CHAPTER 3

Screening of Marine Actinomycetes as Putative Probiotics in *Penaeus monodon* Culture Systems

3.1 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 over-exploited and in decline. Shrimp culture is widespread throughout the tropical world, and *Penaeus monodon*, the black tiger prawn is the most widely cultured species. However, the shrimp aquaculture industry is beset by disease, mostly due to bacteria, (especially the luminous *Vibrio harveyi*) and viruses. This has resulted in huge economic loss to the shrimp industry. The high density of animals in hatchery tanks and ponds is conducive to the spread of pathogens, and the aquatic environment, with regular applications of protein-rich feed, is ideal for culturing bacteria.
(Moriarty, 1997). Moreover, unlike land animals, aquatic animals are surrounded by a milieu that supports opportunistic pathogens independently of the host animal, and so the pathogens can reach high abundance around the animal.

The intensive cultivation conditions for marine shrimp larvae may easily cause microbial problems, both bacterial and viral. Shrimp larvae are small and sensitive, so there is a period with no or low water exchange in the early stages of larval rearing, and this leads to a condition with high larval densities, the accumulation of debris from dead larvae and high loads of organic matter (Skjermo and Vadstein, 1999). Under stress, the marine larvae will lower their feeding activities, the amount of unconsumed feed in the culture pond will increase, and this cause both nutrient enrichment and deterioration of water quality (Sung et al., 1994).

Vibrios are gram negative bacteria prominent in marine environment and are more frequent in sediment than in water (Bhaskar.N.Shetty et al., 1998) and are the normal flora in both cultivated and wild penaeid shrimp culture systems (Bauchau, 1981). But many marine Vibrio spp. are opportunistically pathogenic bacteria. The proliferation of opportunistically pathogenic vibrios are the major cause of mortality in culture systems rich in organic nutrients. In fact, vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in all shrimp producing countries. More than thirty species of Vibrio have been identified and the major species causing vibriosis are Vibrio harveyi, Vibrio alginolyticus, Vibrio parahaemolyticus and Vibrio anguillarum (Goarant et al., 1999).

Larval mortalities associated with the presence of V.harveyi have been reported in Penaeus monodon and Penaeus vannamei in Indonesia (Sunaryanto and Mariam, 1986),
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Thailand (Jiravanichpaisal et al., 1994), India (Karunasagar et al., 1994), Philippines (Baticados et al.) and Australia (Pizzuto and Hirst, 1995). Disease outbreaks attributed to the other *Vibrio* spp. such as *V.alginolyticus, V.damselei, V.parahaemolyticus, V.vulnificus, V.penaeicida* have been observed in nursery and growout ponds of *P. vannamei, P. monodon, P. Japonicus, P. stylirostris,* and *P.orientalis* in Ecuador (Bauchau, 1981), Philippines (Alapide-Tendencia and Dureza, 1997), New Caledonia (Cost et al. (1998) & Mermound et al. (1998)) and in Peoples Republic of China (Sudheesh and Xu, 2001). *V.harveyi*, a luminous species of *Vibrio*, has been recognized as a tropical pathogen of importance especially in shrimp culture (Owens et al. (1992), Karunasagar et al. (1994), Jiravanichpaisal et al. (1994) and Abraham and Manley (1995)). It can elicit disease and significant mortality in shrimp larvae at $10^2$-$10^3$ cfu/ml (Karunasagar et al., 1994).

Several strategies to control vibriosis have been proposed. For instance, vaccines are being developed to control vibriosis, but they generally cannot be used as a universal disease control measure in aquaculture as they are too time and labour intensive. So the addition of substantial amounts of antibiotics and chemotherapeutics remains the method of choice for disease control. But the abuse of antimicrobial chemicals has led to the occurrence of resistant strains and accumulation of chemicals in aquaculture products. A few studies on antibiotic resistance of *Vibrio* spp. in aquaculture suggested that they are resistant to several antibiotics such as erythromycin, kanamycin, pencillin,G and streptomycin.

In 1990, only 4 antibiotics were resistant to *Vibrio* spp., but 9 years later, the number of resistant antibiotics increased to 20 (Eleonar and Leobert., 2001). The presence of antimicrobial agents at low concentration through leaching or continued usage may lead to the development of drug resistant strains and multiple antibiotic resistance (MAR) in
bacteria, which may result in resistance transfer to pathogenic bacteria. Hence developing alternative strategies to the use of antimicrobials in disease control is urgent. One of the successful methods to control vibriosis is the use of probiotics, which are applied in the feed or added to the culture tank or pond as preventive agents against infection by pathogens. Most probiotics proposed as biological control agents in aquaculture are lactic acid bacteria, \textit{(Lactobacillus, Carnobacterium etc)}, non-pathogenic \textit{Vibrio (Vibrio alginolyticus,), Bacillus strains} and \textit{Pseudomonas strains (Verschuere et al., 2000)}, but few studies on actinomycetes as probiotics have been reported.

3.1.1 Probiotics

The term probiotics was first coined by (Parker, 1974). It originated from two Greek word 'pro' and 'bios', which means 'for life'. "Probiotics", "probiont", "beneficial bacteria", or "friendly bacteria" are the terms synonymously used for probiotic bacteria (Rao, 2002). Elie Metchnikoffs work at the beginning of this century is regarded as the first research conducted on probiotics.(Fuller, 1992). The use of probiotics in human and animal nutrition is well documented Fuller (1992), Rinkinen et al. (2003) and recently, they have begun to be applied in aquaculture (Gatesoupe. (1999), (Bachere et al., 1995), Gomez-Gill et al. (2000), Verschuere et al. (2000) and Irianto and Austin (2002)). (Fuller, 1992) defined probiotics as 'A live microbial feed supplement, which beneficially affects the host animal by improving its microbial intestinal balance. The new definition of probiotics is that it is 'a mono or mixed culture of live microorganisms that, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora (Havenaar et al., 1992).
3.1.2 Probiotics in Aquaculture

The theory of ecological prevention and cure in controlling pest of terrestrial higher grade animals and plants has been in practice for long time, and has achieved great success. The use of beneficial digestive bacteria in human and animal nutrition is well documented. *Lactobacillus acidophilus* is used commonly to control and prevent infections by pathogenic microorganisms in the intestinal tract of many terrestrial animals. Recently, the biocontrol theory has been applied to aquaculture also. Many researchers attempt to use some kind of probiotics in aquaculture ponds to regulate the micro flora in water, control pathogenic microbes, to enhance decomposition of undesirable organic compounds, and improve ecological environment in aquaculture. In addition, the use of probiotics can increase the population of food organisms, improve the nutritional level of aquaculture animals, and enhance immunity of cultured animals to pathogens.

In aquaculture systems, the probiotics are applied in two ways i.e.) as gut probiotics to maintain the microbial balance of the animal and thereby reduce the number of pathogenic species in the body and as pond probiotics applied to water / sediment, which will provide a healthy environment for the animals and help in the exclusion of pathogens (Singh and Jayaprakash, 2002). Nogami and Maeda (1992) isolated a bacterial strain from a crustacean culture pond, and was found to improve the growth of crab, *Portunus trituberculatus* larvae and repress the growth of vibrios. Austin et al. (1992) reported a kind of micro algae (*Tetraselmis suecica*) which can inhibit pathogenic bacteria of Atlantic salmon. Smith and Davey (1993) reported that fluorescent *pseudomonads* can competitively inhibit growth of fish pathogen *Aeromonas salmonicida*, by competing for free iron. Garriques and Arevalo (1995) reported that the use of *Vibrio alginolyticus* as a probiotic agent may increase survival and growth in *Penaeus vannamei* post larvae. Douillet and Langdon (1994) have reported that the addition of two probiotic bacteria
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... to oyster larval ponds had enhanced production by providing essential nutrients and digestive enzymes. *Pseudomonas* PS-102, isolated from a brackish water lagoon, showed antagonistic property to a wide range of pathogenic vibrios isolated from penaeid and *Macrobrachium* larval rearing systems and it is proposed that the inhibitory activities is probably due to the ability of the strain to produce siderophores (Vijayan et al., 2005).

3.1.2.1 Modes of Action of Probiotics

Several mechanisms have been suggested as modes of action for probiotic bacteria. Enhancement of colonisation, resistance and/or direct inhibitory effects against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have been shown to inhibit pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms. Several studies on probiotics have been published during the last decade. Some possible benefits linked to the administration of probiotics have already been suggested as i) competitive exclusion of pathogenic bacteria (Garriques and Arevalo (1995), Moriarty (1997), Gomez-Gill et al. (2000) and Vine et al. (2004)) ; ii) source of nutrients and enzymatic contribution to digestion (Prieur et al. (1990) and Garriques and Arevalo (1995)); iii) direct uptake of dissolved organic material mediated by the bacteria (Garriques and Arevalo (1995) and Moriarty (1997)); iv) enhancement of the immune response against pathogenic microorganisms (Rengipipat et al. (2000), Gullian and Rodriguez. (2002), Irianto and Austin (2002), Balcazar et al. (2003) and Balcazar et al. (2004)); v) Colonisation in the gastrointestinal tract; vi) antiviral effects (Kamie et al. (1988) and (Girones et al., 1989)). Moreover they should also be non-pathogenic to the host organism.

Competitive Exclusion

Bacterial antagonism is a common phenomenon in nature; therefore, microbial interac-
tions play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms. The competitive exclusion mechanism (competition for nutrients, space or oxygen), based on the substitution of the pathogen by the beneficial population, has been considered to be important by many authors (Fuller (1989), Moriarty (1998) and Gatesoupe. (1999)). Through bacterial substitution, it is possible to reduce the adherence of pathogenic strains in the host animal and consequently reduce the risk of disease. Some may act by inhibiting the pathogens by producing antibiotics, bacteriocins, lysozymes, proteases, and/or hydrogen peroxide and by altering pH values by producing organic acids. (Verschuere et al., 2000).

In aquaculture, Thalassobacter utilis, has shown inhibitory effects against Vibrio anguillarum. This strain increased the survival of larvae of the crab, Portunus triangulatus, and also reduced the amount of Vibrio sp. in the water used to rear the larvae (Nogami and Maeda (1992) and Nogami et al. (1997)). Gram et al. observed in vitro inhibition of Vibrio anguillarum by Pseudomonas florescens and obtained lower mortalities in probiotic treated fish, Oncorhynchus mykiss. Specific inhibition of V.harveyi by Pseudomonas aeruginosa has been reported earlier by Torrento and Torres (1996). The use of Vibrio anguillarum as probiotics has been recommended to increase survival and growth of white shrimp (Litopenaeus vannamei). Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis in intensive larviculture systems (Garriques and Arevalo, 1995). Recently, a marine bacterial strain, Pseudomonas 12, was isolated from estuarine environmental samples that produced inhibitory compounds against shrimp pathogenic vibrios (Chythanya and Karunasagar., 2002).
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Source of Nutrients and Hydrolytic Enzymes

Some microbes may act as sources of nutrients and enzymes that contribute to digestion, thus promoting growth. In fish, it has been reported that *Bacteriodes* and *Clostridium* spp. have contributed to the hosts nutrition, especially by supplying fatty acids and vitamins. In addition, some may participate in digestion of some bivalves by producing extracellular enzymes like proteases, lipases, carbohydrolases along with growth factors (Prieur et al., 1990). Similar observations have been reported for the microbial flora of adult penaeid shrimp, *P.chinensis*, where a complement of enzymes for digestion help in synthesis of compounds that are assimilated by the animal (Wang et al., 2000).

Influence on Water Quality

They could also act by improving water quality. The rationale is that gram positive bacteria are better converters of organic matter back to CO₂ than gram negative bacteria. It has been reported that use of *Bacillus* sp. improved water quality, survival and growth rates and increased health status of juvenile *P.monodon* besides reducing the pathogenic vibrios (Dalmin et al., 2001).

Enhancement of the Immune System

Balcazar et al. (2003) demonstrated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrio* sp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against the pathogens *Vibrio harveyi* and white spot syndrome virus. This protection was due to a stimulation of the immune system, by increasing phagocytosis and antibacterial activity. Rengipipat et al. (2000) mentioned that the use of *Bacillus* sp. (S11) provided disease protection by activating both cellular and humoral immune defenses in tiger shrimp. In addition, Pan et al. (2000) showed that administration of a lactic acid bacterium *Lactobacillus rhamnosus* (strain ATCC 53103) at
a level of $10^5$ cfug$^{-1}$ feed, stimulated the respiratory burst in rainbow trout (Oncorhynchus mykiss).

**Colonisation in the Gastrointestinal Tract**

Colonisation of the gastrointestinal tract of animals by probiotics is possible only after birth, and before the definitive installation of a very competitive indigenous microbiota. After this installation, only the addition of high doses of probiotic provokes its artificial and temporary dominance. In mature animals, the population of probiotic organism in the gastrointestinal tract shows a sharp decrease within days after intake had stopped. (Fuller, 1992).

According to Conway (1996), a microorganism is able to colonise the gastrointestinal tract when it can persist there for a long time, by possessing a multiplication rate that is higher than its expulsion rate. For example, *Vibrio* sp. normally colonize the hepatopancreas of juvenile white shrimp; however, this normal microflora can artificially become dominated by *Bacillus* sp. (upto 50% of the total) if it is added to the water for 20 days (Gullian and Rodriguez. 2002).

The process of colonization is characterized by attraction of bacteria to the mucosal surface, followed by association within the mucous gel or attachment to epithelial cells. Adhesion and colonization of mucosal surfaces are possible protective mechanisms against pathogens through competition for binding sites and nutrients (Westerdahl et al., 1991), or immune modulation (Salminen et al., 1998). Although there are very few reports on the colonisation efficiency of candidate probionts in shrimp gut, Moriarty (1998) suggested that application of *Bacillus* could displace other bacteria while competing for space in the
gut when present in high numbers.

**Antiviral Effects**

Some bacteria used as candidate probiotics have antiviral effects although the exact mechanism of action is not known. It has been reported that the strains of *Pseudomonas* sp., *Vibrio* spp., *Aeromonas* sp., and groups of coryneforms isolated from salmonid hatcheries, showed antiviral activity against infectious hematopoietic necrosis virus (IHNV) with more than 50% plaque reduction (Kamie et al., 1988). Direkbusarakam et al. (1998) isolated two strains of *Vibrio* spp. NICA 1030 and NICA 1031 from black tiger prawn which displayed antiviral activity against IHNV and *Oncorhynchus masou* virus.

### 3.1.3 Marine Actinomycetes as Probiotics in Shrimp Aquaculture

In the present investigation we have attempted to study the effect of marine actinomycetes as probiotics in shrimp aquaculture. Actinomycetes have long been recognized as prime sources of antibiotics, enzymes and other important metabolites. Over 4000 of the naturally occurring antibiotics discovered are synthesized by this group of microorganisms. Actinomycetes are dominant in marine sediments. Proven by numerous isolates from soil, actinomycetes merit competitive biosynthetic capabilities so that marine actinomycetes also should be considered as the prime candidates in screening as producers of novel products.

Actinomycetes have shown many interesting activities in water, such as degradation of starch and casein and production of antimicrobial agents against both gram negative and gram positive bacteria (Barcina et al. (1987) and Pisano et al. (1992)). Marine actinomycetes have also been proven to be antagonistic to *Vibrio* spp. pathogenic to shrimps (You et al., 2005). Marine actinomycetes exhibits a wide range of enzymatic activity, namely,
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chitinolytic activity (Pisano et al. (1992) and Mahendra et al. (2002)), cellulase activity by thermophilic actinomycete Microbispora, Streptomyces lividans (Kluepfel et al., 1986) and lipase activity by Streptomyces, (Large et al. (1999) and Gandolffi et al. (2000)). With these bioactivities, actinomycetes could play an important role in the food webs of the marine environment.

As potential probionts marine actinomycetes have many advantages: 1) degradation of macromolecules, such as starch and protein in the culture ponds 2) production of antimicrobial agents and 3) formation of heat and desiccation resistant spores. In the present study actinomycetes were screened for antagonistic and hydrolytic properties. The selected isolates were also screened for pathogenicity (in vitro and in vivo) and for ability to colonise in the intestine of shrimps. A study on exclusion of vibrios, both in vitro (Co-culture experiments) and in vivo and its effect on growth and survival of Penaeus monodon post larvae were also done.

3.2 Materials and Methods

3.2.1 Screening of Marine Actinomycetes for Antagonistic Property Against Shrimp Pathogens

3.2.1.1 Microorganisms

Actinomycetes isolates (99 Nos) isolated from the sediment samples collected from the West coast of India and South West coast of India were used to test the antagonistic potential against shrimp pathogens. Slant cultures of the different isolates grown on Marine Actinomycetes growth medium (MAG) (Table 3.1) was used to inoculate 20 ml seed medium
(Marine Actinomycetes Growth medium). The tubes were incubated on a reciprocal shaker at 100 rpm for 7 days at room temperature and were used for the study.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>1g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.4g</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.2g</td>
</tr>
<tr>
<td>Agar</td>
<td>2g</td>
</tr>
<tr>
<td>Sea Water (15 ppt)</td>
<td>100 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3.1: Composition of Marine Actinomycetes Growth Medium (MAG)

3.2.1.2 Pathogens

Eleven pathogenic isolates including ten *Vibrio* spp. (*Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio proteolyticus*, *Vibrio cholerae*, *Vibrio mediterranei*, *Vibrio vulnificus*, *Vibrio nereis*, *Vibrio fluvialis*, *Vibrio anguillarum*) and *Aeromonas hydrophila* isolated from larval rearing systems of *Peneaus monodon* and infected shrimp and prawn samples were used to test the antagonistic potential of actinomycete isolates. These cultures were obtained from the culture collection of National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology.

3.2.1.3 Antagonism Assay

The actinomycete culture broths (7 day old) were centrifuged at 10,000 rpm for 15 min. in a cooling centrifuge & the supernatants impregnated on four mm diameter sterile discs (Himedia) and placed on Nutrient agar (1.5 % NaCl) plates previously swabbed with the target bacterial pathogens (Kirby Baur Disc Asssay). The plates were then incubated at 30°C for 24h and the zone of inhibition around the discs was measured and recorded.
3.2.2 Screening of Marine Actinomycetes for Hydrolytic Enzyme Production

3.2.2.1 Microorganisms

Actinomycetes isolates (99 Nos) isolated from the sediment samples collected from the West coast of India and South West coast of India were used to test the hydrolytic (amylase, gelatinase, lipase, cellulase and chitinase) activity. Slant cultures of the different isolates grown on Marine Actinomycetes Growth Medium was used for the study.

3.2.2.2 Amylase Test

The isolates were spot inoculated onto Starch Agar medium of *Harigan and Maccance* (1972) (Table 3.2) and incubated for 4-5 days at room temperature (28 ± 2°C).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10g</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>10g</td>
</tr>
<tr>
<td>Starch (Soluble)</td>
<td>5g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Sea Water (50 %)</td>
<td>1L</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

*Table 3.2: Composition of Starch Agar Medium*

The production of amylase was tested by flooding the plates with Grams iodine solution. Unhydrolysed starch formed a blue colour and amylolytic colonies developed a clear zone around them. Zone of clearance was measured and recorded.

3.2.2.3 Lipase Test

Production of lipase was tested on Tributyrin Agar medium (*Rhodes*, 1959) (Table 3.3).
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<table>
<thead>
<tr>
<th>Peptone</th>
<th>5g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Extract</td>
<td>3g</td>
</tr>
<tr>
<td>Tributyrin</td>
<td>10 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Seawater (50%)</td>
<td>1L</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Table 3.3: Composition of Tributyrin Agar medium

The isolates were spot inoculated onto plates and incubated for 4-5 days at room temperature. Lipase production was detected by the appearance of halo zone around the colony. Zone of clearance was measured and recorded.

3.2.2.4 Gelatinase Test

Fraziers gelatin agar medium was used for detection of gelatinase activity (Table 3.4).

<table>
<thead>
<tr>
<th>Peptone</th>
<th>5g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3g</td>
</tr>
<tr>
<td>Gelatin</td>
<td>2g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Seawater (50%)</td>
<td>1L</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Table 3.4: Composition of Fraziers Gelatin Agar

The isolates were spot inoculated onto plates and incubated for 4-5 days at room temperature. The plates were flooded with Fraziers mercuric chloride solution and the colonies with halo zone were noted as positive.
3.2.2.5 Cellulase Test

The isolates were spot inoculated onto Cellulose Agar medium of (Riviere, 1961) and incubated for 4-5 days at room temperature (Table 3.5). Cellulase production was detected by the appearance of halo zone around the colony. Zone of clearance was measured and recorded.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose powder</td>
<td>5g</td>
</tr>
<tr>
<td>NaNO3</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>0.5g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Seawater (50 %)</td>
<td>1L</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Table 3.5: Composition of Cellulose Agar medium

3.2.2.6 Chitinase Test

The isolates were spot inoculated onto Chitin Agar medium (Holding and Collee, 1971) and incubated for 4-5 days at room temperature (Table 3.6). Chitinase production was detected by the appearance of halo zone around the colony. Zone of clearance was measured and recorded.

3.2.3 Screening of Marine Actinomycetes for Pathogenicity

3.2.3.1 Microorganisms Used

Based on the hydrolytic and antimicrobial activity three actinomycete strains (L18, L39, L45) (Fig. 3.1) were selected for further study.
Figure 3.1: Slant and slide culture of the selected three strains
3.2.3.2 Identification of the selected three actinomycetes based on 16S rDNA sequencing

Identification of the three strains were done based 16S rDNA sequencing (refer section 2.4.2.4).

3.2.3.3 Pathogenicity Test in vitro

The selected three actinomycete strains (L18, L39, L45) were tested for pathogenicity by observing haemolysis on prawn blood agar as per Chang et al. (2000).

Collection of Haemolymph

Adult *Penaeus monodon* were brought to the laboratory of School of Marine Sciences from a shrimp farm located at Kannamali, Cochin and acclimatised to the laboratory conditions for one week. The shrimps were surface sterilized by washing with ice-cold freshly prepared sodium hypochlorite solution (2000 ppm) followed by 70% ethanol. Haemolymph (1 ml) was collected aseptically from the rostral sinus of the prawn by using a sterile capillary tube and transferred into a sterile eppendorf tube containing 200 μl shrimp anticoagulant solution (Song and Hsieh, 1994).

Haemolysis Assay

One ml haemolymph was mixed with 130 μl 3% (w/v) Rose Bengal stain prepared in

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>5g</td>
</tr>
<tr>
<td>Colloidal chitin</td>
<td>5g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Seawater (50 %)</td>
<td>1L</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table 3.6: Composition of Chitin Agar medium
shrimp anticoagulant solution (3% W/V) in order to stain the haemocytes. Nutrient Agar
( NaCl-1.5 % ) was prepared, autoclaved and allowed the temperature of the media to
drop to 45°C - 50°C. Rose Bengal stained haemolymph (1ml) was added to 15 ml of this
medium with gentle shaking for proper mixing. This was poured in to a petridish and the
plate was rotated clockwise and anti-clock wise so as to ensure thorough mixing and even
spreading of haemocytes throughout the plate. After surface drying, the plate was observed
for the stained intact haemocytes. Actinomycete strains (L18, L39, L45) were inoculated
on to the prawn blood agar plate along with a standard reference haemolytic strain of Vibrio
harveyi (MBCS 6), isolated from a diseased prawn (isolated and characterized by NCAAH,
CUSAT). The plates were incubated for 48 hrs at 28 ± 2°C and observed for haemolysis
around the colonies. Haemolysis was confirmed by microscopic observation of the lysed
haemocytes around the colony.

3.2.3.4 Pathogenicity Test in vivo

Marine actinomycetes (L18, L39, L45) were selected and tested for pathogenicity in vivo.
Two separate experiments (Experiment I and Experiment II) were conducted to exami­
ne the pathogenic effect of actinomycetes administered to Penaeus monodon post larvae.
In experiment I the selected actinomycetes was added directly to water at a recomended
dosage. In experiment II actinomycete biomass was incorporated into feed and then admin­
istered to the animals for checking the pathogenic effect of actinomycetes to shrimps.

Preparation of Actinomycete Biomass

Actinomycete isolates (L18, L39, L45) were inoculated into Marine Actinomycetes
Growth medium (500 ml) and incubated for 7 days at room temperature. Culture broths
were centrifuged at 10,000 rpm for 15 minutes at 4°C in a cooling centrifuge (Remi C-30).
Biomass was kept in a deep freezer at -20°C until used.
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Experimental Animals

*Penaeus monodon* post larvae (PL-40) of the size range 0.035-0.04g were used for the experiment. The larvae were brought from Matsyafed hatchery (Ponnani, Kerala) and was acclimatized to laboratory conditions. These larvae were maintained on control diets for a period of one week.

Experiment I - Challenge Via Rearing Water

Fifteen animals were stocked in Fibre Reinforced Plastic (FRP) tanks of 30 L capacity containing 20 L seawater (Fig. 2.2) The experiments were done in triplicate for each treatment group and control. The actinomycete biomass was suspended in sterile PBS to OD$_{600}$ of 1.0, corresponding to $3 \times 10^9$ cfu/ml. The prawns were challenged at $10^7$ cfu/ml on alternate days for a period of 10 days. Water exchange was done on alternate days. Animals were observed for mortality up to 14 days.

Experiment II - Challenge Via Diet

Fifteen animals were stocked in Fibre Reinforced Plastic (FRP) tanks of 30 L capacity containing 20 L seawater. The experiments were done in triplicate for each treatment group and control. The actinomycete biomass were incorporated separately into the diet at 1:2 ratio (actinomycete biomass: feed). Incorporation was done using binder (Bindex gel) and dried at room temperature (28 ± 2°C) for one hour. Feeding experiment was done for a period of 10 days. Water exchange was done on alternate days. Animals were observed for mortality up to 14 days.
3.2.4 Assessing the Colonisation Property of Actinomycetes in the Intestine of Shrimps

3.2.4.1 Preparation of Actinomycete Biomass

Actinomycete isolates (L18, L39, L45) were inoculated into Marine Actinomycetes Growth Agar plates and incubated for 2-3 days at room temperature and harvested with sterile saline (0.5 % NaCl).

3.2.4.2 Experimental Animals

*Penaeus monodon* adults of the size range 10-12 g collected from a private farm at Cherthala were used for the study. The animals were acclimatized to laboratory conditions for a week.

3.2.4.3 Experimental Setup

The animals were transferred 20 each into rectangular fibreglass tanks of 30L capacity. 50% water exchange was done daily and the water quality parameters were maintained. Animals were maintained on a commercial diet (Higashimaru, Kochi).

3.2.4.4 Evaluation of the Colonisation property of Actinomycetes

The harvested biomass of each actinomycete isolate (L18, L39, L45) was incorporated into feed at 1% (wet wt.) and administered once in a day for a period of seven days and the animals were maintained on control diet for the rest of the experimental period. For the recovery isolations, four animals were collected periodically on 8th, 10th, 15th and 20th day, intestine was removed aseptically, and then homogenized in sterile saline. Serial dilutions were performed and plating was done in triplicate on Marine Actinomycete growth Agar plates employing the pour plate and spread plate technique. Incubation was done at 28±
2°C for 3-5 days. The characteristic actinomycete colonies on the plates were counted and the number was expressed as cfu/mg intestine of the animals.

3.2.5 Exclusion of Vibrios by Marine Actinomycetes

3.2.5.1 Exclusion of Vibrio- in vitro test (Co-culture Experiment)

Co-culture experiments with actinomycete isolates (L18, L39 and L45) and *V. harveyi* were carried out following the method of *Gram et al.*. They were pre-cultured separately in 250 ml flasks at 28°C on a shaker at 120 rpm overnight. From the above cultures, *Vibrio harveyi* was inoculated in 100 ml Nutrient Agar medium flasks, incubated at 28°C, to obtain an initial cell count of $10^3$ cfu/ml, whereas initial levels of actinomycetes (L18, L39 and L45) in those flasks were $10^8$ cfu/ml. Flasks were incubated at 28°C on a shaker at 120 rpm and samples (1 ml) were withdrawn at 24h intervals for determination of cell count.

Counts of pathogen (*Vibrio harveyi*) were also monitored by withdrawing 1 ml samples, which were serially diluted 10 fold and 0.2 ml aliquots spread plated on TCBS agar (Hi media) and Nutrient Agar plates. The plates were incubated at 28°C for 24 hrs and colonies formed on TCBS were counted and expressed as $\log_{10}$ cfu/ml of *V. harveyi* in the co-culture flasks. Actinomycetes do not grow on TCBS and can be differentiated on Nutrient agar as non-luminiscent colonies.

3.2.5.2 Exclusion of Vibrio- in vivo test

**Experimental Animals**

*Penaeus monodon* post larvae (PL-40) of the size range 0.035- 0.04g were used for the experiment. The larvae were brought from Matsyafed hatchery (Ponnani, Kerala) and was
Experimental Design

Fifteen animals were stocked in Fibre Reinforced Plastic (FRP) tanks of 30 L capacity containing 15 L seawater. The experiments were done in triplicate for each treatment group and control group. Both the control group and treatment group animals were fed with a commercial diet (Higashimaru). All probiotics (L18, L39 and L45) were pre-cultured in Marine Actinomycete Growth medium at 28°C for 6 days and the broth was centrifuged at 10,000 rpm for 15 minutes. The biomass was suspended in sterile saline and added to rearing water of the treatment groups every 7th day at a cell density of $10^7$ cfu / ml (approx.) for a period of 28 days. The total plate count and total vibrio count of rearing water were monitored periodically by spread plating 0.2 ml aliquots after 10-fold serial dilution on Nutrient Agar and TCBS medium at zero day (before the addition of actinomycetes), 14th and 28th day. Plates incubated at 28 ± 1°C for 24-72 hours, and those having 30-300 colonies were taken for estimating bacterial counts which were expressed as cfu / ml for water samples.

3.2.5.3 Efficacy of the three selected actinomycetes as putative probionts in terms of growth and survival of Penaeus monodon post larvae when applied in rearing water

Experimental Animals

Penaeus monodon space post larva (PL-40) of the size range 0.035-0.04g were used for the experiment. The larvae were brought from Matsyafed hatchery (Ponnani, Kerala) and was acclimatized to laboratory conditions. They were PCR screened for WSSV. These larvae were maintained on control diets for a period of one week.
Experimental Design

Fifteen animals were stocked in Fibre Reinforced Plastic (FRP) tanks of 30 L capacity containing 15 L seawater. The experiments were done in triplicate for each treatment group and control group. Both the control group and treatment group animals were fed with a commercial diet (Higashimaru). Freshly prepared actinomycete biomass of the three selected actinomycetes were added to the rearing water at a cell density of $10^7$ cfu/ml for a period of 28 days. Initial and final weight of all the animals in the different treatment group and control were taken at the start and at the end of the experiment. The number of animals at the start and end of the experiment (after 28 days) were recorded and the survival was calculated. The individual increase in weight (absolute growth) was calculated using the formulae given below:

\[
\text{Absolute Growth} = \text{Final Weight} - \text{Initial weight}
\]

3.3 Statistical Analysis

The data were subjected to Duncans multiple range analysis to bring out the differences between the various treatment groups.

3.4 Results

3.4.1 Inhibition of Prawn Pathogens by Marine Actinomycetes

The inhibitory activity of actinomycetes against prawn pathogens were studied. Out of the 99 isolates only 32 strains exhibited inhibitory action against the pathogens. Actinomycete L39 inhibited (81.81%) followed by L45 (72.7%) and L18 (45.45%) (Figs. 3.2 & 3.3). Generally the percentage of actinomycete inhibiting the various prawn pathogens were found to be less and in the range 3-17%. *V. splendidus* was the most susceptible pathogen.
(Fig. 3.4) and was inhibited by 17.4%, followed by *V.parahaemolyticus* (14.1%), *V.fluvialis* (9.78%), *V.cholerae* (9.78%) and *V.harveyi* (7.6%). L39 showed maximum inhibitory activity against *V.harveyi* (Fig. 3.5).

### 3.4.2 Hydrolytic Enzyme Production by Marine Actinomycetes

All actinomycete isolates possessed proteolytic (gelatinase) activity. Lipase activity was shown by 96% followed by amylase (95%), chitinase (36%) & cellulase (9.8%) activity (Fig. 3.6). Culture No.s L17, L18, L25, L39, L45, L56 and B451 were capable of producing all the five enzymes. Maximum amylase activity was exhibited by L45 (2.0 cm) (Fig. 3.7), gelatinase by L45 (1.7 cm) (Fig. 3.8), lipase by L18 (1.6 cm) (Fig. 3.9) and chitinase by L18 (0.8 cm) (Fig. 3.10).

### 3.4.3 Identification of the selected three actinomycetes based on 16S rDNA sequencing

Based on partial sequencing of 16S rDNA, the actinomycete cultures were identified as follows (Fig. 3.11 & Table 3.8)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Item</th>
<th>Genera/Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L18</td>
<td><em>Prauseria hordei</em></td>
</tr>
<tr>
<td>2</td>
<td>L39</td>
<td><em>Nocardia alba</em></td>
</tr>
<tr>
<td>3</td>
<td>L45</td>
<td><em>Streptomyces griseus</em></td>
</tr>
</tbody>
</table>

Table 3.7: Actinomycete cultures identified by 16S rDNA sequencing
Chapter 3: Screening of Marine Actinomycetes as Putative Probiotics in Penaeus monodon Culture Systems

Figure 3.2: Inhibitory action of marine actinomycetes to prawn pathogens
Chapter 3: Screening of Marine Actinomycetes as Putative Probiotns in Penaeus monodon Culture Systems

Figure 3.3: Inhibitory action of marine actinomycetes to prawn pathogens
Figure 3.4: Percentage of actinomycetes inhibiting prawn pathogens

Figure 3.5: Inhibitory activity of L39 against *V. harveyi*
Figure 3.6: Hydrolytic enzyme activity (%) of marine actinomycetes

Figure 3.7: Amylase activity by L45
Figure 3.8: Gelatinase activity by L45

Figure 3.9: Lipase activity by L18
3.4.4 Screening of Marine Actinomycetes for Pathogenicity

3.4.4.1 Pathogenicity Test \textit{in vitro} (Haemolytic Property on Prawn Blood Agar)

Based on the above results three isolates (L18, L39 and L45) were selected for further study. \textit{in vitro} pathogenicity test (Haemolytic Property on Prawn Blood Agar) showed that they were non haemolytic (Fig. 3.12). \textit{V. harveyi} used as a reference haemolytic strain showed haemolytic property on haemolymph agar.

3.4.4.2 Pathogenicity Test \textit{in vivo} (Challenge via Rearing water and Diet)

No significant mortality of prawns could be observed by challenge via rearing water and diet. All the treatments showed above 90% survival as the control. The results confirmed the non-pathogenicity of the selected actinomycetes to \textit{Penaeus monodon} post larvae (Fig. 3.13). Even better survival could be recorded in animals maintained on actinomycete
Figure 3.11: Gel Photograph of 16s rDNA amplification of selected three Actinomycetes
\#1 DNA Ladder (1 Kb), Lane 2 - L18, Lane 3 - L39, Lane 4 - L45
3.4.5 Colonisation Capacity of Marine Actinomycetes in the Intestine of Shrimps

Typical colonies of selected actinomycetes were not found on marine actinomycete agar plates during the recovery isolations. This confirms that these isolates could not colonise the intestine of shrimps (Table 3.8).
Figure 3.13: Percentage survival of *P. monodon* post larvae after exposure to actinomycetes strains (Through Rearing water and Diet)
Chapter 3: Screening of Marine Actinomycetes as Putative Probiotics in Penaeus monodon Culture Systems

<table>
<thead>
<tr>
<th>Time of Isolation</th>
<th>L18</th>
<th>L39</th>
<th>L45</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th day</td>
<td>10</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>10th day</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>15th day</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20th day</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.8: Colonisation of actinomycetes (expressed as cfu / 100 mg wet wt.) in the intestine of *P. monodon* fed on actinomycete incorporated diets

3.4.6 Exclusion of vibrios by Marine Actinomycetes

3.4.6.1 Co-Culture Experiments (*in vitro*)

In co-culture experiments with actinomycetes and *V. harveyi*, a gradual decline in vibrio count was observed for all co-cultures, and it became undetectable on the 3rd day for all co-cultures (Fig. 3.14).

3.4.6.2 Exclusion Of vibrios (*in vivo*)

Significant reduction in vibrio count could be observed in all treatment groups. This reduction was maximum with L39 followed by L45 and L18. TPC showed an increase on 14th day in all the treatment groups and remained at the same level till the end of the experiment. TPC and vibrio count remained at the same level throughout the experimental period in the control tank.

3.4.6.3 Efficacy of the actinomycetes as Probiotics in terms of Growth & Survival

The individual increase in weight (production) was more for prawns reared with actinomycete as probiotics compared to control (6.033%), in the order L18 (7.14%), L45 (9.83%), & L39 (10.916%) (Fig. 3.15). The survival rate was also more for actinomycete fed prawns compared to control (77.5%) in the order L18 (85.51%), L45 (86.63%) & L39 (87.58%) (Fig. 3.16). (Fig. 3.17). Duncan's multiple range analysis of variance showed significant
Figure 3.14: Total plate count (TPC) and vibrio count for co-culture of various actinomycetes with *V. harveyi*
variation between the control and the actinomycete treated animals in terms of growth. There was no significant variation between the control and the actinomycete treated animals in terms of survival.

![Graph showing weight gain in mg for different treatments]

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>061.00 ± 2.5</td>
</tr>
<tr>
<td>L18</td>
<td>074.16 ± 2.74</td>
</tr>
<tr>
<td>L39</td>
<td>109.16 ± 2.39</td>
</tr>
<tr>
<td>L45</td>
<td>118.33 ± 3.1</td>
</tr>
<tr>
<td>Ave ± SD</td>
<td></td>
</tr>
</tbody>
</table>

Value with same superscript does not vary significantly (p<0.05)

**Figure 3.15**: Weight gain (Production in mg) obtained in *P. monodon* post larvae reared in the presence of putative probionts
**Figure 3.16:** Average survival obtained in *P. monodon* post larvae reared in the presence of putative probionts.
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Figure 3.17: Total plate count (TPC) and vibrio count in the rearing water of *P. monodon* post larvae bioassay system in the presence of actinomycetes as probiotics
3.5 Discussion

Farming of shrimp has become a significant aquaculture activity in many countries in the tropics, but the growth of this industry has resulted in environmental changes which adversely affect the shrimp and their aquatic environment. The intensification of aquaculture activities has increased the occurrence of diseases (Shariff et al., 2001). The search for technological solutions to problems related to high density aquaculture practices has resulted in proliferation of often unproven and potentially dangerous solutions. Products and procedures such as chlorination, antibiotics and even toxic insecticides are touted cures for problems in hatcheries and on farms. Although some of these products can improve the culture environment or exclude disease carriers, misuse has been shown to compound existing problems.

An alternative method to antibiotic treatment is the application of probiotics. Probiotics are live microorganisms, which when consumed in adequate amounts confer a health benefit to the host, and can be administered as a food supplement or as additive to the water (Moriarty, 1998). The present study was undertaken to evaluate the effects of marine actinomycetes as probiotics in *Penaeus monodon* culture systems. Its hydrolytic, antibacterial, pathogenicity and colonisation properties were also investigated. Actinomycetes, although proven to be a rich source of hydrolytic enzymes and antibiotics had rarely been applied in aquaculture systems as prophylactic agents. Although literature pertaining to probiotic effects of actinomycetes in aquaculture are scanty, there are reports showing marine actinomycetes inhibiting *Vibrio* pathogens of shrimp (You et al., 2005) and fish pathogens (Patil et al., 2001). Mathew (2003), have reported on the efficacy of marine actinomycetes in reducing the vibrio counts and improving water quality in *P. monodon* rearing tanks.
In the current study, 99 isolates of actinomycetes were screened for hydrolytic and antagonistic properties against prawn pathogens. Production of inhibitory substances help in the exclusion of pathogens from culture systems. Maeda and Nogami (1989) reported that bacterial strains possessing vibriostatic activity, could improve the growth of prawn and crab larvae. Vaseeharan and Ramasamy (2003) reported the effect of Bacillus subtilis BT23 in controlling the growth of pathogenic Vibrio harveyi in Penaeus monodon under in vitro and in vivo conditions. By applying these bacteria equilibrium between beneficial and deleterious microorganisms can be produced and the results show that the population of Vibrio sps., were decreased. In this study, the percentage of actinomycetes inhibitory to prawn pathogens were found to be less. However, a few actinomycetes exhibited good inhibitory property against prawn pathogens. Maximum inhibition was exhibited by L39 (81.81%) followed by L45 (72.7%) and L18 (45.45%). The antagonistic property of these actinomycetes can be exploited for the control of vibrios in culture systems, the most prevalent pathogen in penaeid prawns. This is particularly important in the context of ban of many of the antibiotics in aquaculture. Best choice is bioaugmentation, i.e., application of beneficial microbes for the maintenance of desirable microflora and thereby the health of the ecosystem.

All actinomycete isolates possessed proteolytic activity, lipase activity was shown by 96 % followed by amylase activity (95 %), chitinase (36 %) & cellulase (9.8 %) activity. Grey and Williams (1971) reported that actinomycetes could efficiently degrade high molecular weight polymers. Microbes act as source of enzymes which help in digestion thus promoting growth as already reported by Prieur et al. (1990). Prawns being detritus feeders, the diet will consist of components like starch, cellulose, chitin etc. Microbes capable of digesting these high molecular weight compounds will definitely contribute to the nutritional enrichment in the intestine. Water quality improvement is
another criteria of importance for as good probiont. Organic load in culture ponds form faecal matter and uneaten food is a major problem. Actinomycetes are gifted with the specific property of degrading high molecular compounds. Hence these microbes are of importance in water quality improvement. In the present study, few actinomycetes were found to produce all the enzymes tested. These strains can very well be used for cleaning the pond water and maintain water qualities at the desired level.

Probiotics selected should be non-pathogenic. Both *in vitro* and *in vivo* studies revealed that the tested actinomycetes (3 Nos.) were non-pathogenic to shrimps and can be safely applied in culture systems. These isolates were obtained from the marine sediments and there are no reports of marine actinomycetes as pathogens in culture systems. The study on colonisation of actinomycetes in the gut revealed that they were not able to colonise the intestine. *Skjermo and Vadstein* (1999) pointed out that bacterial colonisation depends on several factors such as adhesion properties, bacterial attachment site, stress factors, diet and environmental factors. Colonisation of intestine and other tissues of marine larvae take place during early feeding stages and the diversity of ambient flora determines the microbial diversity in the animal (*Hansen and Olafsen*, 1999). If opportunists are dominant, in ambient water, then they will colonise and proliferate. Therefore, it has been suggested that probiotic treatment at early stages significantly impacts survival positively preventing opportunists from making deleterious effect (*Ringo and Vadstein*, 1998). The intestinal flora in prawns depend on the ambient flora of the culture environment and especially the pond bottom since they are detritus feeders. The diet remain in the intestine only for a few hours and therefore the intestinal flora being a direct reflection of the ambient flora and is transient in nature and depends on the feed intake.
Co-culture experiments with *Bacillus subtilis* BT23 and *V. harveyi* revealed that the growth of *V. harveyi* was inhibited by *B. subtilis* at an initial cell density of $10^5-10^9$ cfu/ml (Vaseeharan and Ramasamy, 2003). They suggest that the antagonist must be present at significantly higher levels than the pathogen and the degree of inhibition increased with the level of the antagonist. A similar trend was observed in the co-culture experiments with *Pseudomonas* MCCB103 and *V. harveyi* were a cell density greater than $10^5$ cfu/ml and $10^6$ cfu/ml could significantly eliminate the pathogen (Pai, 2006). In the co-culture experiment study with actinomycetes and *V. harveyi* (at a cell density of $10^5$ cfu/ml and $10^3$ cfu/ml respectively), a gradual decline in *Vibrio harveyi* count was observed for all co-cultures and became undetectable on the third day of incubation. Application of actinomycetes under rearing conditions also revealed that there was a reduction in *Vibrio harveyi* count while the total bacterial count was more or less steady throughout the experimental period. This suggest that actinomycetes could inhibit the proliferation of *vibrios* in aquaculture systems. The actinomycetes also enhanced the survival and production of the larvae showing that these probiotics can significantly improve yield.

Actinomycetes as probionts thus are a welcome addition to the armament of disease prophylaxis in aquaculture although the technology and science behind it is still very much in a developmental stage. It seems likely that the use of probiotics will gradually increase and if validated through rigorous scientific investigations and used widely, may prove to be a boon for aquaculture industry.