III *Bacillus* sp. Mk22 provided.

2). The ANOVA showed 5% level of significance ($P < 0.05$) between the water and digestive tract.

DISCUSSION

Aquaculture is badly affected by parasites, fungi, bacteria, viruses and non-infectious diseases cause problems. Various toxins and other water quality stressors can also affect crustacean health. With the increasing importance of crustacean aquaculture as well as mounting pressures on fisheries, there is a need for reliable accurate means for developing the health of crustaceans (Sindermann, 1990). Particularly, vibriosis is one of the major diseases in aquatic organisms. This is usually caused by the species belonging to the genus *Vibrio* which are the natural inhabitants of estuarine and marine environments, well known for causing vibriosis in fish worldwide (Schaperclaus, 1986). Several species are known to be pathogenic to aquatic animals as well as humans. They are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments. Even though there are several remedies in controlling *Vibrio*, there exists several constraints such as the prevalence of the antibiotic resistance bacteria. In this present study we aimed to find the potential of the application of halophilic bacteria for controlling the crisis of vibriosis in the culture of *P. monodon*.

The total bacterial count varied between 12 x10⁶ and 45 x10⁶ CFUg⁻¹. 16S rDNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between microorganisms and to identify bacteria from various sources, such as environmental or specimens. This technology is used today in laboratories for routine identifications, not only for slow-growing, unusual or fastidious bacteria but also for bacteria that are poorly differentiated by conventional methods.

Among the five isolates, *Bacillus* sp. Mk22 was selected for molecular level identification and it was subjected to further probiotic study. In phylogenetic analysis based on a comparison of the 16S rDNA sequencing, data showed that the genus, *Bacillus* is phylogenetically homogeneous, forming a distinct lineage within the radiation of the bacteria. The dendrogram placed the strain *Bacillus* sp. Mk22 in a separate line of descent within the

genus *Bacillus*, representing a distinct phylogenetic lineage and forming a distinct cluster. Gracia et al. (1987) reported an endospore forming gram positive *Bacillus* sp from saltpan in USA. Even though Garabito et al. (1997) proposed the new species *Bacillus salexigens* in hypersaline soils located in different soils located in different geographical areas of Spain, Yoon et al. (2004) proposed the transfer of *Bacillus halodenitrificans* a gram variable, endospore forming moderately halophilic rod isolated from a marine solar saltern of the yellow sea in Korea.

Bacillus sp. Mk22 is closely related to the *Bacillus* sp., 32 JD-2009 (FN435894); there is detection of halophilic bacteria and archaea on the extreme salt environment that attacks monuments. There was identification of halophilic bacteria from a salt marsh and two salters in the protected ecosystem of lower Loukkos (Larache, Morocco). *Bacillus* sp T7-9T (AB617553) and *Bacillus* sp., CCMM B656 (FR695466) are all the strains isolated from the saltpan environment in South Korea (Na et al., 2011); and other species related to *Bacillus aquimaris*, with accession number EU099383, have 99% similarity in the species level. *B. aquimaris* is not only present in the saltpan environment but also in the marine environment. *B. aquimaris* is isolated from the Kumta Coast of Karnataka, India; this bacterium grows at pH 7.5- 9.5 and 40°C (Pooja and Jayaraman, 2009). *B. aquimaris* is a relatively novel marine bacterium. Recent reports are available on the culture characteristics, sequence information and phylogeny of the organism (Gontang et al., 2007; Liu and Shao, 2007). Yoon et al. (2004) reported the isolation of endospore-forming, rod shaped moderately halophilic, *B. aquimaris* (TF-12T) from seawater.

Halophilic bacterial forms produce several secondary metabolites such as bacteriocins, bacteriocin like substances and antibacterial lipopeptides and these metabolites have received considerable attention as biological control agents in pharmaceutical industry because they are generally recognized as safe (GRAS), have low toxicity, high biodegradability, and are environmental friendly (Oliveira and Pijoan, 2004). In the present study, the halophilic bacterial strain *Bacillus* sp. showed antagonistic activities against all shrimp pathogens (*V. parahaemolyticus* (16 mm) and *V. harveii* (9 mm)). Lee et

al. (2010) reported a novel analysis of *Bacillus subtilis* SC-8 antagonistic to *Bacillus cereus*. Liu et al. (2008) proved antagonistic activities of volatiles from four strains of *Bacillus* spp. and *Paenibacillus* spp. against soil-borne plant pathogens.

Several early reports are available for mode of action and application of probiotics in ponds (Sugita et al., 1998). In the present investigation, halophilic bacterial strain *Bacillus* sp. was used as probiotic for *P. monodon*. Under laboratory conditions, the result showed excellent production rate (378 ± 1.0 g), wet weight (6.9 ± 0.15 g) and FCR (0.9 ± 0.03). Rengpipat et al. (2000) reported that *Bacillus* sp. will activate both cellular and humoral immune defense against shrimp pathogens in tiger shrimp (*P. monodon*). Balcazar et al. (2006) stated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrio* sp) positively influence the growth and survival of juvenile of white shrimp. The present study also revealed that *Bacillus* sp. was able to colonize both the culture water and the shrimp digestive tract. Based on the above observations, it was concluded that the strain of halophilic *Bacillus* sp. was effective in inhibiting the shrimp pathogens, like *V. paraheamolyticus* and *V. harveyi* both *in vitro* and *in vivo*. The probionts significantly reduce mortality and also do not have any pathogenic effect on the shrimp larvae. Halophilic (*Bacillus*) bacteria were administered to *P. monodon* under laboratory condition; final production, FCR, wet weight and survival were significantly higher in experiments I-III than in the control. Therefore, these bacterial probionts can be used effectively to control the shrimp pathogens and enhance production that may substitute the use of antibiotics in aquaculture.

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REFERENCES

- Ahilan B, Shine G, Santhanam R (2004). Influence of probiotics on the growth and gut microflora load of juvenile Gold fish (*Carassius auratus*). Asian Fish. Sci. 17: 271–278.
- Balcazar JL, Blas I, Ruiz-Zarzuola I, Cunningham D, Vendrell D, Muzquiz JL (2006). The role of probiotics in aquaculture. Vet. Microbiol. 114: 173–186.
- Bruun MS, Schmidt AS, Madsen L, Dalsgaard I (2000). Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture 187:201-212.
- DePaola A (1995). Tetracycline resistance by bacteria in response to oxytetracycline contaminated catfish feed. J. Aquat. Anim. Health 7: 155-160.
- Garabito MJ, Arahall DR, Mellado E, Marquez MC, Ventosa A (1997). *Bacillus salaxigens* sp. nov., a new moderately halophilic *Bacillus* species. Int. J. Syst. Bacteriol. 47: 735-741.
- Gatesoupe FJ (1999). The use of probiotics in aquaculture. Aquaculture 180:147- 165.
- Gontang EA, Fenical W, Jensen PR (2007). Phylogenetic diversity of Gram positive cultures from marine sediments. Appl. Environ. Microbiol. 73: 3272–82.
- Hayashi F, Fuse A, Inoue M, Ishii H, Barcs I, Mitsuhashi S (1993). Frequency of drug resistance in *Vibrio* spp. in Japan. Lett. Appl. Microbiol. 16: 28-31.
- Irianto A, Austin B (2002). Probiotics in aquaculture Review. J. Fish Dis. 25:633-642.
- Lee NK, Yeo IC, Park JW, Kang BS, Hahm YT (2010). Isolation and characterization of a novel analyte from *Bacillus subtilis* SC-8 antagonistic to *Bacillus cereus*. J. Biosci. Bioeng. 110: 298-303
- Liu W, Wei M, Zhu B, You-chen D, Liu F (2008). Antagonistic Activities of Volatiles from Four Strains of *Bacillus* spp. and *Paenibacillus* spp. Against Soil-Borne Plant Pathogens. Agric. Sci. China 7(9):1104-1114.
- Liu Z, Shao ZZ (2007). The diversity of alkane degrading bacteria in the enrichments with deep sea sediment of the South China Sea. Pub. Med. 47: 869-73.
- Lo CF, Leu JH, Chen CH, Peng SE, Chen YT, Chou CM, Wang CH, Kou GH (1996). Detection of *Baculovirus* associated with White Spot Syndrome (WSBV) in penaeid shrimp using polymerase chain reaction. Dis. Aquat. Org. 25:133–141
- Mellado E, Garcia MT, Roldan E, Nieto JJ, Ventosa A (1998). Analysis of the genome of the Gram-negative moderate halophiles Halomonas and Chromohalobacter by using pulsed-field gel electrophoresis. Extremophiles 2: 435-438.
- Miranda CD, Zemelman R (2002). Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture 212(1-4):31-47.
- Na J-M, Kang M-S, Kim J-H, Jin Y-X, Je J-H, Kim J-B, Cho Y-S, Kim J-H, Kim S-Y (2011). Distribution and Identification of Halophilic Bacteria in Solar Salts Produced during Entire Manufacturing Process. Korean J. Microbiol. Biotechnol. 39:(2) 133–139
- Oliveira S, Pijoan C (2004). Haemophilus parasuis: new trends on diagnosis, epidemiology and control. Vet. Microbiol. 99:1-12.
- Pooja S, Jayaraman G (2009). Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. Process Biochem. 44:1088-1094.
- Rengpipat S, Pukpratanporn S, Piyatitivorakul S, Menasaveta P (2000). Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191: 271-288.
- Robert R, Nicolas JL, Miner P (1995). Mortality control of Pecten maximus larvae in hatchery. In Abstracts. 10th. Pectinid Workshop, Cork Ireland. pp. 51-52.
- Sahul Hameed AS, Balasubramanian G (2000). Antibiotic resistance in bacteria isolated from Artemia nauplii and efficacy of formaldehyde to control bacterial load. Aquaculture 183:195-205.
- Schaperclaus W (1986). Fish Diseases. Akademie-Verlag, Berlin. 2: 665-666 .
- Sindermann CJ (1990). Principal diseases of marine fish and shellfish, Vol. 2. Gulf Professional Publishing, Academic Press, San Diego, CA
- Sugita H, Hirose Y, Matsuo N, Deguchi Y (1998). Production of the antibacterial substance by *Bacillus* sp. strain NM12, an intestinal bacterium of Japanese coastal fish. Aquaculture 165:269-280.
- Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.
- Van der Waaij D, Nord CE (2000). Development and persistence of multi-resistance to antibiotics in bacteria; an analysis and a new approach to this urgent problem. Aquaculture 16:191-197.
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000). Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64: 655-671.
- Yoon JH, Oh TK, Park YH (2004). Transfer of *Bacillus* halodenitrificans Denariet et al., 1989 to the genus *Virgibacillus* as *Virgibacillus* halodenitrificans comb. nov. Int. J. Syst. Evol. Microbiol. 54: 2163-2167.