ANTIBODY BASED DIAGNOSTICS FOR DETECTION OF VIBRIOS AND THEIR BIOLOGICAL CONTROL USING ANTAGONISTIC BACTERIA IN MACROBRACHIUM ROSENBERGII LARVAL REARING SYSTEM

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By
N.S. JAYAPRAKASH

SCHOOL OF ENVIRONMENTAL STUDIES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN-682 022, KERALA

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Certificate

This is to certify that the research work presented in this thesis entitled **"Antibody based diagnostics for detection of vibrios and their biological control using antagonistic bacteria in *Macrobrachium rosenbergii* larval rearing system"** is based on the original work done by Mr. N.S. Jayaprakash under my guidance, in the School of Environmental Studies, Cochin University of Science and Technology, Cochin 682022, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Dr. I.S. Bright Singh

(Research Guide)
Reader in Microbiology
School of Environmental Studies
Cochin University of Science and Technology

Cochin-682022
July 2005

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CHAPTER - 1

GENERAL INTRODUCTION

Giant freshwater prawn (*Macrobrachium rosenbergii* de Man, 1879) or scampi is an important commercial candidate species due to increasing demand in domestic and export markets. It is a native prawn of Thailand and other Southeast Asian countries including Vietnam, Kampuchea, Malaysia, Myanmar, Bangladesh, India, Sri Lanka and the Philippines. Production of *M. rosenbergii* is also reported from Israel, Japan, Taiwan, and some countries in Africa, Latin America and the Caribbean (New, 1990). *M. rosenbergii* rose in importance after the life cycle of the prawn was first discovered in Penang, Malaysia in the sixties (Ling, 1969).

Giant fresh water prawn is usually found in rivers, canals, lakes and inundated fields. The prawn becomes capable of reproduction from the age of 5-6 months. It breeds throughout the year with a peak at the beginning of the rainy season. The animal performs a spawning migration from its original freshwater habitat to estuarine regions and spawns in area where salinity fluctuates from 5-15 ppt. Mating is always preceded by moulting of the female prawn. The hard shell male deposits sperm in a gelatinous mass on the ventral median thoracic region of the soft-shelled female. The female releases the eggs within 24 hours of pre-mating moult and fertilization takes place. In the natural habitat, a female spawns 3-4 times in a year producing 1-2 lakh eggs. The fecundity varies according to size of the female.

The female carry eggs in brood pouch beneath the abdomen. Vigorous aeration is provided by the female prawn with the movement of her swimming legs throughout the incubation period. Incubation usually requires about 19-20 days at 25°C to 32°C. The colour of the eggs will be bright orange. As the incubation proceeds, the colour will turn from orange to pale grey and then to dark grey at the time of hatching. Hatching is completed within one or two nights. The larvae are dispersed by rapid movements of the

abdominal appendages of the parent. Immediately after hatching, the larvae settle down and remain motionless for some time. The larvae at this stage are known as pre zoea. After about 5-10 minutes, the larva comes up and transforms into zoea, which is planktonic. Eleven larval stages are associated in the development, which takes about 30-40 days before it transforms to post larvae. Post larvae grow into juveniles, which migrates towards freshwater regions of the habitat where it grow and live till the spawning migration.

Freshwater prawn used to be very common in the natural waters. As a result of over fishing and deterioration of its habitat and spawning grounds, the natural catch has been reduced drastically and the prawn has become a luxury food item; production no longer meets consumer demand. Since it has been possible to produce postlarvae in hatcheries (Fujimura and Okamoto, 1972; Ling, 1977), the prospects of its culture and consequently the number of active prawn farms have significantly increased. Moreover, there is great scarcity of fresh water prawn seed and it is well-accepted fact that rapid development of scientific prawn farming is just impossible without meeting the demand for good quality seeds. This necessitated the establishment of freshwater prawn seed producing centres or hatcheries.

A prawn hatchery can be defined as an artificial facility, where prawn larvae are produced and reared under controlled conditions. The operational activities of the hatchery can be grouped into three broad categories namely preparatory phase, rearing phase and managerial phase.

Preparatory phase is the initial phase of the hatchery operation. Under this phase, all hatchery facilities must be adequately prepared for mother prawn holding, hatching and larval rearing. Conditioning of berried female prawn is vital in ensuring trouble-free holding and hatching of eggs. Adequate preparation is necessary to ensure supply of berried females in time and also to ensure the timely availability of larval feed.

The water supply system should ensure good water quality, adequate and trouble-free supply. The incoming water shall pass through a variety of filters namely sand filters, activated carbon filters, U-V ray filters etc. As a matter of practice, disinfecting of sand filters is done once in every month to reduce the organic load and bacterial flora.

Sufficient number of brood stock must be made available to provide into the breeding module so as to ensure targeted requirement of larvae per cycle. For raising brood stock, spawners are collected from culture ponds of any nearby farms and stocked in a ratio of 1 male: 5 female in specially designed brood stock ponds created close to the hatchery complex. The recommended stocking density of spawners is 2 to 3 prawns/m^2. Spawners are fed with chopped clam meat, trash fish squid etc. at the rate of 5-10% of their body weight per day.

If berried females are readily available in sufficient numbers, the above step can be avoided. Inside the hatchery, on arrival of berried female prawn, the animals will be kept under quarantine conditions and will be examined for disease signs and general health. They will be then conditioned and acclimatized in holding tanks. Each tank is adequately aerated along with sufficient exchange of water. The animals are fed with fresh diets like clam meat, oyster meat and egg etc.

Larval rearing systems can be categorized into green water and clear water systems. Few hatcheries now operate green water system except those in Malaysia. Almost all the hatcheries now operate on a clear water system, with or without the use of a bio-filter, to re-circulate the rearing water. Using the clear water system larvae can be reared at high densities.

The hatched out larvae are transferred into larval rearing tanks filled with water of 4-5 ppt salinity. No feed is required for the nauplii as it can utilize it's own embryonic yolk. It is advisable to follow the clear water system of larval rearing which facilitates the

stocking of higher density of larvae during the operation. The stocking density in larval rearing tanks range from 75-80 larvae/litre of water. To ensure high survival rate, exchange of water on a daily basis is a must.

As the rearing of larvae progress, the salinity should be gradually increased to a level of 12-15 ppt. The larvae should be fed as per scientific feeding schedule. There is presently no nutritionally complete artificial diet for consistently successful larval rearing of *M. rosenbergii*. For this reason, live feed organisms such as the newly hatched *Artemia* (brine shrimp) nauplii still seem to be the only solution. It is better if the artemia cysts are sterilized and decapsulated before used for hatching. Most larvae begin to feed one day after hatching. In addition to the live feed, supplementary feeding should be started after about 10 days of larval cycle. Ingredient composition of a standard supplementary diet includes prawn meat, clam meat, fish or squid, chicken eggs, beef liver powder etc. The custard made out of these ingredients is sieved through suitable mesh sizes according to the stage and size of the larvae. The suggested mesh sizes are 300-400 microns, 400-600 microns and 600-1000 microns for I to IV, V to VIII and IX to XII stage of larvae. The larval rearing operation is continued up to 30 to 35 days till it reaches state XI of the larval stage.

Beyond 35th day, the rearing can be in larval tanks or post larval tanks at much lower density. This stage continues up to 40-50 days to achieve PL 15 stage before it is sold to farmers for stocking in culture ponds. During post larval rearing, diets comprising of artemia, specially prepared feed etc. should be provided adequately so as to prevent cannibalism.

For ensuring water quality, 100% exchange is envisaged daily. Dissolved oxygen should be maintained at the saturation level through continuous aeration. The other area of importance in the hatchery operation is routine hatchery management which includes maintenance of water quality, maintenance of aeration equipment,

monitoring of health condition of hatchery workers, monitoring of general sanitary procedures followed in the hatchery etc.

The commercial success of scampi hatcheries in many parts of the world prompted a spurt in the development of such hatcheries, in India too. It was from the late 80's its culture potential got realized in India due to the rise in export of prawn and prawn products. According to Kurup (1994), it is one of the most ideal candidate species for freshwater and low saline areas of the Indian subcontinent. India is earning a huge sum of foreign exchange (1253 million US dollar; FAO 2003) through the export of fish and fishery products, of which shrimp wild caught and farmed contributes, 69.4 percentage (FAO, 2003). As the wild catches of shrimp are dwindling and severe disease problems are persisting in the shrimp culture systems, India has started giving much importance to the culture of freshwater prawns, especially *M. rosenbergii.* It is cultured in 34630 ha area in the country. The average production per ha ranges from 880 kg to 1250 kg and 62% of the scampi culture operation is in Andhra Pradesh. There are 71 hatcheries operating in various states supplying 183 billion scampi seeds to the farmers in India (Bojan, 2003).

Rapid development of prawn farming with large scale, high stocking density and supplementary feeding demanded good quality uninterrupted prawn seed supply. However, production of healthy prawn seed is still a concern of aquaculturists world over. Freshwater prawn culture comprises three phases i) hatchery, ii) nursery, and iii) pond growout. The hatchery and nursery stages are labor intensive and require relatively high expertise for success. A limited number of postlarvae and juvenile suppliers currently exist, and an increase in demand will eventually lead to more enterprises that deal exclusively in the production and sale of seedstock. Although it often appears that *M. rosenbergii* is less susceptible to diseases than penaeid shrimps, it might be a result of the lower stocking densities used in culture and less transfer of brood stock. Recently emerged white muscle syndrome is an example.

Larval stages of *M. rosenbergii* seems to be more susceptible to vibriosis in hatcheries as a result of their high stocking densities with heavy organic loading associated with daily feeding and their extended period of larval development (Singh, 1990). In nature, *M. rosenbergii* larvae after metamorphosis and settlement remain in brackish water for 1 to 2 weeks as post larvae and after reaching a size of 2 to 3cm migrate slowly towards the freshwater habitats. The time taken for a larval batch to metamorphose varies according to feeding and environmental conditions, particularly the temperature variations, the optimum being 26 to 31°C (New, 1995).

With the rapid development in hatchery production of juveniles and the number of prawn growout farms, good husbandry and environmental management have often been neglected. Consequently, disease problems develop as prawns were stressed and weakened under adverse environmental conditions. This intensification of prawn culture industry has resulted in a concomitant appearance of infectious and non-infectious diseases. Several bacterial related diseases are periodically observed in prawn culture causing mortality and severe losses in cultured stocks. Among them 'vibriosis' is one of the most prevalent diseases causing high mortality not only in larval cultures but also in growout systems (Egidius, 1987; Lightner, 1988; Austin and Austin, 1993). Often acting as opportunistic pathogen or secondary invader, they induce mortality, which range from very limited to 100% especially in affected population under stress (Lightner, 1988). According to Rosenberry (1998), vibriosis still continues to be a hazardous disease in shrimp/ prawn culture.

A bulk of literature is available on vibriosis in penaeids (Sinderman, 1974; Lightner, 1984; Singh *et al.* 1985; Anderson *et al.* 1988; Singh, 1990; Takahashi *et al.* 1991; Hipolito *et al.* 1996). In general, *Vibrio* spp. are one of the major pathogens, which cause high mortality among economically important species of farmed marine fish and shrimp (Ruangpan and Kitao, 1991). These organisms occur widely in aquatic environment and as part of the normal flora of coastal seawater. They also exist as normal flora in fish and shellfish but have also been recognized as opportunistic pathogens in many marine animals (Austin and Austin, 1993). Li *et al.* (1999), showed 7 *Vibrio*

species associated with vibriosis in silver sea bream (*Sparus sarba*) and of these species *V. alginoliticus, V. vulnificus, V. parahaemolyitcus* are dominant. Among the various species of *Vibrio* associated with the diseases *V. parahaemolyticus* (Vera *et al.* 1992; Mohney *et al.* 1994; Ponnuraj *et al.* 1995; Anand *et al.* 1996) luminous and non-luminous *V. harveyi* (Lavilla-Pitogo *et al.* 1990; Karunasagar *et al.* 1994; Liu *et al.* 1996), *V. alginolyticus* (Yang *et al.* 1992; Hameed, 1994), *V. anguillarum* (Lightner, 1984; Vera *et al.* 1992; Mohney *et al.* 1994; Anand *et al.* 1996) are the most commonly isolated species. In addition, Austin and Austin (1993), have categorized *V. alginolitycus, V. anguillarum, V. carchariae, V. cholerae, V. damsela, V. ordalii* and *V. vulnificus* as the seven major pathogenic species of *Vibrio to* fishes.

In this context, it has been observed that very little information is available on the microbiology of nonpenaeids in general and *M. rosenbergii* in particular. Diseases that have been reported in larval, juvenile and adult *M. rosenbergii* include fouling protozoans such as *Zoothamnium, Vorticella* and *Epistylis*; fungal pathogens such as *Lagenidium, Sirolpidium* and *Fusarium*; bacteria such as *Vibrio, Aeromonas, Leucothrix* etc., and also non-infectious diseases such as stress and neurotic signs, moult failure and so on.

Shell disease is commonly observed in larvae, post- larvae and adults in prawns. It is variously termed as 'black spot', 'brown spot', 'burnt spot' or chitinolytic bacterial disease. This is caused by the invasion of chitinolytic bacteria, which break down the chitin of the exoskeleton, leading to erosion and melanization (dark brown to black pigmentation) at the site of infection. Several chitinolytic bacteria (Gram negative rods) such as *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., *Spirillium* spp. etc are involved in the process of exoskeleton breakdown. The disease reduces value of the harvested prawns, apart from causing mortality. Normally, the disease is managed in captive and cultured populations by reducing over crowding, proper husbandry and system hygiene.

Filamentous bacteria such as *Leucothrix mucor, Thriothrix* spp., *Flexibacter* spp. etc., sometimes cause mortality in prawn larvae subsequent to discolouration of gills and associated secondary infections. The larvae become moribund, with reduced motility, poor feeding and growth. Better husbandry and hygiene standards will improve the situation.

Larval mid cycle disease (MCD) disease generally occurs in the early larval stages (IV to XI). Anderson *et al.* (1990), reported mass larval mortality of *M. rosenbergii* cultured in Malaysia at about 16 days after hatching. The clinical signs were similar to bacterial necrosis. Larvae loose their appetite and the healthier larvae eat moribund individuals. Affected larvae are often blue-grey in colour and swim weakly, often in spirals. The etiologic agent has not been identified but it is considered to be infectious in nature. A possible cause may be the bacterium *Enterobacter aerogenes* (Brock, 1988). Proper sanitation procedures have proven to be effective in eliminating the disease (Brock, 1983). Attention should also be paid to nutrition, ensuring that good quality artemia are used (Johnson, 1982).

Bacterial necrosis, having the clinical signs as bluish colour or discoloration, empty stomach, weak larvae falling to the bottom of the tank, and brown spots on antennae and newly formed appendages is a major disease, affecting larvae. Mixed bacterial infections were observed, with filamentous *Leucothrix* spp., and non-filamentous bacilli and cocci present on the setae, gills and appendages. The disease is more serious in younger larvae. Aquacop (1977), has reported bacterial necrosis affecting *Macrobrachium* larvae (stages IV-V) in Tahiti causing up to 100% mortality in 48 hours.

Brock (1983), reviewed several diseases associated with *M. rosenbergii*, some during larval stages and the others during the advanced stages. Exuvia entrapment is a disease primarily of stage XI larvae and early post larvae with death usually occurring at

the time of metamorphosis or moult (Brock, 1983). Idiopathic muscle necrosis known otherwise as spontaneous muscle necrosis is observed in larvae -juveniles and adults of *M. rosenbergii* (Brock, 1983). Colorni (1985), studied the bacteria associated with the larvae of *M. rosenbergii* fed with *Artemia* nauplii and Huang *et al.* (1981), attempted to even vaccinate *M. rosenbergii* with *V. anguillarum* even though it was unsuccessful.

On several occasions, mortality in shellfishes has been associated with an increase in the *Vibrio* population (Sung *et al.* 1999; Sung *et al.* 2001). Among the different species of vibrios, *Vibrio alginolyticus* has been isolated frequently from diseased prawn as the aetiologic agent of vibriosis and has been described as the principal pathogen of both penaeids and non-penaeids (Lightner, 1988; Baticados *et al.* 1990; Limsuwan, 1993; Felix and Devaraj, 1993; Mohney *et al.* 1994; Lee *et al.* 1996).

According to Anderson *et al.* (1989), *Alcaligens* and *Vibrio* species are the most commonly encountered genera from larval rearing system of *M. rosenbergii.* Singh (1990), observed while working out the microbiology of a typical freshwater prawn larval rearing system at the Regional Shrimp Hatchery -Azhikode, Kerala, that there existed a profound relationship between the abundance of the members of family Vibrionaceae (Baumann and Schubert, 1984) and the mortality of larvae during the mid larval cycle. The association of *Aeromonas* and *Vibrio* in sizeable percentages with eggs of *M. rosenbergii* led to the failure of completion of the embryonic development and subsequent hatching. *Aeromonas* formed the major flora of the sick culture systems and *Pseudomonas* those of the healthy ones. Larvae with *Pseudomonas* as the major intestinal flora metamorphosed successfully while the ones with *Aeromonas* failed to do so. In the larvae representing the sick pool, though found apparently healthy at the time of sampling, with their characteristic response to light, tendency to remain at the top of the water column and zigzag motion, progressive mortality was observed over the entire larval rearing period (Singh, 1990). Later Bhat and Singh (1998), worked out the numerical taxonomy of the family Vibrionaceae associated with the larvae of *M. rosenbergii* and observed the possibility of identifying new species within the family based on the phenotypic and genotypic dissimilarities with the type strains.

Bhat *et al.* (1998), tried to segregate the pathogenic strains of the family and noted that all the strains used as representatives of the family were pathogenic. This implied that larvae of *M. rosenbergii* in hatchery had to be protected from the invasive death of vibrios or the vibrios as a whole had to be avoided in the system. However, to make the larval rearing technique foolproof and to make the practice rewarding an environment friendly and sustainable technology to exclude vibrios from the larval rearing system has necessarily to be included in the husbandry practices.

The existing methods of application of chemotherapeutics including antibiotics have proved to be unsustainable and environment unfriendly as the pathogens develop drug resistance, the effluent discharged contains residual antibiotics and more than that the drug resistance is liable to be transferred to human pathogens too (Brown, 1989). Karunasagar *et al.* (1994), reported mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi*.

Antibiotics like streptomycin, erythromycin, and chloramphenicol are used to treat infections while oxytetracycline and penicillin are commonly used as prophylactic agents (Tjahjadi *et al.* 1994). Luminous vibrios isolated from shrimp hatcheries on Java Island, Indonesia, have demonstrated multiantibiotic resistance to antimicrobials like ampicillin, tetracycline, amoxicillin, and streptomycin (Tjahjadi *et al.* 1994). Bacteria present in aquaculture settings may be transmitted to humans who come in contact with this ecosystem. For example, *Vibrio spp.* are part of the normal warm marine flora and cause wound infections in persons with open wounds or abrasions exposed to seawater or marine life (Blake *et al.* 1979).

In general, pathogenic species are normally present in low numbers when compared with the more abundant saprophytes, but their presence in a certain environment always means a risk of transmission to higher organisms including man, especially if they become concentrated by filter-feeding organisms living in the same habitat. *V. vulnificus* has been the focus of much attention during the last decade due to its role as both a human (Hoyer *et al.* 1995) and fish pathogen (Tison *et al.* 1982; Biosca

et al. 1991; Arias *et al.* 1997). This species causes two kinds of clinical manifestations in humans, fetal septicemia after consumption of seafood harbouring the bacterium (Levine and Griffin, 1993), or severe wound infections from exposure to seawater or the handling of fish/ shellfish.

1.1 Detection of vibrios

Very less number of groups of workers has ever been involved in studying the microbial involvement in the unsuccessful completion of the larval cycle of *M. rosenbergii* in India. The documented literature are from Singh, (1990); Bhat and Singh, (1998); Bhat *et al.* (1998) and Vici *et al.* (2000). Even now for the detection of pathogenic vibrios, standard culture methods based on general as well as selective media are followed. Karunasagar *et al.* (1994) used a PCR based technique for the detection of pathogenic marine vibrios such as *V. parahaemolyticus, V. holisae, V. cholerae*, but specifically oriented towards seafood industry.

As vibriosis is a major disease in hatcheries, prompt and specific identification of bacteria is mandatory. The routine microbiological and biochemical analyses need three working days. The rapid methods includes DNA hybridization, immunomagnetic separation and polymerase chain reactions are, however, relatively costly, needing specialized equipment, highly trained personnel and expensive specialist reagents. They are also unsuited to large-scale commercial operations involving the analysis of very large number of samples.

Frequent disease outbreak and high mortality due to vibriosis in hatcheries makes it mandatory that improved rapid detection methods be developed to evaluate water quality and assess the risk of disease. Correlations between microbial quality, measured by conventional culture techniques, and *Vibrio* related diseases have been established. The direct detection of pathogens rather than reliance on culture methods may more accurately assess larval rearing water quality. Fluorescent antibody assay (Xu *et al.* 1984; Brayton and Colwell, 1987; Brayton *et al.* 1987; Hasan *et al.* 1994; Marcelo-Noales *et al.*

2000) and enzyme immunoassay (Chen *et al.* 1992; Biosca *et al.* 1997) can be usefully applied to both direct and specific detection of bacteria in environmental samples.

The normal identification of pathogenic vibrios in the environment has involved a four-step strategy: (i) collection of the samples, (ii) recovery of vibrios from the samples, (iii) identification of the vibrios recovered, and (iv) confirmation that they are pathogenic (Spira, 1984). The preliminary differentiation of *Vibrio*-like organisms from other gram-negative bacteria has involved the growth of isolates on thiosulfate-citrate-bilesalts-sucrose (TCBS) agar selective medium, on which most vibrios will grow. Further differentiating characteristics include salt requirement for growth, Gelatinase production, sensitivity to vibriostatic compound O/129 (2,4-diamino-6,7-diisopropyl pteridine phosphate), and an oxidase-positive reaction. Identification of *Vibrio* species that possess similar morphological, physiological, and biochemical characteristics still remains a problem because of the large number tests that are involved and that usually give an identification with a probability level of less than 100%. Alternative serological identifications of *Vibrio* species with polyclonal antisera have resulted in relatively quick results (Adams and Siebeling, 1984). Rapid identification of pathogenic strains facilitates better management of infection and understanding of disease etiology.

1.1.1 Fluorescent antibody technique

Since the introduction of fluorescent antibody technique (FAT) by Coons *et al.* (1942), it became a very attractive technique due to the rapidity with which the detection of the specific bacterial pathogen could be achieved. FAT has been recognized as a sensitive and specific diagnostic method (Bullock *et al.* 1980; Laidler, 1980) and is widely used for the detection and identification of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (Fryer and Sanders, 1981). This technique has found a position now in the diagnosis of fish/prawn diseases in aquaculture systems.

Application of FAT and IFAT has been reported for bacteria such as *Aeromonas hydrophila* (Kawahara *et al.* 1987), *Aeromonas salmonicida* (Sakai *et al.* 1986),

Renibacterium salmoninarum (Laidler, 1980; Lee and Gordon, 1987), *Yersinea ruckeri* (Johnson *et al.* 1974). Lallier *et al.* (1990), developed both ELISA and IFAT for diagnosis of furunculosis, bacterial kidney disease and vibriosis and evaluated the method using 29 different bacterial strains. Huq *et al.* (1990), used a fluorescent monoclonal antibody technique for the detection of *Vibrio cholerae* serum 1 in the aquatic environment and compared it with the culture methods and observed that the FAT was very much superior in enumerating the specific pathogen in water. de la Pena *et al.* (1993), developed an IFAT for the detection of *Vibrio penaeicida* which shared a common antigens with 75 strains and with minimal cross reactivity with other *Vibrio* species. La Patra *et al.* (1989), used FAT for rapid diagnosis of infectious hematopoietic necrosis. Hung and Ling (1996), could localize *Vibrio* antigen using IFAT which was delivered by immersion to tiger shrimp (*Penaeus monodon*) and the method was very sensitive to demonstrate the antigen as it was absorbed through the digestive- circulatory system. Kitao and Kimura (1974), developed a method for rapid diagnosis of pseudotuberculosis in yellow tail by means of FAT. It has to be emphasized at this point that no FAT or IFAT has ever been attempted for the rapid detection of vibrios in the larval rearing system of prawns.

The importance of FAT in detecting specific bacterial strains directly from water-sediment systems without involving a standard culture practice was demonstrated very much by Voytek *et al.* (1998), when they worked out the abundance of ammonium oxidizing bacteria in Lake Bonney, Antartica by immunofluorescence, PCR and *in situ* hybridization. Through these techniques, distribution and relative abundance of nitrifying bacteria could be assessed, and especially IFAT was extremely useful. The fluorescent-monoclonal antibody staining procedure has been used successfully for direct detection of viable but not culturable *V. cholerae* O1 in environmental water samples. Results of such studies showed the presence of higher *V. cholerae* O1 counts than could be obtained by conventional culturing methods (Brayton *et al.* 1987; Xu *et al.* 1984).

The advantage of developing a rapid detection method by polyclonal antibodies is that they are very much less expensive to produce. But the monoclonal antibody selection

is expensive and time consuming. The disadvantage of a monoclonal lies in the high cost of isolating the right clone and cloning the corresponding cell line. Polyclonal antibodies recognize multiple epitopes, making them more tolerant of small changes in the nature of the antigen. Polyclonal antibodies are often the preferred choice for detection of denatured proteins. Monoclonal antibodies react with one epitope on the antigen, however, they are more vulnerable to the loss of epitope through chemical treatment of the antigen than are polyclonal antibodies. Polyclonal antibodies may be generated in a variety of species, including rabbit, goat, sheep, donkey, chicken and others, giving the users many options in experimental design (Harlow and Lane, 1988).

1.2 Microbial antagonism

The health of organisms in nature depends primarily on the inherent resistance to microbial invasion and the biological equilibrium between competing beneficial and deleterious microorganisms at the interface of the organism as mediated by the environment. Antagonism, which is a means of biological control, is a microbial technique, which uses the interaction of microorganisms to repress the growth of deleterious organisms or pathogens. Although the useful activities may not last long, addition of profitable microorganisms to a certain environment can be expected to produce good results to repress the growth of pathogenic microbes in that environment. In the case of *M. rosenbergii* it is seen that the larval stage is susceptible to vibriosis and hence exclusion of pathogenic microorganisms from its environment will create favourable conditions for faster larval development rate thus increasing the post larval yield. In biocontrol using antagonistic organisms, exclusion of pathogenic organisms is achieved without resorting to chemical control methods.

1.2.1 Probiotics in aquaculture

The theory of ecological prevention and cure in controlling the insect pest of terrestrial higher-grade animals and plants has been in practice for long time, and has achieved remarkable success. The use of beneficial digestive bacteria in human and

animal nutrition is well documented (Fuller, 1989). *Lactobacillus acidophilus* is used commonly to control and prevent infections by pathogenic microorganisms in the intestinal tract of many terrestrial animals. Recently, the biocontrolling theory has been applied to aquaculture. Many researchers attempt to use some kind of probiotics in aquaculture to regulate the microflora of water, control pathogenic microorganisms, to enhance decomposition of the undesirable organic substances in the culture environment, and improve ecological conditions of the culture environment. In addition, the use of probiotics can increase the population of food organisms, improve the nutrition of the cultured animals and improve their immunity to pathogenic microorganisms. In addition, the use of antibiotics and chemicals can be reduced and frequent outbreaks of diseases can be prevented (Verschuere *et al.* 2000).

Recently, the use of probiotics to improve and maintain healthy environment for prawn culture has become popular. Probiotics were used to supply beneficial bacterial strains to rearing water that will help to increase microbial species composition in the environment and to improve water quality. Probiotics are considered to be able to make cultured animals healthier by inhibiting the growth of pathogenic bacteria in the same habitat. This has led to new strategy for prevention of disease outbreak and improvement of seed quality. However, effectiveness of probiotics in aquaculture is still a debate due to different observations in different areas and in different cultured species (Maeda, 1999).

1.2.2 Kinds of Probiotics

'Probiotics' generally includes bacteria, cyanobacteria, micro algae, fungi, etc. Some Chinese researchers translate it into English as 'Normal micro biota' or 'Effective microbiota'; it includes photosynthetic bacteria, *Lactobacillus, Actinomycetes,* nitrifying and denitrifying bacteria, bifidobacterium, yeast, etc. In English literature, probiotic bacteria are generally called the bacteria, which can improve the water quality of aquaculture, and (or) inhibit the pathogens in water thereby increasing production.

'Probiotics', 'Probiont', 'Probiotic bacteria' or 'Beneficial bacteria' are the terms synonymously used for the above group of organisms.

Probiotics can be defined as cultures (single or mixed) of selected strains of bacteria that are used in culture and production systems (tanks, ponds and others) to modify or manipulate the microbial communities in water and sediment, reduce or eliminate selected pathogenic species of microorganisms, and generally improve growth and survival of targeted species (Jory, 1998). Probiotic protection can be due to different mechanisms such as nutrition, competition or production of antibacterial substances.

The mechanism of action of the probiotic bacteria has not been investigated yet. According to some publications (Austin *et al.* 1995; Moriarty, 1997; Verschuere *et al.* 2000), in aquaculture, the mechanism of action of the probiotic bacteria may have several facets, viz., 1. competitive exclusion of pathogenic bacteria or production of substances that inhibit growth of pathogens, 2. supply of essential nutrients to enhance nutrition to the cultured animals, 3. supply of digestive enzymes to enhance digestion of the cultured species, 4. direct uptake or decomposition of organic matter or toxic material in water improving its quality. Microorganisms and microbial byproducts have been proved to be inhibitory against many pathogenic organisms.

The trend of using probiotics in aquaculture is increasing as evidenced by the research results pouring in indicating their ability to increase production and prevent disease in farm animals. The development of suitable probiotics for biocontrol in aquaculture would result in less reliance on chemicals and antibiotics and result in a better environment. The above description clearly indicates the strong possibility of developing a 'natural' method for excluding or suppressing vibrios in the larval rearing system of *M. rosenbergii*.

1.2.3 *Bacillus* as probiotic

Bacillus spores had been tested for probiotic efficacy against opportunistic strains of *Vibrio* sp. in turbot larvae *Scophthalmus maximus* by Gatesoupe (1991).

Food additives containing live lactic bacteria or *Bacillus* spores decreased the amount of vibrionaceae in the rotifers and improved the survival rate of turbot *(Scophthalmus maximus)* (Gatesoupe, 1991).

Bacillus S 11 bacterium isolated from black tiger shrimp habitats was added to shrimp feed as fresh cells, in normal saline solution and a lyophilized form and after a 100-day feeding trial the animals (*Penaeus monodon*) were challenged with pathogen *Vibrio harveyi*. 100% survival was seen compared to only 26% in controls (no probiotics used) (Rengpipat and Rukpratanporn, 1998).

Selected *Bacillus* species as probiotic controlled the luminous *Vibrio* sp. in penaeid culture ponds. *Vibrio* count was low in sediments and water where probiotic *Bacillus* was used (Moriarty, 1998).

Bacillus is an important component of Bio Start Twin Pack used successfully in shrimp farming. A Thai bacillus isolate (Strain S11) used as probiotic by passage through *Artemia spp.* has significantly shortened development time and fewer disease problems than controls (Rengpipat and Rukpratanporn, 1998).

1.2.4 Lactic acid bacteria as probiotic

Gatesoupe (1994), investigated whether the artificial maintenance of a high concentration of lactic acid bacteria (LAB) in rotifers might increase their dietary value for turbot larvae, particularly when the fish were infected with pathogenic *Vibrio*. The inoculum concentration of LAB had a decisive effect on survival rate and the optimum was between 10^7 and $2x10^7$ colony forming units (CFU) daily added per ml of the

enrichment medium (53% survival rate after 72 h of challenge, versus 8 % for the infected control group without LAB).

Jiravanichpaisal *et al.* (1997), reported the use of *Lactobacillus sp.* as probiotic bacteria in the giant tiger shrimp (*P. monodon* Fabricius).

A dry feed containing lactic acid bacteria (*Cornobacterium divergens*) isolated from Atlantic cod (*Gadus morhua*) intestines was given to cod fry. After three weeks of feeding the fry was exposed to a virulent strain of *Vibrio anguillarum.* Improvement in disease resistance was observed and the lactic acid bacteria dominated in the intestinal flora on surviving fish (Gildberg, 1997).

An increase in resistance against pathogenic *Vibrio* was observed in turbot larvae when fed on rotifers which were fed on a medium enriched with lactic acid bacilli (Gatesoupe, 1994) and in *P. monodon* fed on a diet supplemented with the above probiotic (Jiravanichpaisal *et al.* 1998).

1.2.5 *Vibrio* as probiotic

The use of live bacterial isolate *Vibrio alginolyticus* as a probiotic to manipulate the microbial flora in the commercial production of *P. vannamei* could significantly increase growth rate and survival by the competitive exclusion of potentially pathogenic bacteria, thus reducing the need to use antibiotics and chemotherapeutants (Jory, 1998).

Garriques and Arevalo (1995), reported the use of *V. alginolyticus* as a probiotic agent which might increase survival and growth in *P. vannamei* postlarvae by competitively excluding potential pathogenic bacteria, and effectively reducing or eliminating the need for prophylactic use of antibiotics in intensive larval culture system. They believed that in nature a very small percentage of *Vibrio sp.* was truly pathogenic and the addition of bacteria *V. alginolyticus* as a probiotic to mass larvae culture tanks

resulted in increased survival rates and growth over the controls and the antibiotic prophylaxes.

Austin *et al.* (1995), reported a probiotic strain of *V. alginolyticus*, which did not cause any harmful effect in salmonids. By using the cross-streaking method, the probiont was observed to inhibit the fish pathogens. When the freeze-dried culture supernatant was added to the pathogenic bacteria such as *V. ordalii*, *V. anguillarum*, *A. salmonicida* and *Y. ruckeri*, showed a rapid or steady decline in the number of culturable cells could be observed, compared to the controls. Their results indicated that application of the probiont to Atlantic salmon culture led to a reduction in mortalities when challenged with *A. salmonicida* and to a lesser extent *V. anguillarum* and *V. ordalii*. The observation with this probiotic *Vibrio* is encouraging, and it appears that there is tremendous potential for the use of such probiotics in aquaculture as part of a disease control strategy.

1.2.6 *Pseudomonas* as probiotic

The probiotic effect of an antibacterial strain *Pseudomonas fluorescens* AH2 was tested by challenging rainbow trout (*Oncorynchus mykiss* Walbaum) with a pathogen *Vibrio anguillarum*. It was found that the probiotic increased the survival rate of the trouts (Gram *et al.* 1999).

Smith and Davey (1993), reported that fluorescent strain pseudomonad bacteria could competitively inhibit the growth of fish pathogen *Aeromonas salmonicida*. Their results showed that the fluorescent pseudomonad was capable of inhibiting the growth of *A. salmonicida* in culture media and that this inhibition is probably due to competition for free iron. In a challenge test of the Atlantic salmon by *A. salmonicida,* a statistically significant reduction in the frequency of stress-induced infection in the group of fish bathed in the bacterium fluorescent pseudomonad compared to the control group was observed.

Inhibition of one or several target organisms (*Escherichia coli, Aeromonas sobria, Pseudomonas fluorescens, Listeria monocytogenes, Shewanella putrefaciens* and *Staphylococcus aureus*) by *Pseudomonas* strains isolated from spoiled iced fish and newly caught fish were assessed by screening target organisms in agar diffusion assays. This suggests that microbial interaction (e.g. competition and antagonism) may influence the selection of a microflora for some chilled food products (Gram, 1993).

Artemia fed on diet of *Pseudomonas* survived successfully (Gorospe and Nakamura, 1996) and *Pseudomonas* served as a source of protein and amino acid (Gorospe *et al.* 1996).

Pseudomonas flourescens was found to inhibit (*in vitro*) *Aeromonas salmonicida*, which played a central role in stress inducible furunculosis in Salmon (Smith and Davey, 1993).

In vitro vibriostatic property of a *Pseudomonas* isolate was confirmed by Torrento and Torres (1996). *Saprolegnia* was inhibited by *P. flourescens* from catfish pond water (Bly *et al.* 1997).

1.2.7 Algae as probiotic

Austin *et al.* (1992), reported a kind of micro alga (*Tetraselmis suecica*), which can inhibit pathogenic bacteria of fish. *Tetraselmis suecica* was observed to inhibit *Aeromonos hydrophila, A. salmonicida, Serratia liquefaciens, Vibrio anguillaram, V. salmonicida* and *Yersnia ruckeri* type I. When used as a food supplement, the algal cells inhibited laboratory-induced infection in Atlantic salmon. When used therapeutically, the algal cells and their extracts reduced mortality caused by *A. salmonicida, Serratia liquefaciens, V. anguillaram, V. salmonicida* and *Yersnia ruckeri* type I. They suggested that the microalgae might be producing bioactive compounds having significant role in the control of fish diseases.

A commercial spray dried preparation of *Tetraselmis suecia* -a microalgae used extensively in aquaculture -used for mollusc and prawn diets, was observed to rapidly inhibit prawn pathogenic strains of *Vibrio spp.* namely *V. alginolyticus, V. anguillarum, V. parahaemolyticus, V. alginolyticus, V. vulnificus* (Austin and Day, 1990). They were found to reduce bacterial number in water (Austin *et al.* 1992) and on the walls of the fish holding facilities (Maeda, 1992).

1.2.8 Photosynthetic bacteria as probiotic

Zhenguo *et al.* (1994), investigated three strains of photosynthetic bacteria used in prawn (*P. chinensis*) diet preparation and their effect. Addition of the photosynthetic bacteria in the food or culture water was found to improve growth of the prawn and quality of the water.

Jingjin *et al.* (1997), reported application of photosynthetic bacteria in the hatchery rearing of *P. chinensis.* They used a mixture of several kinds of photosynthetic bacteria (*Rhodomonas sp.*) as water cleaner and auxiliary food. Their results showed that the water quality of the pond treated with the bacteria was remarkably higher, the fouling on the shell of the larvae reduced, the metamorphosis time of the larvae one day or even earlier, and the production of post-larvae more than that of the control.

1.2.9 Other bacterial probiotics

Aeromonas media, strain A 199, was used as probiotic to control the pathogen *Vibrio tubiashii* in Pacific oyster (*Crossostreae gigas*) and a wide range of fish/shellfish pathogen *in vitro*. The larvae challenged with the pathogen *Vibrio* alone died whereas larvae challenged with both pathogen and probiotic survived. Moreover, the larvae challenged with the probiotic alone also survived (Gibson, 1999).

A bacterium (*Roseobacter* sp., BS 107) found as part of the dominant flora in scallop larval cultures and collectors exhibited antibacterial activity (Ruiz-Ponte *et al.* 1999).

A bacterium (*Weissella hellenica* DS-12) isolated from the intestinal contents of flounder (*Paralichthys olivaceus*) from a fish farm was found to be having antimicrobial activity against fish pathogens like *Edwardsiella, Pasteurella, Aeromonas* and *Vibrio*. The strain was Gram positive, and catalase negative coccoid rods (Cai *et al.* 1998).

Seawater isolates of *Planococcus* were seen to inhibit fish pathogenic *Serratia liquefaciens* (Austin and Billaud, 1990).

Antibiotic production was observed from the bacterial strains isolated from 5 species of green and brown marine algae with *Enteromorpha intestinalis* being the source of the highest number of producer strains and all the above strains were assigned to the *Pseudomonas-Alteromonas* group (Lemos *et al.* 1985).

Solvent extract of six strains of actinomycetes isolated from mangrove environment was found to inhibit the growth of fish bacterial pathogens and filamentous and non-filamentous fungi (Ratnakala and Chandrika, 1996).

Bacteria isolated from the gastrointestinal tract of cultured halibut larvae showed antagonistic activity against pathogenic *Vibrio* sp. The high fraction of isolates with the ability to inhibit growth of the pathogenic *Vibrio* sp. among the total number of isolates indicates that pathogen inhibition may be an important mutualistic role of the intestinal flora of early stages of halibut (Bergh, 1995).

According to Dopazo *et al.* (1988), antibiotic production give marine bacteria an antagonistic capability against fish pathogens.

A large fraction of the intestinal bacteria isolated from start-feeding larvae and fry of Atlantic halibut (*Hippoglossus hippoglossus* L.) was shown to possess pathogen-inhibiting ability. The results indicated that the composition of the intestinal flora of the larvae from first-feeding onwards played an important part in the defence against colonization and growth of opportunistic pathogens (Lee, 1995).

Bacterial isolates from turbot (*Scophthalmus maximus*) were shown to inhibit the *in vitro* growth of the fish pathogen *Vibrio anguillarum*. Inhibition of these strains on the pathogen was studied by measuring the colonization potential by the capacity of the strains to adhere and to grow in turbot intestine mucus epithelium. The antagonistic strains isolated from the intestine showed greater capacity for adhesion to grow in fish intestinal mucus than did the pathogen. All isolates released metabolites into the culture medium that had inhibitory effects against *V. anguillarum* (Olsson *et al.* 1992).

Autochthonously obtained, 11 non-pathogenic strains of heterotrophic marine bacteria are used as a supplementary feed for microalgae in rearing larval *P. monodon* (Mohammed, 1996). Three natural marine bacterial isolates prevented the growth of antibiotic resistant *V. harveyi* in *P. monodon* larvae (Tjahjadi *et al.* 1994).

Nogami and Maeda (1992), isolated a bacterial strain from a crustacean culture pond. The bacterial strain was found to improve the growth of crab (*Portunus trituberculatus*) larvae and repress the growth of other pathogenic bacteria, especially *Vibrio spp.*, but would not kill or inhibit useful micro algae in seawater when it was added into the culture water. Among the bacterial population present in the culture water of the crab larvae, the count of *Vibrio spp.* and pigmented bacteria decreased or even became undetectable when the bacteria were added into culture water. The production and survival rates of crab larvae were greatly increased by addition of the probiotic bacteria into the rearing water. They also suggested that the bacterium might improve the physiological state of the crab larvae by serving as a nutrient source during its growth. The organism may have a profound effect on crab larval culture as a biocontrolling agent in future.

Maeda and Nogami (1989), reported a few biocontrolling methods in aquaculture employing bacterial strains possessing vibriostatic activity, which had improved the growth of prawn and crab larvae. By applying these bacteria in aquaculture, a biological equilibrium between the competing beneficial and deleterious microorganisms could be established and the results showed that the population of *Vibrio spp.*, which frequently caused large-scale damage to the larval production, was decreased. Survival rate of the crustacean larvae in these experiments was higher than those without the addition of the antagonistic bacterial strains.

Sugita *et al.* (1996), reported the antibacterial abilities of intestinal bacteria in freshwater cultured fish. They isolated bacteria from the intestine of 7 kinds of freshwater cultured fishes, and investigated the antibacterial abilities of these organisms to 18 fish or human pathogenic bacteria. Their results indicated that the bacteria isolated from the intestine of 7 freshwater cultured fishes possessed the antibacterial abilities, and their presence could protect the fish against the infection by pathogenic bacteria.

Maeda and Liao (1992), reported the effect of bacterial strains, obtained from soil extracts, on the growth of prawn larvae of *P. monodon*. Higher survival and molt rates of prawn larvae were observed on treating with soil extract, and the bacterial strains isolated.

Douillet and Langdon (1994), reported the use of probiotics for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunbeerg). They added probiotic bacteria as a food supplement to xenic larval cultures of the oyster *Crassostrea gigas* which consistently enhanced growth of larvae during different seasons of the year. Probiotic bacteria were added, at 0.1 million cells/ml, to cultures of algal-fed larvae. Consequently, the proportion of larvae that were set to produce spat also increased. Manipulation of bacterial population present in bivalve larval cultures was a potentially useful strategy for the enhancement of oyster production. They suggested that the mechanisms of action of probiotic bacteria could be categorized as their efficacy in providing essential nutrients

that are not present in the algal diets or enhancement of the oyster's digestion by supplying digestive enzymes or removal of metabolic substances released by bivalves or algae.

Maeda and Liao (1994), reported microbial processes in aquaculture environment and their importance in increasing crustacean production. They suggested that bacteria, protozoa and other microorganisms use organic matter produced by photosynthetic microalgae and play a significant role in the aquatic food chain. They also described the presence of a bacterial clump, stained with a fluorescent dye, inside the digestive organ of the crab *Portunus trituberculatus*. Using molecular techniques, they concluded that there existed a relationship between the 'Zoea syndrome' and the presence of bacterial pathogens, *V. harveyi,* as type E22. Besides, the bacterial strain, *Vibrio alginolyticus* was also found to grow under all experimental conditions. For controlling these pathogens, they used bacterial strain *V. alginolyticus* as probiotics in rearing facilities. Their study unequivocally demonstrated that the use of probiotics in aquaculture facilities could be an effective method to prevent disease outbreaks caused by pathogens in shrimp hatcheries.

1.3 Feasibility and future of the application of probiotics in aquaculture

Based on the previous research results on probiotics we can suggest that the use of probiotic bacteria in aquaculture has tremendous scope and the study of the application of probiotics in aquaculture has a glorious future. At present, the probiotics are widely applied in United States of America, Japan, European countries, Indonesia and Thailand, with commendable results. India has yet to realize the potential of the probiotic industry in aquaculture. As on today, the Indian aquaculture sector consumes probiotics worth of Rs. 500 crores annually (Pakshirajan, 2002). Irony of the situation is that more than 90% of the probiotics are either directly imported or manufactured here with the alien strains. We have yet to realize the diversity of our aquatic microbial flora as source of novel organisms to be used as probiotics in the diverse sphere of aquaculture.

Main concern of the work undertaken here was to develop an appropriate microbial technology to protect the larvae of *M. rosenbergii* in hatchery from vibriosis. This technology precisely is consisted of a rapid detection system of vibrios and effective antagonistic probiotics for the management of vibrios. The following chapters describe how these objectives were achieved.

CHAPTER - 2

ISOLATION OF VIBRIOS ASSOCIATED WITH *MACROBRACHIUM ROSENBERGII* LARVAL REARING SYSTEMS

2.1 Introduction

Augmentation of growout systems of scampi during the past decade demanded hatchery production of seed, which in turn, resulted in the expansion of scampi hatcheries in India. With the rapid development in hatchery production of juveniles, good husbandry and environmental management have often been neglected. Consequently, disease problems develop as prawns are stressed and weakened under adverse environmental conditions. Ironically, in spite of two and a half decades of research in production systems and production processes, several issues are still left unresolved. One among them is vibriosis which has been hampering the scampi seed production culminating in low yield due to mass mortality. It is one of the most important diseases in penaeid and nonpenaeid larvae and is often reported as a limiting factor in hatcheries (Felix and Nanjaiyan, 1992; Abraham *et al.* 1993; Lightner, 1996; Nayak and Mukherjee, 1997).

Vibrios are ubiquitous in marine and estuarine environments and are associated with fish and other poikilothermic animals, existing as part of the normal microbiota and as primary or secondary pathogens as well (Anderson *et al.* 1989; Cahill, 1990; Austin and Austin, 1993). On several occasions, mortality in finfish and shellfishes have been associated with an increase in the *Vibrio* populations (Sung *et al.* 2001). Several species of *Vibrio* are associated with surfaces and internal organs of marine invertebrates and vertebrates (Huq *et al.* 1983; Colwell and Grimes, 1984; Ortigosa *et al.* 1994) and have

been isolated from lesions or haemolymph in most of the reported bacterial infections in shrimps.

V. alginolyticus (Felix and Devaraj, 1993), *V. anguillarum* (Nammalwer and Thangaraj, 1980), *V. cholerae* (Premanand *et al.* 1996), *V. fluvialis* (Ponnuraj *et al.* 1995), *V. parahaemolyticus* (Abraham *et al.* 1993), *V. mimicus* (Karunasagar *et al.* 1990), *V. vulnificus* (Karunasagar *et al.* 1992), *V. damsela* (Aravindan and Kalavati, 1997), *V. harveyi* (Abraham and Manley, 1995) *Vibrio proteolyticus* CW8T2 (Verschuere *et al.* 2000) have been isolated so far from larval shrimps. Among the different species of vibrios, *Vibrio alginolyticus* has been isolated frequently from diseased shrimp as the aetiologic agent of vibriosis and has been described as the principal pathogen of both penaeids and non-penaeids (Lightner, 1988; Baticados *et al.* 1990; Limsuwan, 1993; Felix and Devaraj, 1993; Mohney *et al.* 1994; Lee *et al.* 1996). *Aeromonas* sp. may occasionally be involved in bacterial disease syndrome in prawns (Yasuda and Kitao, 1980; Lightner, 1983).

In prawns, vibrios are known to be pathogenic (Anderson *et al.* 1989) and systemic infections and necrotic appendages due to *Vibrio* have been reported in hatcheries (New, 1995). Miyamoto *et al.* (1983), analysed quantitative and qualitative changes in the bacterial population of larvae and culture medium in two *M. rosenbergii* hatcheries in Hawaii. According to that study, 13 genera of bacteria were identified including *Vibrio, Aeromonas* and *Pseudomonas*. Fujioka and Greco (1984), enumerated *Vibrio* spp., which included *V. fluvialis*, *V. alginolyticus* and *V. Cholerae* non O1, in the larval culture medium of *Macrobrachium* sp. One year later, Colorni (1985), described *Aeromonas liquifaciens* and *V. anguillarum* associated with the larvae of *M. rosenbergii.* Anderson *et al.* (1989), made an estimation of aerobic heterotrophic bacterial flora associated with tank water, tank sediment, tank surface, larval surface and larval slurry in three *M. rosenbergii* hatcheries in Malaysia. According to them, *Vibrio* species and *Alkaligens* are the most commonly encountered genera from larval rearing system of *M. rosenbergii.* Phatarpekar *et al.* (2002), carried out quantitative and qualitative analyses of bacterial flora, associated with larval rearing of the *M.*

rosenbergii, along with important water quality parameters, over a larval cycle. They detected *Vibrio* spp. in eggs and water.

Anderson *et al.* (1990), reported mass larval mortality of *M. rosenbergii* cultured in Malaysia at about 16 days after hatching and the clinical signs were similar to bacterial necrosis.. Later, Lombardi and Labao (1991a, b), documented the association of vibrios with necrosis (black spot) and gill obstruction. However, a definite aetiology of this necrosis has not been identified so far. Singh (1990), while researching the microbiology of a typical freshwater prawn larval rearing system at the Regional Shrimp Hatchery - Azhikode, Kerala, observed a profound relationship between the abundance of the members of family Vibrionaceae (Baumann *et al.* 1984) and mortality of larvae during the mid-larval cycle. The same observation was later made by Hameed *et al.* (2003), who observed that *Vibrio* species comprised the dominant taxon in eggs, larvae and post-larvae of *M. rosenbergii*.

Vibrio spp. appears to be more virulent in the larval stages due to their ability to produce exotoxins/ exoenzymes and/or due to their invasiveness (Elston and Leibovitz, 1980; Nottage and Birkbeck, 1987a, b; Santos *et al.* 1992; Birkbeck and Gallacher, 1993; Toranzo and Barja, 1993; Riquelme *et al.* 1995). Prominent virulence factors of vibrios have been correlated with their extracellular, protease, lipase, DNase, chitinase enzymes and haemolysins (Reid *et al.* 1980; Janda *et al.* 1988; Wong *et al.* 1992; Austin *et al.* 1993). Liu *et al.* (1996), considered that proteases, phospholipases, haemolysins or exotoxins might be important for pathogenicity. Virulence factors associated with their pathogenicity in *Vibrio* species appear to be strain-specific and due to the production of cytotoxic substances such as enterotoxins and haemolysins (Chowdhury *et al.* 1987; Stelma *et al.* 1992; Okuda *et al.* 1997). Different strains of *Vibrio* have been shown to have one or more mechanisms for expressing virulence in the fish host. Crosa *et al.* (1977), discovered that high virulence strains of *V. anguillarum* contained a large plasmid that enabled the bacterium to obtain iron necessary for its metabolism, even though the host produced factors that bind iron. Haemolysins, cytolysins, proteases, and other extracellular toxic substances have been demonstrated among some vibrios (Kodama *et*

al. 1984; Inamura *et al.* 1985; Kodama *et al.* 1985; Kothary and Kreger, 1985; Moustafa *et al.* 1985).

Smith and Merkel (1981), postulated that the virulence of *V. vulnificus* and *V. alginolyticus* was due to the production of collagenase. Reports concerning extracellular proteases produced by pathogenic vibrios of shrimps have been published (Lee *et al.* 1997; Liu *et al.* 1997). Proteases present in the ECP of bacteria have been implicated as virulence determinants in fish diseases (Inamura *et al.* 1984; Kanemori *et al.* 1987). Sudheesh and Xu (2001), reported the toxicity of the extracellular protease of *V. parahaemolyticus* to tiger prawn, *Penaeus monodon.* Pacha (1968), suggested proteolytic enzymes of *Flavobacterium psychrophilum* to be important virulence factors, and in a recent study by Bertolini *et al.* (1994) different protease profiles were associated with differences in virulence. Alkaline serine proteases produced by *V. alginolyticus* have been reported to be toxic for kuruma prawns (*Penaeus japonicus*) (Liu *et al.* 1997).

Bacterial haemolysins have been suggested to be important factors of pathogenic vibrios by causing haemorrhagic septicemia in the host and many of these haemolysins are well characterized in *V. parahaemolyticus* (Nishibuchi and Kaper, 1985), *V. cholerae* (Baba *et al.* 1991), *V. mimicus* (Kim *et al.* 1997) and *V. vulnificus* (Yamamoto *et al.* 1990). *V. harveyi*, which was the most pathogenic isolate to salmonids, produced extracellular product with the highest titer of haemolytic activity towards Atlantic salmon and rainbow trout erythrocytes (Zhang and Austin, 2000). Ruangpan and Kitao (1991), and Chang *et al.* (1996), suggested that haemolytic activity might be correlated to their pathogenicity, since factors such as adhesion and invasion (Ruangpan *et al.* 1994) have been excluded. Although there is still much disagreement about the relationship between haemolysis and pathogenicity (Inamura *et al.* 1984; Chung and Kou, 1985; Kodama *et al.* 1985), haemocytolytic assay is still commonly used to differentiate among suspected pathogens (Ullah and Arai, 1983; Chen *et al.* 1995; Lee *et al.* 1995). Chang *et al.* (2000), used prawn blood agar haemolysis to screen for bacteria pathogenic to cultured tiger prawns *Penaeus monodon.*

Elevated levels of shell disease related with chitinolytic bacteria have been reported amongst crustaceans living in degraded environmental conditions, such as aquaculture systems (Delves-Broughton and Poupard, 1976; Prince *et al.* 1995) or polluted environments (Young and Pearce, 1975; Sawyer, 1991). Cipriani *et al.* (1980) reported that chitinolytic activity is fundamental to lesion progression; microbial proteases and lipases may be involved in exoskeletal breakdown, particularly in the initial stages of shell disease. Vogan *et al.* (2002), reported the involvement of chitinolytic bacteria in shell disease syndrome in the edible crab (*Cancer pagurus*).

The most common method employed to control vibriosis was treatment of diseased fish with antibiotics and antimicrobial chemicals. Commonly used substances are oxytetracycline, sulphonamides, nitrofuran derivatives, trimethoprim and quinolines, and antibiotic therapy for the control of vibriosis has met with variable success. Many shrimp hatcheries use antibiotics as prophylactic agents to prevent bacterial infection of larvae (Baticados and Paclibare, 1992; Supriyadi and Rukyani, 1992; Karunasagar *et al.* 1994). The most common way to resolve the vibriosis problem is by the use of feed plus antibiotics. However, the continued use of chemotherapeutic agents may be harmful for the larvae due to the alteration of the natural equilibrium among the bacterial populations in the culture environment as well as to the possible selection of resistant pathogenic strains (Bourne *et al.* 1989; Kerry *et al.* 1994). The most significant difficulty caused by the continued and indiscriminate use of antibiotics has been the development of serious drug resistance (Frappaolo and Guest, 1986).

Although vibriosis has been reported in various species of cultured shrimps and fishes, reports on bacterial diseases of freshwater prawn are limited. The available ones deal with total bacterial count and scanty data on *Vibrio* sp. Knowledge on species diversity and their pathogenicity are not available at all. Precisely, the information on *Vibrio* species related to prawn hatcheries is essential to find out appropriate control measures.

This chapter describes isolation of *Vibrio* and *Aeromonas* from larval rearing system of *M. rosenbergii*, their phenotypic characterization, determination of pathogenicity, relationship between virulence characteristics of the isolates and haemolysis in prawn blood agar and also with hydrolytic properties such as lipase, protease and chitinase. Antibiotic sensitivity of the *Vibrio* isolates and *Aeromonas* to selected antibiotics is also dealt with.

2.2 Materials and Methods

2.2.1 Bacterial isolates

To satiate the requirement of a comprehensive selection of vibrios from a variety of locations, three distinct sources were used.

First set of isolates were the ones obtained during the period 1985-1992 from larval rearing system of *M. rosenbergii* (Azhikode, Kerala), isolated and identified to family Vibrionaceae by Singh (1990), and further investigated by Bhat (1998), and Bhat and Singh (1998), and maintained at the Centre for Fish Disease Diagnosis and Management, School of Environmental Studies, Cochin University of Science and Technology.

Second set of isolates of *Vibrio* was obtained during the period 2001 from the *M. rosenbergii* larval production facility of M/S Rosen Fisheries, Trichur, Kerala. The samples were obtained from a tank (5-ton capacity with 500,000 mysis as initial stocking density) where majority of the larvae (stage nine) had displayed anorexia, inactivity, poor growth and morbidity. The prominent sign was severe necrotic appendages. For isolating bacterial aetiology of the larval necrosis, water and moribund necrotic larval samples (around 200 nos.) were collected. Collection of moribund larvae was made in sterile (autoclaved at 15 lbs for 15 min) polystyrene bottles having sterile seawater (15ppt) as the transport medium. Water sample (100 ml) from the same tank was also collected in a sterile bottle. Both the samples were placed in ice-chest at 4°C and transported to the laboratory within two hours of collection.

In the laboratory sterile seawater (15ppt) was used uniformly for washing the larvae, homogenization, dilution and preparation of media, unless otherwise specified. The larvae (30 nos.) were washed gently and repeatedly for three times, transferred to a sterile glass tissue homogenizer and homogenized well to a fine paste. Homogenized sample was diluted serially to 10^{-6}. Water sample was also serially diluted to 10^{-6}.

Aliquots of 0.2 ml were spread plated on ZoBell's marine agar 2216E plates and incubated at 28±1°C for 48 h. The plates, which had well-distributed colonies within the range of 30 to 300, were selected for isolation of the colonies. Instead of random isolation of the colonies a different pattern of differential selective isolation based on colony morphology was adopted. Accordingly, colonies were grouped based on their morphology following Cappuccino and Sherman (1999). Subsequently, from each group 10% of the colonies were isolated into ZoBell's agar slants. The colonies were repeatedly streaked onto ZoBell's marine agar plates till purity was attained. All Gram negative isolates were identified to genera following Oliver (1982), and all Gram positive isolates following McFaddin (1980). Isolates of *Vibrio* were further identified to species level following Alsina and Blanch (1994), and Baumann and Schubert (1984).

Third set of bacterial isolates was obtained during the period 2002 from a batch of *M. rosenbergii* post-larvae (PL-1), which experienced heavy mortality in the hatchery of Matsyafed, Quilon, Kerala. Samples were transported to the laboratory and processed as above for the isolation of associated bacteria. After two days of growth the plates, which showed well-distributed colonies (30 – 300) were selected for isolation. All the colonies developed on a plate (37 colonies) were isolated, purified and characterized for the presumptive identification of *Vibrio*.

Before carrying out the biochemical tests, purity of bacterial isolates was confirmed by streaking thrice on ZoBell's agar plates. The pure colonies were maintained on ZoBell's agar slants for further biochemical characterization.

Composition of ZoBell's Marine 2216 E agar per litre:

Peptone	5.0g
Yeast extract	1.0g
Ferric phosphate	0.1g
Agar	20.0g
Aged-seawater (15ppt)	1000ml

pH 7.0 ± 0.2

Those cultures, which were Gram negative, motile, oxidase positive and fermentative on MOF medium, were presumptively identified as *Vibrio* and brought under study.

2.2.2 Identification to Species

All those bacterial isolates, which could grow on thiosulphate citrate bile sucrose agar (TCBS), Gram negative, motile, kovacs oxidase positive, fermentative on marine oxidation fermentation medium were further identified following Alsina and Blanch (1994). Briefly, starting from the combination of the arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase tests (A/L/O), it was possible to proceed to six blocks or 'keys' of biochemical reactions, for a total of 38 tests.

2.2.3 Biochemical Tests

2.2.3.1 Marine Oxidation Fermentation Test

MOF medium (Himedia Laboratories, Bombay) was used for the present work. Transferred 22g of MOF medium to 1000 ml of distilled water, added 15g agar and sterilized by autoclaving at 15 lbs for 15 min. To the above sterile basal medium, 1% glucose was added and transferred at 4 ml aliquots aseptically into sterile tubes. The tubes with the medium were again autoclaved at 10 lbs for 10 min and converted to slants with a long butt. The tubes were stabbed and streaked and incubated at 28±1°C.

The results were recorded as follows:

O - Oxidation (yellow colouration in the butt)

F - Fermentation (yellow colouration through out the tube)

(F) - Fermentation with gas production

Alk / N - alkaline reaction (pink or purple colouration in the slant and no reaction in the butt)

2.2.3.2 Kovac's Oxidase Test

According to the methods recommended by Kovacs (1956), the organisms were freshly grown on ZoBell's marine agar. A platinum loop was used to make a thick smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl paraphenylene diamine dihydrochloride. A positive result was recorded when the smear turned violet within 10 seconds, indicating the formation of indophenol, the oxidation product of tetramethyl paraphenylene diamine dihydrochloride.

2.2.3.3 Motility Test

Motility was tested either by inoculating in soft agar medium or by hanging drop method.

A) In soft agar medium

Yeast extract	1g
Peptone	5g
Ferric phosphate	0.1g
Aged seawater(15ppt)	1000ml
Agar	3g
pH	7.0 ± 0.2

Melted the medium and poured into tubes in 3 ml aliquots and autoclaved at 15 lbs for 15 min. Stab inoculated and incubated at 28±1°C for 24 to 48 h. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility. A thick growth along the line of inoculation was considered negative. All negative cultures were subjected for observation by hanging drop method.

B) Hanging drop method

The organisms were grown in ZoBell's broth of the above composition devoid of agar. Vaseline was applied on the four corners of a glass cover slip and a loopful of 18 to 24 h old culture was placed at the center. A cavity slide was kept over the drop in such a way that the drop came within the cavity. The vaseline helped to adhere the coverslip to the slide and the whole preparation was inverted quickly so that the drop of culture was seen hanging from the cover slip. The slide was placed under oil immersion objective (100X) and observed for actual displacement of the cells that could very well be differentiated from brownian movement.

2.2.3.4 Growth on TCBS medium

TCBS agar (Himedia Laboratories, Bombay) was used for the present study. Suspended 89.0 g of TCBS medium in 1000 ml of distilled water and boiled to dissolve the medium completely. Cooled to 50°C and poured into sterile petri plates. Cultures were streaked onto the prepared plates and incubated at 28±1°C for 48 h and observed for growth.

2.2.3.5 Arginine Dihydrolase Test

The ability of certain organisms to produce an alkaline reaction in arginine containing medium under relatively anaerobic conditions has been used by Thornley (1960), to differentiate between certain Gram-negative bacteria, especially *Pseudomonas* spp. The alkaline reaction is thought to be due to the production of ornithine, CO_2 and NH_3 from arginine.

Thornley's medium has the following composition per litre:

Peptone	1g
NaCl	5g
K_2HPO_4	0.3g

Phenol red	0.01g
L(+) Arginine hydrochloride	10g
pH	7.2

The basal medium prepared in distilled water without L (+) arginine hydrochloride was heated to dissolve the solids. pH was adjusted and sterilized by autoclaving at 15 lbs for 15 min. L (+) arginine hydrochloride was added and dispensed as 3 ml aliquots in sterilized culture tubes. The medium in the culture tubes were overlaid with liquid paraffin to a height of 5 mm and autoclaved again at 10 lbs for 10 min. As control, tubes containing only the basal medium overlaid with liquid paraffin were included.

The test organisms were stab inoculated into the medium through the liquid paraffin layer. Color changes are recorded after incubation at 28±1°C for upto 7 days. The control tube without the amino acid remained yellow; but a subsequent change to purple in the tests indicated that alkaline degradation products were produced.

2.2.3.6 Lysine and Ornithine Decarboxylase Test

The amino acid decarboxylase test demonstrates the bacterial decarboxylation of amino acids. In this test, the decarboxylation or the elimination of a molecule of carbon dioxide from the amino acid results in the formation of an amine with one carbon atom less. Decarboxylases are generally induced by growing the bacteria at a low pH. This is achieved by cultivating the test organisms in a fermentable carbohydrate medium.

One percent of the L-amino acid [L (+) Lysine dihydrochloride or L (+) Ornithine monohydrochloride], was incorporated in Falkow's medium (modified from Falkow, 1958), containing per litre:

Peptone	5g
Yeast extract	3g

Glucose	1g
Bromocresol purple (0.2% solution)	10ml
pH	6.7

The basal medium prepared in distilled water without L (+) lysine dihydrochloride or L (+) ornithine monohydrochloride, was heated to dissolve the solids. pH was adjusted and sterilized by autoclaving at 15 lbs for 15 min. L (+) lysine dihydrochloride or L (+) ornithine monohydrochloride was added and dispensed in 3 ml aliquots in sterilized culture tubes. The medium in the culture tubes was overlaid with liquid paraffin to a height of 5 mm and autoclaved again at 10 lbs for 10 min. As control, tubes containing only the basal medium overlaid with liquid paraffin were included.

The test organisms were stab inoculated into the medium through the liquid paraffin layer. Color changes were recorded after incubation at 28±1°C for upto 7 days. As a result of the bacterial fermentation of the glucose in the medium, the indicator turned yellow. The control tube without the amino acid remained yellow; but a subsequent change to violet or purple in the tests indicated that alkaline degradation products were produced in the course of decarboxylation of the particular aminoacid.

2.2.3.7 Growth at 0, 3, 6, 8 and 10% NaCl

Growth at 0, 3, 6, 8 and 10% NaCl was tested by observing growth in 1% tryptone (Tryptone without NaCl, Himedia Laboratories, Bombay) broth at pH 7 containing varying amounts of analytical grade sodium chloride. The medium was dispensed in 3 ml aliquots in to tubes and sterilized at 15 lbs for 15 min. These broth media were inoculated with a 24 h culture and the growth was detected visually by observing turbidity.

2.2.3.8 Indole Production

Certain bacteria produce indole by decomposition of tryptophan, which is present in tryptone broth. The liberated indole reacts with the Kovac's reagent to produce red color at the top of the medium (Cowan and Steel, 1965).

Composition of test medium per litre:

Tryptone water	15g
Sodium chloride	15g
pH	7.0 ± 0.2

Dispensed the medium in 3 ml aliquots into culture tubes, sterilized by autoclaving at 15 lbs for 15 min. The tubes were inoculated and incubated at 28±1°C for two to four days. To each tube added about 0.5 ml of Kovac's reagent.

Preparation of Kovac's reagent

ρ - dimethyl amino benzaldehyde	5g
Amyl alcohol	75ml
Conc.HCl	25ml

2.2.3.9 Voges-Proskauer Test

The test was carried out with cultures grown in glucose-phosphate peptone water, which has the following composition per litre:

Glucose	5g
K_2HPO_4	5g
Peptone	5g
pH	7.0 ± 0.2

The medium was dispensed in 5 ml aliquots in culture tubes and sterilized by autoclaving at 10 lbs for 10 min. The inoculated tubes were then incubated at 28±1°C for 7 days until good growth was obtained.

Some organisms, after producing acids from glucose, are capable of converting the acids to acetylmethyl carbinol or 2, 3 butanediol, which are neutral substances. Aeration in the presence of alkali then converts the products to diacetyl, which in turn reacts with the peptone constituents producing a pink coloration. Two modifications of the test viz. O'Meara's modification (O'Meara, 1931) and Barrit's modification (Barrit, 1936) are in use and both enhance the development of color. For the present study Barrit's modification was followed. To 2 ml of the culture, added 0.5 ml 40% KOH solution and agitated thoroughly. Then 0.5 ml 6% alcoholic solution of ∝- naphthol was added and maintained for 2 h. Positive test was indicated by the development of pink colouration.

The pale pink colouration normally appears within 5 min but may reach maximum red color intensity within an hour. Negative tubes were examined and confirmed after a longer period.

2.2.3.10 Utilization of Citrate

Simmon's citrate agar medium (Himedia Laboratories, Bombay) was employed for the test. Suspended 24.28 g of the above medium in 1000 ml of distilled water and boiled to dissolve the medium completely. Dispensed 3 ml aliquots of the medium into tubes and sterilized by autoclaving at 15 lbs for 15 min. The tubes were converted to slants and cultures to be tested were streaked on to slants using a sterile inoculation loop. The inoculated slants were incubated at 28±1°C for 24 to 48 h and the tubes were observed for color change. A positive reaction is indicated by an intensive blue color in the medium along with the intensive growth of the organisms on the slant.

2.2.3.11 Gelatinase Test

Gelatinase activity was examined by plate as well as tube test.

i) Plate test

When proteolytic organisms are grown on a plate of nutrient medium into which gelatin (1.2%) is incorporated, zones of gelatinase activity around the colonies could be demonstrated, if the plates are flooded with acid mercuric chloride solution which reacts with gelatin in the medium to produce an opacity; where the gelatin has been hydrolyzed, the medium remains clear (Frazier, 1926).

The composition of the medium per litre contains:

Peptone	5g
Yeast extract	1g
Beef extract	5g
NaCl	15g
Gelatin	12g
Agar	20g
pH	7.0 ± 0.2

The prepared medium was sterilized by autoclaving at 15 lbs for 15 min and poured into petriplates. The test organisms were spot inoculated and the plates incubated at 28±1°C for three to five days.

Gelatinase production was tested by flooding the plates with 5 ml of mercuric chloride solution, of the following composition:

$HgCl_2$	12g
2M HCl	96 ml

ii) Tube test:

The composition of the medium per litre contains:

Peptone	5g
Yeast extract	1g
NaCl	15g
Gelatin	20g
pH	7.0 ± 0.2

The prepared medium was melted, dispensed in 3 ml aliquots into culture tubes and sterilized by autoclaving at 15 lbs for 15 min. The tubes were stab inoculated and incubated at 28±1°C for three to five days. A control tube was maintained without inoculation. Gelatin at the concentration used melts at about 24°C and is therefore liquid at room temperature. Gelatin liquefaction was tested for at intervals by removing the nutrient gelatin cultures from the incubator and holding them at 4°C for 2h before reading the results. Gelatin liquefaction was indicated by liquid nature of the medium that was observed when the culture tubes were made upside down and whereas in the control tube the medium remained solid.

2.2.3.12 Production of Acid from Compounds

MOF medium (Himedia Laboratories, Bombay) was used for the present work. Transferred 22 g of MOF medium to 1000 ml of distilled water, added 15g agar and sterilized by autoclaving at 15 lbs for 15 min. To the above sterile basal medium, 1% sucrose or arabinose was added and transferred at 4 ml aliquots aseptically into sterile tubes. The tubes with the medium were again autoclaved at 10 lbs for 10 min and converted to slants with a long butt. The tubes were stabbed and streaked and incubated at 28±1°C. The production of acid is detected by change in the color of the medium from pink to yellow.

2.2.3.13 Utilization of Sole Carbon Sources

The basal medium, a modification of Baumann *et al.* (1971) given by Oliver (1982), composed of the following per litre was used:

Solution A

NH_4Cl	5g
NH_4NO_3	1g
Na_2SO_4	2g
K_2HPO_4	3g
KH_2PO_4	1g
NaCl	10g

Solution B

$MgSO_4 . 7H_2O$	0.1g
$MgCl_2. 6H_2O$	4g
Yeast extract	0.01g
Agar	20g
pH	7.0

Yeast extract was prepared in the form of a 0.1% stock solution in the distilled water and added. Solution A and B were sterilized by autoclaving separately at 15 lbs for 15 min, cooled to room temperature and then mixed together (otherwise the basal medium becomes turbid).

Glucosamine or arabinose was added to a final concentration of 0.2% (w/v) and dispensed into sterile tubes in 3 ml aliquots and again autoclaved at 10 lbs for 10 minutes. Control tubes without the carbon source were also maintained for each culture.

The test and control media tubes were inoculated with an uniform quantity of inoculum and incubated at 28±1°C for 24 to 48 h. The tubes were checked for turbidity against control. In cases of negative tubes, they were incubated further for 7 days.

2.2.3.14 Sensitivity to Antibiotics

Sensitivity to Ampicillin (10μg) and O/129 (10μg) were examined by the standard disc diffusion method on nutrient agar plates supplemented with 1.5% (w/v) NaCl. The plates were swab inoculated with the test organisms. Readymade Ampicillin (10μg) discs (Himedia Laboratories, Bombay) and O/129 (10μg) discs (prepared in the laboratory by impregnating O/129 solution of known concentration on to sterile discs) were placed on the swabbed plates. The plates were incubated at 28±1°C for 24 h. Sensitivity to Ampicillin and O/129 compound resulted in the production of clear zones of no growth around the discs.

2.2.3.15 Growth at Different Temperature

Growth at 4°C and 41°C was tested in ZoBell's broth (15ppt). The medium was dispensed in 3 ml aliquots into culture tubes and sterilized by autoclaving at 15 lbs for 15 min. These tubes were inoculated with a 24 h culture and incubated at respective temperature cabinets. Observations were made daily for a week visually reading turbidity.

2.2.4 Pathogenicity of Vibrios

Isolates of *Vibrio* spp. and *Aeromonas* spp. were subjected to the following assays to reveal their pathogenicity.

2.2.4.1 Haemolytic Assay on Prawn Blood Agar

Haemolytic assay of all the bacterial isolates on prawn blood agar was done according to the basic protocol of Chang *et al.* (2000), with modifications. Haemolymph required for the assay was drawn from wild caught adult (30±10g) *M. rosenbergii* using sterile capillary tubes of 2mm outer diameter and 1mm inner diameter. Prior to blood collection, the animals were maintained in the laboratory in freshwater for a week on scampi diet with 100% exchange of water. To facilitate aseptic collection of blood the area beneath the rostrum spine was disinfected with sodium hypochlorite (200ppm) by allowing the solution flow through the area for two minutes. This was followed by administration of 70% ethanol in the same pattern. The area was washed with sterile distilled water subsequently and wiped dry with sterile absorbent cotton swabs.

To prevent clotting of haemolymph, citrate-EDTA buffer containing 0.1M glucose, 30mM trisodium citrate, 26mM citric acid, and 10mM EDTA dissolved in double distilled water was used. The pH of the buffer was adjusted to 4.6 and osmolality to 350 mOsm (by adding sodium chloride) and sterilized at 10 lbs for 10 min. The capillary tubes were rinsed with citrate-EDTA buffer prior to blood collection. One milliliter collected haemolymph was transferred to a sterile tube containing 0.2 ml citrate-EDTA buffer and stained by addition of 140 µL of 2% (w/v) Rose Bengal (dissolved in citrate-EDTA buffer) with gentle rotation to achieve complete mixing. The basal agar medium comprised 10 g Bacto peptone, 5 g sodium chloride and 15 g Bacto agar dissolved in 1000 ml distilled water, adjusted to pH 6.8, autoclaved at 15 lbs for 15 min, and cooled to 45–50°C in a water-bath. Aseptically, 1 ml of the stained haemolymph preparation was added to 15 ml of this prepared basal medium followed by gentle mixing and pouring into petri dishes. The isolates of *Vibrio* and *Aeromonas* were streak inoculated onto the prawn blood agar and incubated at 28±1°C for 3 to 7 days. The plates were observed for clearing zone around the growth and lysis of haemocytes under microcope with 10X objective lens.

2.2.4.2 Hydrolytic properties

a) Production of Lipase

Tributyrin or glyceryl tributyrate is commonly used for recording lipolytic activities. Tributyrin agar medium used for the test had the following composition per litre:

Peptone	5g
Beef extract	5g
Yeast extract	1g
NaCl	15g
Tributyrin	10g
Agar	20g
pH	7.0 ± 0.2

Tributyrin was first mechanically blended into the nutrient broth to form a stable emulsion, agar added and sterilized by autoclaving at 15 lbs for 15 min. Plates were poured while mixing well each time.

Test organisms were spot inoculated and the inoculated plates were incubated at 28±1°C for 3 to 4 days. A positive result was indicated by zone of clearing around the colonies of lipolytic organisms, where the tributyrin has been hydrolyzed (Rhodes, 1959).

b) Production of Gelatinase

Gelatinase activity was examined by plate test and tube test as mentioned earlier.

c) Production of Chitinase

Chitinolytic bacteria hydrolyze chitin to N-acetyl-D-glucosamine. This hydrolysis can be easily tested by incorporation of colloidal chitin into a suitable basal medium (Holding and Collee, 1971).

Purified colloidal chitin (Lingappa and Lockwood, 1961) was made by treating crude chitin alternately with 1N – NaOH and 1N – HCl several times, and then with ethanol until all foreign materials have been removed. The purified chitin was then dissolved in cold con. HCl, filtered through glass wool, precipitated in distilled water until neutral pH. This colloidal chitin was then added to the medium having the following composition per litre:

Peptone	5g
Beef extract	5g
NaCl	15g
Colloidal chitin	20g (or 2% v/v)
Agar	20g
pH	7.0 ± 0.2

The medium was sterilized by autoclaving at 15 lbs for 15 min and poured into plates. The test cultures were spot inoculated and incubated for 7 days at 28±1°C. Hydrolysis of chitin was represented by halo zone around the colonies.

2.2.4.3 Antibiotic Sensitivity

Antibiotic sensitivity of bacterial isolates was tested against commonly used antibiotics in aquaculture systems on nutrient agar plates supplemented with 1.5% sodium chloride by disc diffusion method. For this, the plates were swabbed with overnight grown bacterial suspension culture using sterile cotton swabs. The readymade

antibiotic discs (Himedia Laboratories, Bombay) were placed on the seeded plates and incubated at 28±1°C for 24 h. The antibiotic discs tested were,

Ampicillin	: 10 mcg
Streptomycin	: 10 mcg
Rifampicin	: 2 mcg
Neomycin	: 30 mcg
Erythromycin	: 10 mcg
Kanamycin	: 30 mcg
Chloramphenicol	: 10 mcg
Ciprofloxacin	: 5 mcg
Oxytetracycline	: 30 mcg
Novobiocin	: 30 mcg
Furazolidone	: 50 mcg
Nitrofurantoin	: 100 mcg

Sensitivity to the antibiotics resulted in the production of halozone around the antibiotic discs. The halozone diameters were measured and the results recorded.

2.2.4.4 Pathogenicity of the isolate of *V. alginolyticus* on the larvae of *M. rosenbergii*

To evaluate pathogenicity of the isolates of *V. alginolyticus* on *M. rosenbergii* larvae, one representative isolate (MRNL 3) was used. Larvae of *M. rosenbergii* at stage nine were brought to the laboratory from the scampi hatchery of M/s Rosen Fisheries, Trichur, Kerala in oxygen filled polythene bags. They were maintained in 15ppt seawater and were fed with freshly hatched *Artemia* nauplii. The experiment was conducted as follows: Seawater (15ppt) was autoclaved and transferred (1L) in to 2L capacity round plastic containers and aerated at a rate of 1L per minute. Apparently healthy larvae, characterized by their photosensitivity, were removed from top of the holding tank and distributed at 30 each to twelve experimental containers, nine as tests and three as controls. Larvae in both the sets of containers were fed uniformly with freshly hatched

300 *Artemia* nauplii per larva of *M. rosenbergii* daily. Overnight cultures of *V. alginolyticus* (MRNL 3) was harvested from ZoBell's marine agar plates to sterile seawater (15ppt) and diluted to a concentration equivalent to optical density (OD) 1.0 at 600 nm. This standard suspension of bacteria contained ~1 X 10^{10} cfu/ml, as determined by standard dilution and plating method. The containers with larvae demarcated as 'tests' were challenged by inoculating the rearing water to a final number of 10^6, 10^7 and 10^8 cfu/ml in triplicate. Three control tanks were maintained without challenge. The larvae were observed for four days and assessed mortality daily for determining the cumulative mortality at the end of the experiment. During this period no water exchange was provided and the water quality parameters such as temperature, pH, ammonia and nitrite were within the ranges recommended by New and Singholka (1982), and Correia *et al.* (2000) i.e. 28°C to 29°C, pH 7.5 to 8, ammonia and nitrite < 0.1ppm.

2.3 Results and Discussion

From the first set of bacterial isolates, which comprised heterotrophic bacteria already isolated and identified as vibrios (Singh, 1990; Bhat, 1998) from larval rearing system of *M. rosenbergii* and maintained at the Centre for Fish Disease Diagnosis and Management, thirty five strains could be segregated based on the isolates obtained from the moribund larvae and apparently healthy ones (Table 1). This comprised the representatives of the bacteria belonging to vibrios from the larval rearing system of *M. rosenbergii.*

As second set, two hundred and sixty bacterial colonies could be counted on ZoBell's marine agar plates developed from diseased *M. rosenbergii* larvae (stage 9) and the rearing water collected from a hatchery tank (M/S Rosen Fisheries, Thrissur, Kerala) which had showed high mortality and signs of necrosis. Before isolation from the ZoBell's marine agar plates, differential counting of bacterial colonies from larval macerates and water samples were made based on their morphological characteristics. After recording morphological characters and pigmentation, representative types, constituting at least 10% of the total number of the type of colonies on plates, were isolated, purified and subjected to biochemical tests.

Generic composition of heterotrophic bacteria and their relative frequency recovered from both necrotic larvae and water sample from the larval rearing system are shown in Table 2. In this study, colony morphology was adopted as the basis of grouping the bacterial colonies to facilitate isolation of the organisms representing each group. Accordingly, eight (A to H) morphological types could be documented. Among them, the morphological type 'A', (small, circular, convex, entire, translucent colony) could be isolated only from the necrotic larvae and not even a single colony from the rearing water at the dilution plated. This morphological type was identified as *Vibrio.*

Seven different morphological types were observed in the plate that was seeded with larval macerates (Table 2). Gram-negative isolates were identified to generic level

using the taxonomic key by Oliver (1982), whereas Gram-positive isolates were identified following McFaddin (1980). Altogether, seven genera were identified; the most frequently isolated was *Vibrio*. The other genera isolated, from larvae, in the decreasing order of dominance, were *Cytophaga, Aeromonas, Alteromonas, Pseudomonas, Alcaligenes* and *Bacillus* (Table 2). Bacteria isolated were predominantly Gram-negative, although Gram-positive ones were *Bacillus*.

Meanwhile, six different morphological types were observed in the plates that were seeded with water sample (Table 2). Altogether, six genera were identified and the most frequently isolated ones were found to be *Cytophaga* followed by *Alcaligenes, Aeromonas*, *Pseudomonas* and *Alteromonas* and *Micrococcus* in the decreasing order of dominance (Table 2). Bacteria isolated were predominantly Gram-negative, and the Gram-positive isolate was *Micrococcus. Vibrio* species isolated from the above set of bacterial cultures is presented in Table 2a.

As third set, thirty-seven bacterial colonies could be obtained from the larval sample collected from diseased *M. rosenbergii* post larvae from a hatchery at Kollam, Kerala. All the bacterial colonies were isolated from the ZoBell's marine agar plate and identified to generic level using the taxonomic key for identification by Oliver (1982). Altogether, four genera were identified and the most frequently isolated one was *Vibrio* followed by *Enterobacteriaceae, Pseudomonas* and *Alcaligenes* (Table 3). *Vibrio* species isolated from the above set of bacterial cultures are presented in Table 3a.

For identifying to species level, the method proposed by Alsina and Blanch (1994), was employed for all the above bacterial isolates which were negative rods, motile, kovacs oxidase positive, growth on TCBS agar and fermentative on marine oxidation fermentation medium.

In total, fifty-five isolates of *Vibrio* and six isolates of *Aeromonas* could be segregated from the lot recovered during the periods 1985-1992, 2001 and 2002, from three scampi hatcheries (Table 4). All the above bacterial isolates were maintained on

ZoBell's marine agar vials either by overlaying with sterile liquid paraffin or dry wax sealing at room temperature. Running cultures were maintained on ZoBell's marine agar slants at room temperature and also at 4°C. The genus was composed of nine species such as *V. cholerae* (12 isolates), *V. nereis* (11), *V. vulnificus* (8), *V. alginolyticus* (8), *V. mediterranei* (7), *V. parahaemolyticus* (4), *V. splendidus* II (2), *V. proteolyticus* (2), and *V. fluvialis* (1). Among these, the association of *V. cholerae*, *V. alginolyticus* and *V. fluvialis* with the larvae of *M. rosenbergii* has been reported earlier (Fujioka and Greco, 1984). Meanwhile *V. alginolyticus* has been described as the principal pathogen of non-penaeids in general (Lightner, 1988).

Dominance of *Vibrio* among the other genera in the necrotic larvae (sampled during 2002) and the failure in isolating them from the rearing water strongly point to their possible role in the necrosis. Logically, failure in isolating *Vibrio* from water at that dilution suggests its low count also. Meanwhile, in the necrotic tissue the organism might have multiplied and facilitated easy isolation. Such selective colonization of *Vibrio* and *Aeromonas* was documented by Dumontet *et al.* (2000), while studying the prevalence and diversity of *Aeromonas* and *Vibrio* spp. in coastal waters of southern Italy. He observed the colonization of *V. cholerae* non-O1, *V. alginolyticus*, *V. fluvialis* and *Aeromonas caviae* on copepods selectively.

On challenging the larvae with a representative isolate of *V. alginolyticus* (MRNL 3) at 10^6, 10^7 and 10^8 cfu/ml, percentage mortalities of 80, 87 and 100 were observed respectively within 96 h (Table 5). Meanwhile, in the control group the corresponding mortality was 37% only. Chi-square test showed that the mortality was significantly higher ($P < 0.01$) in the entire test groups challenged than the control. Signs preceding larval death included anorexia, settling to the bottom and weak swimming. The significantly higher mortality observed in the challenged set of larvae demonstrated pathogenicity of *V. alginolyticus* to the larvae of *M. rosenbergii* in a confined system. In penaeid prawns, *V. alginolyticus* has already been described as the principal pathogenic *Vibrio* species (Lightner, 1996). Several authors have also pointed out the importance of

V. alginolyticus as a potential shrimp pathogen (Baticados *et al.* 1990; Limsuwan, 1993; Mohney *et al.* 1994; Felix and Devaraj, 1993).

Haemolytic assay of 61 bacterial isolates on prawn blood agar was done according to Chang *et al.* (2000) with modifications. According to the above method, Rose Bengal reagent specifically stains prawn haemocytes but not the other proteins of the haemolymph. Thus, prawn blood agar appeared rose red because of well-distributed, stained haemocytes. When the haemocytes were destroyed by haemolytic bacteria, a clear zone appeared around the colonies and could easily be recognized by the naked eye (Fig. 1). Microscopic observation revealed intact dyed haemocytes in prawn blood agar away from haemolytic zone around bacterial colonies, while only haemocyte debris was seen adjacent to the colonies. The haemolytic activity of 61 bacterial isolates inoculated on prawn blood agar are shown in Table 6. Haemolytic assay revealed that all isolates of *V. alginolyticus, V. parahaemolyticus* and *Aeromonas* sp were haemolytic. Besides producing halozone around the growth, the haemocytes in the halozone were lysed leaving only debris, while the haemocytes away from the growth were intact. Meanwhile, among *V. nereis*, *V. vulnificus* and *V. mediterranei* both haemolytic and non-haemolytic isolates could be seen. However, all isolates of *V. cholerae*, *V. splendidus II*, *V. proteolyticus* and *V. fluvialis* from larval rearing systems investigated during 1985 to 1992 were non-haemolytic. Bacterial haemolysin has been suggested as an important virulent factor of pathogenic vibrios (Chang *et al.* 1996). A step further, the haemolytic assay has been recognized to be used to differentiate the virulent strains among suspected pathogens also (Chang *et al.* 2000). The importance of haemolysin as a virulent factor in pathogenic vibrios in fishes has been rightly pointed out by Zhang and Austin (2000). According to them *V. harveyi*, which is the most pathogenic isolate to salmonids, produced extracellular products with the highest titer of haemolytic activity towards Atlantic salmon and rainbow trout erythrocytes. In crustaceans, Chang *et al.* (2000), could effectively use prawn blood agar haemolysis to screen out bacteria pathogenic to cultured tiger prawn, *Penaeus monodon.*

All isolates of *Vibrio* and *Aeromonas* were lipolytic and all but two isolates of *V. splendidus* II were proteolytic. However, chitinolytic property was not widespread among the *Vibrio* isolates (Table 6). Toranzo *et al.* (1983), and Ellis (1991), suggested that production of gelatinase, lipase, and chitinase might be considered as the probable expression of virulence factors in vibrios. A comprehensive search for virulence factors among vibrios revealed unequivocally the role of proteases, lipases, chitinase and plasmids coding for iron chelators apart from haemolysins in initiating an infectious death (Reid *et al.* 1980; Moustafa *et al.* 1984; Nottage and Birkbeck, 1986; Wong *et al.* 1992). Among these hydrolytic enzymes, the importance of protease has been demonstrated in *V. alginolyticus*, (Lee *et al.* 1997), and *V. parahaemolyticus* (Sudheesh and Xu, 2001). Amongst crustaceans living in aquaculture systems elevated levels of shell disease related with chitinolytic bacteria have been reported (Prince *et al.* 1995). Vogan *et al.* (2002), reported that, chitinolytic activity is fundamental to lesion progression and microbial proteases and lipases may be supporting the process of exoskeletal breakdown, particularly in the initial stages of shell disease. However, if haemolysis can be considered as a virulent determinant, the proteolytic, lipolytic and chitinolytic properties may serve as the supplementary factors only helping the organisms to invade the host. Further studies at molecular level on the importance of haemolytic activity and the hydrolytic properties of *Vibrio* might help in elucidating the possible virulence factors.

In order to select an appropriate chemotherapy to protect the larvae from vibriosis, 12 commonly used antibiotics in aquaculture systems were screened against all the bacterial isolates (61 isolates). Although variations in the resistance patterns could be observed among the bacterial isolates, practically all of them proved to be resistant to erythromycin, which is the commonly employed antibiotic in hatcheries as a prophylactic agent. Among the *Vibrio* isolates, incidence of bacterial resistance to oxytetracycline was higher (40%) followed by ampicillin (24%) and streptomycin (22%). Incidence of resistance to kanamycin, ciprofloxacin and furazolidone was the least observed among the individual antibiotics. It is known that the prophylactic use of antibiotics during larval rearing results in increasing the frequency of antibiotic-resistant bacteria in aquaculture

systems. This is true in the present study and our observations agree with the previous work on the antibiotic-resistant bacteria isolated from *M. rosenbergii* larvae (Hameed *et al.* 2003). According to them 95% of the bacterial isolates were resistant to erythromycin and oxytetracycline. All the isolates of *V. alginolyticus,* obtained from the diseased necrotic larvae, were found to be resistant to ampicillin, streptomycin, erythromycin and oxytetracycline (Table 7), which indicated that their use in larval cultures might turn out to be little use. Moreover, if these resistance factors are carried in mobile genetic elements, they can spread rapidly within the bacterial population in the hatchery (Kerry *et al.* 1994). Moreover, *Vibrio spp* present in aquaculture settings may be transmitted to humans who come in contact with this ecosystem (Blake *et al.* 1979). For example, *V. cholerae, V. vulnificus* and *V. parahaemolyticus* have been associated with infections among persons working in aquaculture (Bisharat and Raz, 1996). Considering all the above negative impact in the larval production systems, environment and human health, antibiotic application in prawn hatcheries has to be discouraged either as therapy or prophylaxis. Instead, biological control methods such as application of antagonistic probiotics have to be resorted to for sustainable larval production.

Table 1. *Vibrio* sp isolated from *Macrobrachium rosenbergii* postlarvae (Regional Shrimp Hatchery, Azhikode, Kerala)

Code	Genus/ Species	Number of strains
MRCS 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39	*V. cholerae*	12
MRCS 15, 18, 22, 32, 34, 36	*V. mediterranei*	6
MRCS 24, 28, 29, 30, 31, 33	*V. nereis*	6
MRCS 1, 6, 8, 9	*V. parahaemolyticus*	4
MRCS 3, 5	*V. splendidus* II	2
MRCS 4, 10	*V. proteolyticus*	2
MRCS 26	*V. fluvialis*	1
MRCS 2, 7	*Aeromonas* sp	2

Table 2. Relative frequency of heterotrophic bacteria recovered from larvae and water sample (M/S Rosen Fisheries, Trichur, Kerala)

Morphology	Number of colonies from larval system (10^4 dilution)	Number of colonies from water sample (10^3 dilution)	Group	% of occurrence (larval sample)	% of occurrence (water sample)
A	75	0	Vibrio	57.7	0
B	20	16	*Aeromonas*	15.4	12.3
C	3	9	*Pseudomonas*	2.3	6.9
D	3	33	*Alcaligenes*	2.3	25.4
E	22	66	*Cytophaga*	16.9	50.8
F	6	5	*Alteromonas*	4.6	3.8
G	1	0	*Bacillus*	0.8	0
H	0	1	*Micrococci*	0	0.8

Legend:

A – Small, circular, convex, entire, translucent colony
B – Moderate, circular, umbonate, entire, translucent colony
C – Moderate, circular, convex, entire, brown colony
D – Small, circular, convex, entire, pale white colony
E – Small, circular, convex, entire, yellow colony
F – Small, irregular, raised, undulate, white colony
G – Moderate, irregular, flat, rhizoidal, white colony
H – Small, circular, convex, entire, white colony

Table 2a. *Vibrio* sp isolated from necrotic *Macrobrachium rosenbergii* postlarvae (Rosen Fisheries, Trichur, Kerala)

Code	Species	Number of strains
MRNL 1, 2, 3, 4, 5, 6, 7, 8	*V. alginolyticus*	8

Table 3. Relative frequency of bacteria recovered from larval macerates on ZoBell's agar plates (10^4 dilution) (Prawn larval hatchery, Kollam, Kerala)

Group	Number of colonies	Percentage of occurrence
Vibrio	14	37.8
Pseudomonas	8	21.6
Alcaligenes	6	16.2
Enterobacteriaceae	9	24.3

Table 3a. ***Vibrio*** **sp isolated from** ***Macrobrachium rosenbergii*** **postlarvae (Prawn larval hatchery, Kollam, Kerala)**

Code	Species	Number of strains
MRQL 27	*V. mediterranei*	1
MRQL 5, 9, 10, 19, 20, 23, 33, 36	*V. vulnificus*	8
MRQL 13, 29, 32, 34, 35	*V. nereis*	5

Table 4. *Vibrio* and *Aeromonas* isolated from *Macrobrachium rosenbergii* larval rearing systems

Code	Genus/ Species	Source	Number of strains
MRCS 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39	*V. cholerae*	Postlarvae (Azhikode, Kerala)	12
MRCS 15, 18, 22, 32, 34, 36	*V. mediterranei*	Postlarvae (Azhikode, Kerala)	6
MRQL 27	*V. mediterranei*	Postlarvae (Kollam, Kerala)	1
MRQL 5, 9, 10, 19, 20, 23, 33, 36	*V. vulnificus*	Postlarvae (Kollam, Kerala)	8
MRCS 24, 28, 29, 30, 31, 33	*V. nereis*	Postlarvae (Azhikode, Kerala)	6
MRQL 13, 29, 32, 34, 35	*V. nereis*	Postlarvae (Kollam, Kerala)	5

Contd....

Contd....

Code	Genus/ Species	Source	Number of strains
MRCS 1, 6, 8, 9	*V. parahaemolyticus*	Postlarvae (Azhikode, Kerala)	4
MRCS 3, 5	*V. splendidus II*	Postlarvae (Azhikode, Kerala)	2
MRCS 4, 10	*V. proteolyticus*	Postlarvae (Azhikode, Kerala)	2
MRCS 26	*V. fluvialis*	Postlarvae (Azhikode, Kerala)	1
MRNL 1, 2, 3, 4, 5, 6, 7, 8	*V. alginolyticus*	Diseased, moribund larvae (Thrissur, Kerala)	8
MRCS 2, 7	*Aeromonas* sp.	Postlarvae (Azhikode, Kerala)	2
MRNL 9, 10, 16, 17	*Aeromonas* sp.	Diseased, moribund larvae (Thrissur, Kerala)	4

Table 5. Percentage mortality after 96 h of larvae of *M. rosenbergii* challenged with *V. alginolyticus* (MRNL 3)

	Control	10^6 cfu/ml	10^7 cfu/ml	10^8 cfu/ml
Number of dead larvae*	11±3	24±3	26±2	30±0
% mortality	37±10	80±10	87±6	100±0

* Mean and SD of 3 replicates

Table 6. Lipolytic, proteolytic, chitinolytic, and haemolytic activities of *Vibrio* isolates

Code	Lipase	Gelati-nase	Chiti-nase	Haemo-lytic	Isolate
MRCS 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39	+	+	+	–	*V. cholerae*
MRCS 15	+	+	–	+	*V. mediterranei*
MRCS 22, 32, 34, 36	+	+	+	+	"
MRQL 27	+	+	–	–	"
MRCS 18	+	+	+	–	"
MRQL 5, 33	+	+	+	+	*V. vulnificus*
MRQL 10	+	+	–	–	"
MRQL 9, 19, 20, 23, 36	+	+	+	–	"
MRCS 31, 33	+	+	+	+	*V. nereis*
MRCS 24 ; MRQL 35	+	+	–	+	"
MRCS 29, 30	+	+	+	–	"
MRCS 28; MRQL 13, 29, 32, 34	+	+	–	–	"
MRCS 1, 6, 8, 9	+	+	+	+	*V. parahaemolyticus*
MRCS 3, 5	+	–	–	–	*V. splendidus* II
MRCS 4, 10	+	+	+	–	*V. proteolyticus*
MRCS 26	+	+	+	–	*V. fluvialis*
MRNL 1, 2, 3, 4, 5, 6, 7, 8	+	+	+	+	*V. alginolyticus*
MRNL 9, 10, 16, 17	+	+	+	+	*Aeromonas* sp
MRCS 2, 7	+	+	+	+	*Aeromonas* sp

Table 7. *Vibrio* and *Aeromonas* isolates from *M. rosenbergii* larval rearing systems and their antibiotic resistance

Isolate	Total number of isolates	Code	Antibiotic resistance
V. cholerae	12	MRCS 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39	All (E); MRCS 19 (N); MRCS 20 (S,N,K); MRCS 35 (S)
V. nereis	11	MRCS 24, 28, 29, 30, 31, 33; MRQL 13, 29, 32, 34, 35	All (E); MRCS 28 (R, N, Cf); MRQL 13, 29, 32, 34, 35 (C,O)
V. vulnificus	8	MRQL 5, 9, 10, 19, 20, 23, 33, 36	All (E); MRQL 9 (O)
V. alginolyticus	8	MRNL 1, 2, 3, 4, 5, 6, 7, 8	All (E, A, S, O)
V. mediterranei	7	MRCS 15, 18, 22, 32, 34, 36; MRQL 27	All (E); MRCS 22, 32 (S); MRQL 27 (C, O)
V. parahaemolyticus	4	MRCS 1, 6, 8, 9	All (E, A); MRCS 1, 9 (O)
V. splendidus II	2	MRCS 3, 5	All (E, R, O, Nv, Nf); MRCS 3 (A); MRCS 5 (C, Fr)
V. proteolyticus	2	MRCS 4, 10	All (E, O)
V. fluvialis	1	MRCS 26	E, O
Aeromonas sp	6	MRNL 9, 10, 16, 17; MRCS 2, 7	All (E); MRNL 9 (R, C, O); MRNL 10, 16, 17 (A); MRCS 2, 7 (A); MRCS 7 (O)

Note:

* Letters in the parenthesis refers to the antibiotics

A– Ampicillin (10 mcg); S– Streptomycin (10 mcg); R– Rifampicin (2 mcg); N– Neomycin (30 mcg); E– Erythromycin (10 mcg); K– Kanamycin (30 mcg); C– Chloramphenicol (10 mcg); Cf– Ciprofloxacin (5 mcg); O– Oxytetracycline (30 mcg); Nv– Novobiocin (30 mcg); Fr– Furazolidone (50 mcg); Nf– Nitrofurantoin (100 mcg)

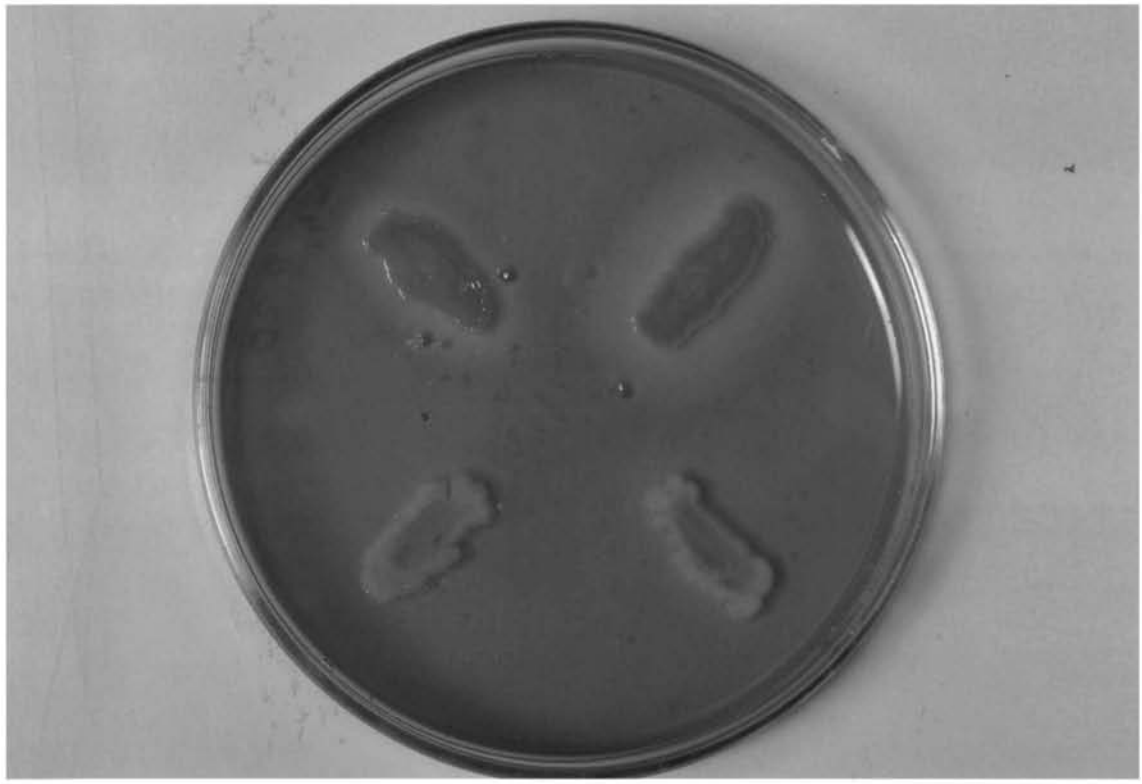


Fig. 1. Demonstration of haemolysis by *Vibrio* sp.
The clearing zone indicates the lysed haemocytes

CHAPTER - 3

DEVELOPMENT OF INDIRECT FLUORESCENT ANTIBODY TECHNIQUE (IFAT) BASED ON POLYVALENT *VIBRIO* ANTIGEN

3.1 Introduction

On several occasions, mortality in finfish and shellfishes has been associated with an increase in the *Vibrio* population (Sung *et al.* 2001). In prawns (non-penaeids), vibrios are known to be pathogenic and systemic bacterial infections and necrotic appendages due to *Vibrio* are common in hatcheries (Anderson *et al.* 1989; New, 1995). Among the different species of vibrios, *V. alginolyticus* has been isolated frequently from diseased prawns as the aetiologic agent and has been described as the principal pathogen of both penaeids and non-penaeids (Lightner, 1988; Baticados *et al.* 1990; Limsuwan, 1993; Felix and Devaraj, 1993; Mohney *et al.* 1994; Lee *et al.* 1996). The normal identification of pathogenic vibrios in the environment involves a four-step strategy: (i) collection of the samples, (ii) recovery of vibrios from the samples, (iii) identification of the vibrios recovered, and (iv) confirmation that they are pathogenic (Spira, 1984). Identification of *Vibrio* species that possess similar morphological, physiological, and biochemical characteristics still remains a problem because of the large number tests that are involved with low probability level.

Since the introduction of fluorescent antibody technique (FAT) by Coons *et al.* (1942), it became a very attractive technique due to the rapidity with which the detection of the specific bacterial pathogen could be achieved. FAT has been recognized as a sensitive and specific diagnostic method (Bullock *et al.* 1980; Laidler, 1980) and is widely used for the detection and identification of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease of salmonid fish (Fryer and Sanders, 1981).

dela Pena *et al.* (1993), developed an IFAT for the detection of *V. penaeicida* which shared a common antigens with 75 strains and with minimal cross reactivity with other *Vibrio* species. Both direct as well as indirect fluorescent antibody techniques (FAT and IFAT) are now available for the detection of plant pathogenic microbes and pathogens of veterinary and human importance.

However, no fluorescent antibody assay technique has ever been attempted for the rapid detection of vibrios and in particular, *V. alginolyticus* in the larval rearing system of *M. rosenbergii*. In order to meet this requirement, an indirect fluorescent antibody technique (IFAT) based on polyclonal antibodies raised against *V. alginolyticus* was designed for detection and monitoring the pathogen in *M. rosenbergii* larval rearing systems.

The advantage of polyclonal antibodies is that they are very much less expensive to generate. The disadvantage of monoclonal antibodies lies in the high cost of isolating the right clone and cloning the corresponding cell line. Meanwhile, polyclonal antibodies recognize multiple epitopes, making them more tolerant of small changes in the nature of the antigen. Therefore, they are often the preferred choice for detection of denatured proteins. Monoclonal antibodies react with only one epitope on the antigen, however, they are more vulnerable to the loss of epitope through chemical treatment of the antigen than are polyclonal antibodies. Polyclonal antibodies may be generated in a variety of species, including rabbit, goat, sheep, donkey, chicken and others, giving the users many options in experimental design (Harlow and Lane, 1988).

3.2 Materials and Methods

V. alginolyticus strain was selected from a total of 55 cultures (9 species of *Vibrio*) representing the family vibrionaceae which were subjected to identification to species based on Alsina and Blanch (1994), for the development of IFAT. The selection was based on the fact that the above *V. alginolyticus* strains were fresh isolates from diseased system and had caused severe larval mortality in our laboratory pathogenicity tests. Isolates of other *Vibrio* species and bacterial genera used in this study are given in Table 1. All isolates were grown either in ZoBell's agar or broth prepared in 15 ppt seawater and were incubated at 28±1°C unless otherwise indicated. *Escherichia coli* (ATCC 11775), *Salmonella typhi* (MTCC 531), *Pseudomonas aeruginosa* (MTCC 741), *Aeromonas hydrophila* (MTCC 646) and *V. cholerae* (MTCC 3906) were grown in nutrient agar or broth and incubated at 37°C.

3.2.1 Raising of antisera

Clinically healthy NewZealand white rabbits (4-6 months old) weighing approximately 1.5 kg, procured from College of Veterinary and Animal Husbandry, Kerala Agriculture University, Mannuthy, Thrissur, were used for raising antiserum. They were housed in stainless steel cages and kept in a well-ventilated animal room. They were provided with pelleted feed and drinking water *ad libitum*.

Prior to immunisation, the rabbits were bled through marginal ear vein and the serum was tested for the presence of anti *Vibrio* antibodies by slide agglutination technique. This serum served as control. The rabbits were acclimatised for two weeks before immunisation. Fur was removed using fine scissors at injection sites a day before injection. Preparation of the antigen was as follows: 18 h broth culture of an isolate of *V. alginolyticus* (MRNL 3) was harvested by centrifuging (6000rpm for 10 min) and killed with 0.4% formalin in 0.85% saline. After overnight incubation at 28±1°C, cells were washed thrice in phosphate buffer saline (PBS) containing NaCl 8g; KCl 0.2g; Na_2HPO_4 1.15g; KH_2PO_4 0.2g; double distilled water 1000 ml, pH 7.4. The above washed cells

were adjusted to OD 1 at A_{600nm} using PBS to get ~ 10^9 cfu/ml. A 1:1 mixture of cell suspension and Complete Freund's Adjuvant (Bangalore Genei, India) was mixed thoroughly and the rabbits were injected 1.0 ml intradermally (maximum of 0.1 ml at a site). Freund's Incomplete Adjuvant (FIA) was used for subsequent antigen boosts given intramuscularly. The immunisation schedule was followed as given in the Table 2.

3.2.2 Blood collection

One week after the last injection of immunisation schedule, the rabbits were bled through marginal ear vein. For this, the collection site was shaved, cleaned with 70% alcohol and washed with sterile distilled water before attempting to make a cut. At the end of the procedures it was made sure that all the bleeding had stopped and the site of withdrawal was treated with an antibiotic spray. Blood (~ 10ml) was collected in a single bleed in clean sterile screw cap tubes and maintained in slanting position for 2 hours at room temperature. Then the above was maintained at 4°C overnight. Serum was collected in Eppendorf tubes, centrifuged at 3000rpm for 10 min at 4°C to remove RBC and other particulates. The supernatant was collected in fresh sterile Eppendorf tubes. A portion of the antiserum was purified using the IgG purification kit (Bangalore Genei, India) as per the protocol given by the manufacturer. Both un-purified as well as purified antisera were maintained at -20°C until further use.

3.2.3 Antiserum titre

The agglutination titre of both the un-purified and purified antisera was determined by slide agglutination method. The overnight grown culture of *Vibrio alginolyticus* from ZoBell's agar plates were harvested using PBS and adjusted to OD 1.0 at A_{600nm} to get a cell count of ~10^9 cfu/ml. Three separate drops (30 µl each) of above culture suspension was placed on a clean glass slide. To one of the suspensions a drop (30 µl) of control serum was added and mixed. To the other two suspensions one drop (30 µl) each of antisera (un-purified and purified) separately diluted to the concentrations 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048 1:4096 and 1:8192

were added and mixed. The slides were rocked gently for one to two minutes and observed visually and then confirmed under low power microscope (10X objective) for agglutination. The titre of the antiserum was expressed as the reciprocal of the last dilution, which showed agglutination.

3.2.4 Testing for cross-reactions

A series of cross-reaction tests was done to verify the specificity of the antiserum by slide agglutination method. Strains of *Vibrio* used in the tests were *V. cholerae* (12 isolates), *V. nereis* (11), *V. vulnificus* (8), *V. alginolyticus* (8), *V. mediterranei* (7), *V. parahaemolyticus* (4), *V. splendidus* II (2), *V. proteolyticus* (2), *V. fluvialis* (1) *V. cholerae* (MTCC 3906) and *V. harveyi* (3). Cross-reactivity was also checked for other genera like *Aeromonas* sp. (6 isolates), *Bacillus* sp. (1), *Pseudomonas* sp. (2), *Micrococcus* sp. (1), *A. hydrophila* (MTCC 646), *P. aeruginosa* (MTCC 741), *E. coli* (ATCC 11775) and *Salmonella typhii* (MTCC 531).

3.2.5 Indirect Fluorescent Antibody Technique (IFAT)

Diluted un-purified (1:2 to1:4096) and purified antisera (1:2 to 1:32), obtained from immunised rabbits, were used for IFAT. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Sigma, US), diluted to a final concentration of 1:20 with PBS, was used to react with the anti-vibrio test sera.

One milliliter suspension of *V. alginolyticus* (~10^2 cells) was filtered through a black polycarbonate filter for epifluorescence (Millipore, US) using syringe and placed on a clean glass slide. A 20 µl of anti-vibrio test serum (primary antibody) was dropped on each membrane filter, which had been placed in a moist chamber (in a petriplate with water-soaked cotton to maintain humidity). Each filter was incubated for 30 min and then gently rinsed twice with 20 ml of PBS (7.4 pH) in a petriplate and transferred on to a new slide. A 20 µl aliquot of secondary antibody (FITC conjugated anti-rabbit antibody, Sigma, US) was spread on the filter and incubated under dark for 30 min. The filters were

gently rinsed twice with 20 ml of PBS in a petriplate and transferred on to a new slide. One drop of immersion oil was placed on to the filter and covered with coverslip.

Filters were observed at X 1,000 magnification by using an Olympus microscope (Model C X 41) equipped with a 50-W mercury lamp, a blue excitation filter (460 to 485 nm) and a yellow-green fluorescence barrier filter (520 to 560 nm). The fluorescent cells were counted on a minimum of 20 fields per slide. If no fluorescent cells were observed on 20 fields, a total of 50 fields were examined to establish that a result was negative. The above IFAT procedure was followed for other species of *Vibrio* including *V. cholerae* (12 isolates), *V. nereis* (11), *V. vulnificus* (8), *V. alginolyticus* (8), *V. mediterranei* (7), *V. parahaemolyticus* (4), *V. splendidus* II (2), *V. proteolyticus* (2), *V. fluvialis* (1), *V. cholerae* (MTCC 3906) and *V. harveyi* (3). Cross-reactivity was also checked by IFAT for other genera like *Aeromonas* sp. (6 isolates), *Bacillus* sp. (1), *Pseudomonas* sp. (2), *Micrococcus* sp. (1), *A. hydrophila* (MTCC 646), *P. aeruginosa* (MTCC 741), *E. coli* (ATCC 11775) and *Salmonella typhii* (MTCC 531).

3.3 Results and Discussion

The homologous agglutination titre of the un-purified antiserum raised against *V. alginolyticus* was found to be 4096 by the slide agglutination method whereas the antibody titre of the purified antiserum was 32. This suggested that the antibody titre declined on purification. Results of cross-reactivity of the antiserum to other bacterial isolates are tabulated in Table 3. The undiluted un-purified antiserum was found to react with all the isolates of *V. alginolyticus* and *V. parahaemolyticus* whereas the diluted antiserum (1:128) with PBS did not react with *V. parahaemolyticus*. Meanwhile, elimination of cross-reactions of the purified antiserum was obtained when it was diluted by 32 times. Cross-reactions of both un-purified and purified antiserum with other genera like *Aeromonas*, *Pseudomonas*, *E. coli*, *Micrococcus*, *Bacillus* and *Salmonella* tested, were not observed. The observed cross-reaction with *V. parahaemolyticus* may arise from the fact that, they share common antigens, and can be avoided by diluting the antiserum. Non cross-reactions of antiserum to other genera explain it's antigenic specificity unique to the bacterial species in question.

Un-purified and purified antiserum, obtained from immunised rabbits, diluted to 1:128 and 1:32 respectively with PBS was found to give good fluorescence in IFAT when compared to other dilutions (Fig. 1). The results of IFAT with the bacterial isolates are tabulated in the Table 3. It showed that only the *V. alginolyticus* strains could be detected by IFA technique. Rapid identification of pathogens is crucial for effective disease control in aquaculture. Detection of pathogens is important not only in infected prawn (clinically and sub-clinically), but also in the environment eg. between harvesting and re-stocking, and as an 'early warning system'. Fluorescent-antibody techniques have previously been used successfully to detect various allochthonous and autochthonous bacterial species in the environment (Brayton *et al.* 1987; Muyzer *et al.* 1987; Howgrave-Graham and Steyn, 1988). dela Pena *et al.* (1993), had developed an IFAT for the detection of *V. penaeicida* which shared a common antigens with 75 strains and with minimal cross reactivity with other *Vibrio* species and isolates that were pathogenic to fish.

Very less number of groups of workers has ever been involved in studying the microbial involvement in the unsuccessful completion of the larval cycle of *M. rosenbergii* in India. The documented literatures are from Singh (1990), Bhat and Singh (1998), Bhat *et al.* (1998), and Vici *et al.* (2000). Even now for the detection of pathogenic vibrios, standard culture methods based on general as well as selective media are followed. However, for the detection of pathogenic marine vibrios such as *V. parahaemolyticus, V. holisae* and *V. cholerae*, Karunasagar *et al.* (1994) used a PCR based technique, but specifically oriented towards seafood industry. It has to be emphasized at this point that no IFAT has ever been attempted for the rapid detection of vibrios in the larval rearing system of prawns.

As vibriosis is one of the major diseases in prawn hatcheries, rapid and specific identification of the pathogen happens to be mandatory for successful seed production. It has to be pointed out that the routine microbiological assay for diagnosis needs three working days. Meanwhile, the rapid methods such as DNA hybridization, immunomagnetic separation and polymerase chain reactions are relatively expensive apart from the requirement of sophisticated infrastructure and highly trained personnel. The recurring cost becomes exorbitant which such techniques are adapted in large-scale commercial operations involving the analysis of very large number of samples. In this context the fluorescent antibody technique (FAT) by Coons *et al.* (1942), offers an attractive alternative method for the rapid, sensitive and specific detection of bacterial pathogen (Bullock *et al.* 1980; Laidler, 1980). Application of FAT and IFAT has been reported for bacteria such as *Aeromonas hydrophila* (Kawahara *et al.* 1987), *Aeromonas solmonicida* (Sakai *et al.* 1986), *Renibacterium salmoninarum* (Laidler, 1980; Lee and Gordon, 1987), *Yersinea ruckeri* (Johnson *et al.* 1974). Lallier *et al.* (1990), developed both ELISA and IFAT for diagnosis of furunculosis, bacterial kidney disease and vibriosis and evaluated the method using 29 different bacterial strains. Huq *et al.* (1990), used a fluorescent monoclonal antibody technique for the detection of *Vibrio cholerae* O1 in the aquatic environment and compared it with the culture methods and observed that the FAT was very much superior in enumerating the specific pathogen in water. La Patra *et al.* (1989), used FAT for rapid diagnosis of infectious hematopoietic necrosis. Hung

and Ling (1996), could localize *Vibrio* antigen using IFAT which was delivered by immersion to tiger shrimp (*Penaeus monodon*) and the method was very sensitive to demonstrate the antigen as it was absorbed through the digestive- circulatory system. Kitao and Kimura (1974), developed a method for rapid diagnosis of pseudotuberculosis in yellow tail by means of FAT. The importance of FAT in detecting specific bacterial strains directly from water-sediment systems without involving a standard culture practice was demonstrated very much by Voytek *et al.* (1998), when they worked out the abundance of ammonium oxidizing bacteria in Lake Bonney, Antartica by immunofluorescence, PCR and in situ hybridization. Through these techniques, distribution and relative abundance of nitrifying bacteria could be assessed, and especially IFAT was extremely useful. The fluorescent-monoclonal antibody staining procedure has been used successfully for direct detection of viable but not culturable *V. cholerae* O1 in environmental water samples. Results of such studies showed the presence of higher *V. cholerae* O1 counts than could be obtained by conventional culturing methods (Brayton *et al.* 1987; Xu *et al.* 1984).

The fluorescent assay technique has been shown previously to be useful in detecting viable but nonculturable *V. cholerae* O1 in water samples (Tamplin *et al.* 1990). Results of several studies have shown that various biological and physicochemical factors influence the growth, survival, and distribution of *V. cholerae* in aquatic environments (Feachem *et al.* 1981; Miller *et al.* 1984). However, the non-culturable but viable forms of *V. cholerae* pose a problem for the accuracy of enumeration of these organisms in the natural environment if only the conventional culture methods are employed.

Venkateswaran *et al.* (1989), reported that the incidence of *V. cholerae* non-O1 was below detectable levels in zooplankton populations. By combining the FA technique and culture methods, those factors involved in maintaining the viability and survival of *Vibrio cholerae* in aquatic environments can be elucidated.

Direct FA staining, coupled with microscopic observation, makes it possible even to detect viable but non-culturable *Vibrio* where all conventional methods of culturing

fail. Hence, the IFAT developed in this study is a viable alternative method in a commercial framework.

Because the antibody titre of the unpurified serum was 1:128 without loosing sensitivity to a cell number of 10^2 cfu/ml, while purification the titre declined to 1:32 without offering any specific advantage to the final results. Therefore, it is resqlved to utilize the antiserum without purification. This approach facilitates substantial reduction in the cost much required to extend to commercial hatchery systems. Steps are underway to make antiserum available commercially to hatcheries to incorporate in the detection of *V. alginolyticus* post isolation on thio sulphate citrate bile sucrose agar (TCBS) where it grows as yellow colonies. The yellow colonies can be picked up and subjected for the analysis as described. This will yield results on the abundance of the pathogen in terms of CFU.

The fluorescent assay can be used as a rapid test for the confirmation of the diagnosis vibriosis by *V. alginolyticus* even directly from the rearing water thus assisting hatchery technicians in managing the disease. The other applications of this technique are as an inexpensive test, it could replace more expansive culture methods and as an aid of surveillance, it can assist epidemiologists for detecting *V. alginolyticus* in areas where bacteriological laboratories are not available. Finally, as a simple test, it can provide confirmation of *V. alginolyticus* even without fully trained microbiology technicians.

Table 1. Bacterial isolates used in this study

Isolate	Total number of isolates	Code
V. cholerae	12	MRCS 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39
V. nereis	11	MRCS 24, 28, 29, 30, 31, 33; MRQL 13, 29, 32, 34, 35
V. vulnificus	8	MRQL 5, 9, 10, 19, 20, 23, 33, 36
V. alginolyticus	8	MRNL 1, 2, 4, 3, 5, 6, 7, 8
V. mediterranei	7	MRCS 15, 18, 22, 32, 34, 36; MRQL 27
V. parahaemolyticus	4	MRCS 1, 6, 8, 9
V. splendidus II	2	MRCS 3, 5
V. proteolyticus	2	MRCS 4, 10
V. fluvialis	1	MRCS 26
V. harveyi	3	MCCB 6, 12, 24
Aeromonas sp	6	MRCS 1, 2; MRNL 9, 10, 16, 17
A. hydrophila	1	MTCC 646
Bacillus sp	1	MCCB 101
Pseudomonas sp	2	MCCB 103
P. aeruginosa	1	MTCC 741
Micrococcus sp	1	MCCB 104
E. coli	1	ATCC 11775
Salmonella typhi	1	MTCC 531
V. cholerae	1	MTCC 3906

Note:
MRCS- Isolates from Regional Prawn Hatchery, Azhikode, Kerala.
MRNL- Isolates from M/S Rosen Fisheries, Trichur, Kerala.
MRQL- Isolates from Matsyafed Hatchery, Quilon, Kerala.
MTCC- Microbial type culture collection, IMTECH, Chandigarh
ATCC- American type culture collection, US
MCCB- Microbial culture collection, CFDDM, CUSAT

Table 2. Schedule of immunisation

Day	Quantity of antigen ($\sim 10^9$ cells/ml)	Adjuvant	Route of injection
0	1 ml	1 ml of Freund's Complete Adjuvant	Intra-dermal
7	0.5 ml	0.5 ml of Freund's Incomplete Adjuvant	Intra-muscular
14	0.5 ml	0.5 ml of Freund's Incomplete Adjuvant	Intra-muscular
21	0.5 ml	0.5 ml of Freund's Incomplete Adjuvant	Intra-muscular
28	Bleeding		

Table 3. Bacterial strains used in cross-screening of polyclonal antibodies produced against *V. alginolyticus*

Isolate	Total number of isolates	Code	Slide agglutination		IFAT (1:100 diluted antiserum)
			Undiluted antiserum	1:100 diluted antiserum	
V. cholerae	12	MRCS 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39	–	–	–
V. nereis	11	MRCS 24, 28, 29, 30, 31, 33; MRQL 13, 29, 32, 34, 35	–	–	–
V. vulnificus	8	MRQL 5, 9, 10, 19, 20, 23, 33, 36	–	–	–
V. alginolyticus	8	MRNL 1, 2, 3, 4, 5, 6, 7, 8	+	+	+
V. mediterranei	7	MRCS 15, 18, 22, 32, 34, 36; MRQL 27	–	–	–
V. parahaemolyticus	4	MRCS 1, 6, 8, 9	+	–	–
V. splendidus II	2	MRCS 3, 5	–	–	–
V. proteolyticus	2	MRCS 4, 10	–	–	–
V. fluvialis	1	MRCS 26	–	–	–
V. harveyi	3	LB 6, 12, 24	–	–	–
Aeromonas sp	6	MRCS 1, 2; MRNL 9, 10, 16, 17	–	–	–
A. hydrophila	1	MTCC 646	–	–	–
Bacillus sp	1	MCCB 101	–	–	–
Pseudomonas sp	2	PS-1, MCCB 103	–	–	–
P. aeruginosa	1	MTCC 741	–	–	–
Micrococcus sp	1	MCCB 104	–	–	–
E. coli	1	ATCC 11775	–	–	–
Salmonella typhii	1	MTCC 531	–	–	–
V. cholerae	1	MTCC 3906	–	–	–

Note:

MRCS- Isolates from Regional Prawn Hatchery, Azhikode, Kerala.
MRNL- Isolates from M/S Rosen Fisheries, Trichur, Kerala.
MRQL- Isolates from Matsyafed Hatchery, Quilon, Kerala.
MTCC- Microbial type culture collection, IMTECH, Chandigarh
ATCC- American type culture collection
MCCB- Microbial culture collection bacteria, CFDDM, CUSAT

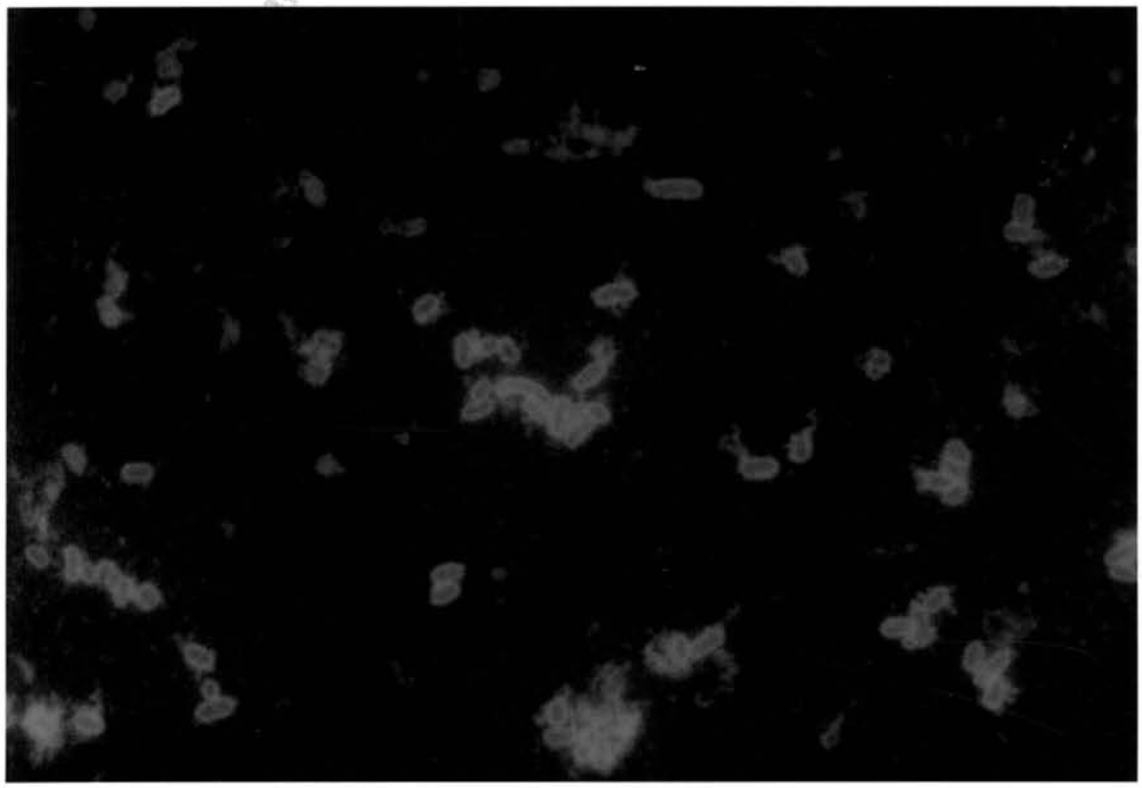


Fig. 1. Brightly fluoresced cells of *Vibrio alginolyticus* observed under fluorescent microscope (objective 100X)

CHAPTER - 4

DEVELOPMENT OF ANTAGONISTIC BACTERIAL SYSTEMS AS VIBRIOSTATIC AND VIBRIOCIDAL AGENTS IN THE LARVAL REARING SYSTEM OF *MACROBRACHIUM ROSENBERGII*

4.1 Introduction

Among heterotrophic bacteria associated with larviculture and grow-out phases of crustaceans, *Vibrio* is a dominant genus responsible for much of the observed mortality (New, 1995; Sung *et al.* 1999). Vibriosis has been a major hindrance to prawn culture in India and in other prawn farming countries (Baticados *et al.* 1990; Singh, 1990; Lightner, 1996). To control vibrios, prophylactic and therapeutic use of antibiotics has been the choice in commercial hatcheries however leading to resistance and its possible spread in the environment (Weston, 1996; Hameed *et al.* 2003).

As an alternate management measure the introduction of selected bacterial cultures/ products as probiotics with antagonistic properties has been proposed and applied (Gomez-Gil *et al.* 2000). Several bacterial isolates which are common members of non-pathogenic microflora of fish and shellfish culture systems have been shown to inhibit fish and prawn pathogens by *in vitro* assay. This has been demonstrated for lactic acid bacteria (Gatesoupe, 1994), *Carnobacterium* (Robertson *et al.* 2000), *Bacillus* (Rengpipat *et al.* 2000), *Vibrio* (Austin *et al.* 1995), *Planococcus* (Austin and Billaud, 1990) and *Pseudomonas* (Chythanya *et al.* 2002). Gomez-Gil (1995) and Verschuere *et al.* (2000), claimed that certain strains of bacteria associated with *Artemia* and prawn culture systems have the capability to control pathogens by means of competitive

exclusion or by the production of inhibitory compounds. The antibacterial effect may be due to production of antibiotics (Williams and Vickers, 1986), bacteriocins (Vandenbergh, 1993), hydrogen peroxide or alteration of pH by producing organic acids (Sugita *et al.* 1997). Among antagonistic bacterial cultures known to be useful in aquaculture, *Micrococcus* has not been recognized so far, even though its presence has been documented by Austin and Allen (1982) and Prieto *et al.* (1987) in dehydrated *Artemia* cysts, cyst-hatching water, and *Artemia salina*. Lalitha and Surendran (2004), reported *Micrococcus* in the environment of the farmed freshwater prawn *M. rosenbergii*.

Fluorescent pseudomonads have been used as biocontrol agents in several rhizophere studies (O'Sullivan *et al.* 1992) where their inhibitory activity has been attributed to a number of factors, such as the production of antibiotics, hydrogen cyanide (Westerdahl *et al.* 1991), or iron-chelating siderophores (Loper and Buyer, 1991). As with their terrestrial counterparts, aquatic pseudomonads are often antagonistic against other microorganisms (Lemos *et al.* 1985; Gram, 1993), including fish pathogenic bacteria (Smith and Davey, 1993) and fungi (Bly *et al.* 1997). Gram *et al.* (1999), demonstrated the protection of rainbow trout administered with *P. fluorescens* AH2 when challenged with *V. anguillarum*. Another study by Smith and Davey (1993), demonstrated that bathing Atlantic salmon in a suspension of *P. fluorescens* reduced subsequent mortality from stress-induced furunculosis. Chythanya *et al.* (2002), has reported inhibition of shrimp pathogenic vibrios by an estuarine strain of *Pseudomonas*.

In the present work, a large collection of heterotrophic bacteria from different environments was screened for antagonistic property against *Vibrio* spp. isolated from prawn larval rearing systems. The most promising bacteria were segregated and characterised. The *in vitro* antagonistic properties of the selected antagonistic isolates were evaluated using disc diffusion assay as well as by co-culture methods. Pathogenicity of selected antagonistic bacterial isolates towards *M. rosenbergii* larvae in a bioassay system was also evaluated in order to explore its potential use as 'probiotic with antagonism' for controlling vibrios in prawn larviculture.

4.2 Materials and Methods

4.2.1 Isolation of heterotrophic bacteria

To obtain efficient antagonistic bacteria the following protocol was used. The primary isolation was a general screening of heterotrophic bacteria from various samples as given in the Table 1. For isolation of bacteria from sediment samples, one gram of sediment was weighed and mixed thoroughly in 100 ml of diluents. One ml from the above suspension was serially diluted and plated on ZoBell's agar. Tissue samples from fishes, prawns and shrimps were homogenized in a tissue homogenizer followed by serial dilution and plating. Water samples were serially diluted and plated. All the inoculated plates were incubated at 28±1°C for a week. Bacterial colonies were randomly selected from the plates and maintained as stock cultures for further tests.

4.2.2 Primary screening of environmental bacterial isolates for antagonism

Primary screening for antagonistic property with the heterotrophic bacteria (812 isolates) which were initially isolated and maintained as stock cultures were screened against 9 species of vibrios (representatives of the total number of 55 strains) and 1 *Aeromonas* species (representative of 6 strains) which were previously isolated, characterized and maintained as stock cultures. The antagonistic property was tested by disc diffusion method.

4.2.2.1 Disc diffusion method

The heterotrophic bacterial isolates were inoculated in ZoBell's broth (15 ml aliquots) and incubated at 28±1°C for 7 days. Vibrios were grown overnight in ZoBell's broth and swabbed on ZoBell's agar plates. 5 mm discs punched from Whatman filter paper No. 1 and autoclaved and dried in hot air oven at 80°C for overnight were dipped in the 7 days incubated heterotrophic cultures and placed on the *Vibrio* swabbed plates. The

plates were incubated at 28±1°C for 48 h and observed for clearing zone. The formation of clearing zone around the discs was considered the positive indication of the antagonistic activity. Isolates, which gave even faint clearing zone, were also segregated.

4.2.3 Secondary screening

Secondary screening of the selected antagonistic bacteria were carried out against all isolates of target *Vibrio* (55 isolates, comprising 9 species) and *Aeromonas* (6 isolates) in order to confirm the antagonistic potential of the primarily screened organisms following the procedure described above. In this step, only those, which gave very good and wide clearing zone, were selected for further experiments.

From the above screening, the minimum number of antagonistic bacteria required for the total inhibition of the pathogens was segregated. The segregated bacterial isolates SAB 273, 274, 301, 303 and 306 were identified based on biochemical tests given in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). For the Gram-negative isolate (SAB 306), the biochemical tests carried out were listed in Table 2 and for the Gram-positive the biochemical tests carried out were given in Table 3. The media compositions of all the biochemical tests were as given in chapter 2 (section 2.3.3). The compositions of three otherwise done are given below.

Starch hydrolysis

Nutrient agar with the following composition was used as basal medium for demonstrating amylase production.

Ingredients	Amount g/L
Peptone	5.0
Beef extract	5.0
Yeast extract	1.0
NaCl	15.0

Soluble starch	5.0
Agar	20.0
pH	7.0±0.2

The test medium was prepared according to the above composition, autoclaved at 15 lbs for 15 minutes and poured into tubes. The plates containing the medium was spot inoculated with the test organism, and the plates were incubated until good growth was obtained at 28±1°C for 24-72 h. The tubes were flooded with Gram's iodine solution prepared in the following manner.

Ingredients	Amount
Iodine	1.0g
KI	2.0g
Distilled water	100 ml

Amylase producing or starch utilizing organisms showed a halo zone around and beneath them. The colour of the zones dependent on the degree of hydrolysis of the starch, when it was hydrolyzed to the stage of dextrin, then the zones were reddish brown, and when the breakdown had gone further, they were colourless.

Reduction of nitrate

Tests for reduction of nitrate were carried out by inoculating organisms into suitable nutrient broth medium containing 0.1% (w/v) KNO_3. The cultures were incubated until good growth was obtained during which time a sample was examined periodically to detect whether reduction of nitrate has occurred and to determine the stage at which it takes place.

Composition of nutrient broth per litre

Peptone : 5g
Beef extract : 5g
Yeast extract : 1g
KNO_3 : 1g
pH : 7.0 ± 0.2

Autoclaved at 15lbs for 15 min in 5ml aliquots, inoculated with the test culture and incubated at 28±1°C for 48h.

Preparation of reagents

Solution A: Sulphanilic acid : 1g
5N glacial acetic acid : 100ml

Solution B: Dimethyl alpha-naphthylamine : 0.6g
5N glacial acetic acid : 100ml

The presence of nitrite could be determined by adding to 5ml of the culture 0.5ml of reagent A, followed by 0.5ml of reagent B. the development of a red colour indicated that the nitrate has been reduced to nitrite. When the nitrite test was negative, the presence of residual nitrate could be shown by adding approximately 1mg of zinc dust per ml of the culture. The development of red colour indicated that the nitrate had not been reduced by the organisms.

The production of nitrogen gas was detected by incorporating inverted Durham's tubes into the liquid medium.

Catalase test

The principle of this test is that when organisms containing catalase are mixed with hydrogen peroxide, gaseous oxygen is released. The test organisms are grown on a slope of nutrient agar. A thick smear of the test organism was made from a 24h culture on a clean glass slide and a drop of hydrogen peroxide is placed on it. Immediate formation of gas bubbles indicated the liberation of oxygen and positive catalase test.

In total five cultures were segregated based on the maximum antagonistic spectrum and were identified as *Micrococcus* sp. (SAB 273, 274, 301 and 303) and a *Pseudomonas* sp (SAB 306). One isolate of *Micrococcus* sp (SAB 274 relabeled as MCCB104) from the above four cultures was taken randomly for subjecting to further experiments along with the *Pseudomonas* isolate (SAB 306 relabeled as MCCB103).

4.2.4 Antagonism assay of cell-free culture supernatant

Micrococcus MCCB104 and *Pseudomonas* MCCB103 were grown separately in ZoBell's broth for 5 days on a shaker (90 rpm) at room temperature (approx 28°C). The cells were pelleted by centrifugation (10,000g, 10 min), the pH of the supernatant adjusted to 7.0 and then passed through 0.2 μm membrane filters (Sartorius, Germany). Inhibitory activity on the target bacterial cultures was detected by disc diffusion method as described earlier and the zone of inhibition around the discs were recorded after 24 h using a HiAntibiotic ZoneScale (Himedia, India).

4.2.5 Time course of growth and production of the antagonistic substance

Commencement of the antagonistic substance production, its peak activity and duration of sustained activity were determined during the growth cycle of *Micrococcus* MCCB104 and *Pseudomonas* MCCB103. The culture was inoculated to an absorbance of 0.01 at 600 nm (approx 10^3 cfu/ml) in 100 ml ZoBell's broth (pH 7.0) and incubated on a magnetic stirrer at room temperature (approx 28°C). Starting at zero hour and then at

periodic intervals, aliquots of 2 ml culture were drawn aseptically of which 1 ml was used for monitoring growth (A_{600nm}) and 1 ml centrifuged at 10,000 x g at 4°C for 10 min, to pellet the cells. The supernatant was filtered and the antagonistic activity tested against an isoalte of *V. alginolyticus* (MRNL 3), in triplicate, as mentioned earlier. The plates were incubated at 28±1°C for 24 h and observed for zones of clearing around the discs. This was continued until the culture entered the decline phase. Antagonistic activity was quantitatively expressed in terms of diameter (mm) of the zone of inhibition around the discs.

4.2.6 Effect of pH, temperature and NaCl on growth

The influence of pH, temperature and NaCl concentration, on growth of *Micrococcus* MCCB104 and *Pseudomonas* MCCB103 was studied. Sterilized nutrient broth supplemented with 1.5% (w/v) NaCl, pH adjusted to 4, 5, 6, 7, 8, 9, 10 and 11 was inoculated with 0.1 ml overnight culture and incubated at 28±1°C for 24 h. Similarly, nutrient broth (pH 7, NaCl 1.5% w/v) was inoculated as described above and incubated at 4°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45°C for 24 h. Influence of different concentrations of sodium chloride was assessed by observing growth at 28±1°C in 1% tryptone broth (pH 7) containing 0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5% NaCl (w/v).

4.2.7 Co-culture experiments

Co-culture experiment with *Pseudomonas* was carried out following the method of Gram *et al.* (1999). Cultures used were the antagonistic *Pseudomonas* MCCB103 and an isolate of *V. alginolyticus* (MRNL 3). They were precultured separately in ZoBell's broth at 28±1°C on a shaker overnight. From the above cultures, *V. alginolyticus* was inoculated into 100 ml ZoBell's broth in four conical flasks (250 ml volume) to get an initial cell count of about 10^3 cfu/ml, whereas the cell counts of *Pseudomonas* MCCB103 in those flasks were initially adjusted to 10^5, 10^6, 10^7 and 10^8 cfu/ml respectively. All

combinations were maintained in duplicate. The flasks were incubated at room temperature (28±1°C) on a shaker (100 rpm), and samples (1 ml) were withdrawn at 24 h intervals for determination of cell count. Counts of *V. alginolyticus* were estimated by using H & L medium (Hugh and Leifson, 1953). Tubes containing 4 ml of H & L medium were inoculated with 1 ml aliquots of serially diluted culture and were covered with liquid paraffin and incubated at 28±1°C. The fermentative growth of *V. alginolyticus* caused a change in the pH indicator of the medium. The highest dilution which showed growth was used to calculate the count of *V. alginolyticus* in the sample following Gram *et al.* (1999). The cell counts of *Pseudomonas* MCCB103 was regularly monitored on *Pseudomonas* isolation agar (Hi media, Bombay, India) by spread plate method.

Co-culture experiment between *Micrococcus* MCCB104 and an isolate of *V. alginolyticus* (MRNL 3) were carried out as follows. *Micrococcus* and *V. alginolyticus* were precultured separately in ZoBell's broth at 28±1°C on a shaker (100 rpm) overnight. From the above, *V. alginolyticus* was inoculated into 100 ml ZoBell's broth in conical flasks (250 ml volume) to provide an initial cell density of about 10^3 cfu/ml, whereas the initial levels of *Micrococcus* MCCB104 were 10^5, 10^6, 10^7 and 10^8 cfu/ml respectively. All combinations were in duplicate. The flasks were incubated at 28±1°C on a shaker (100 rpm), and samples (1 ml) were withdrawn daily for *V. alginolyticus* count. The cell count was taken by spread plating 0.2 ml aliquots of serially diluted broth suspensions on thiosulphate citrate bile salts sucrose agar (TCBS agar) (Himedia, India) plates. The plates were incubated at 28±1°C for 2 days and colonies were counted and expressed as CFU of *V. alginolyticus* in the co-culture. This procedure was specifically chosen because *Micrococcus* MCCB104 failed to grow on TCBS agar plates.

4.2.8 Preliminary characterization of the antagonistic substance

The extracellular product having the antagonistic property was evaluated for the presence of bacteriocin, lipid, carbohydrate, hydrogen peroxide, acid and alkali by disc diffusion method as described earlier. To examine the presence of bacteriocin, the culture supernatant of *Micrococcus* MCCB104 and *Pseudomonas* MCCB103, filtered through

0.2 μm membrane filter (Sartorius, Germany) and neutralized to pH 7 was incubated at 37°C for 1 h with the proteolytic enzymes proteinase K (1 mg/ ml), pronase E (2 mg/ ml), α-chymotrypsin (5 mg/ ml), trypsin (50 mg/ ml) and lysozyme (1 mg/ ml). For lipids and carbohydrates, the cell free supernatant was treated with lipase (1 mg/ ml) and α-amylase (1 mg/ ml); and for hydrogen peroxide the cell-free supernatant was treated with catalase (2 mg/ ml). To examine alkali and acid production, the supernatant was neutralized to pH 7.0 with the addition of 1 M HCl or 1 M NaOH. Heat sensitivity of the inhibitory substance was tested by heating the cell-free supernatant in a waterbath for 30 min at 60°C and 80°C, and autoclaving for 15 min at 121°C. Each treated and untreated supernatant was tested for activity against an isolate of *V. alginolyticus* and the presence of an inhibitory zone around the discs was determined after incubation for 24 h at 28±1°C.

4.2.9 Pathogenicity of *Micrococcus* MCCB104 and *Pseudomonas MCCB103* against *M. rosenbergii* larvae

Pathogenicity of *Micrococcus* MCCB104 and *Pseudomonas* MCCB103 was assessed on larvae of *M. rosenbergii* (stage PL 1) brought from a commercial hatchery in Kerala. They were maintained for three days in 10 ppt seawater and were fed with sterile lab-made egg custard. Apparently healthy larvae were then distributed 50 each in 15 L fiberglass tanks containing 10 L 10 ppt autoclaved seawater. *Micrococcus* MCCB104 and *Pseudomonas* MCCB103 cultures grown for 24 h was scraped from the surface of ZoBell's agar plates into sterile saline and centrifuged at 3000*g* for 10 min. The pellet was resuspended in fresh saline and adjusted to an A_{600nm} value of 1.0 (corresponding to 10^9 cfu/ml). The above suspension was added to the larval rearing water to give a final cell count of 10^8 cfu/ml. The control tank did not have any bacterial inoculum supplemented. The above experiment was done in triplicate. Larvae were monitored for four days for mortality.

4.2.10 LD_{50} of *Pseudomonas* MCCB103 and *Micrococcus* MCCB104 in mice

Male BALB/c mice (12 weeks of age, 20±2 g weight) were brought from College of Veterinary and Animal Husbandry, Mannuthy, Thrissur, Kerala, India. The mice were housed in cages (Tarson, India) and provided with food and water *ad libitum*. 24 h grown cultures were scraped from the ZoBells agar plates (15ppt salinity) into sterile saline, centrifuged (3000g for 10 min) and pelleted. The pellet was resuspended in saline and again centrifuged. The above procedure was repeated twice and the final pellet was diluted until an optical density equivalent to 1.0 at A_{600nm} (corresponding to 10^9 cfu/ml) was obtained. Appropriate dilutions needed for animal trials were prepared from this bacterial suspension. The actual number of viable bacterial cells that were administered to the animals was determined by 10-fold serial dilution and plating out onto prepared ZoBell's agar plates.

Mice were lightly anesthetized with Halothane (Nicholas Piramal India Ltd., Chennai, India) in a glass desiccator and challenged with culture strain at doses of 10^9, 10^8, 10^7, 10^6 and 10^5 cells. Bacterial preparation was administered dropwise to the external nares of each mouse with a micropipette (Socorex, Swiss) with fine tips. Mortality was recorded and LD_{50} was determined following Reed and Muench (1938).

4.2.11 Hydrolytic potential of the antagonistic bacterial cultures

The hydrolytic properties such as the production of amylase, cellulase, lipase, gelatinase and chitinase were carried out in order to find the maximum potential use of the selected antagonistic cultures.

i) Amylase activity

Nutrient agar with the following composition was used as basal medium for demonstrating amylase production.

Peptone	: 5.0
Beef extract	: 5.0
Yeast extract	: 1.0
Sodium chloride	: 5.0
Soluble starch	: 5.0
Agar	: 20.0
pH	: 7.0 ± 0.2

The test medium was prepared according to the above composition, autoclaved at 15 lbs for 15 minutes and poured into petri plates.

The plates containing the medium was spot inoculated with the test organism, and the plates were incubated until good growth was obtained at 28±1°C for 3 to 5 days. The plates were flooded with Gram's iodine solution prepared in the following manner.

Iodine	: 1.0g
KI	: 2.0g
Distl. Water	: 100 ml

Amylase producing organisms showed a halo zone around and beneath them. The colour of the zones dependent on the degree of hydrolysis of the starch.

ii) Cellulase activity

The ability of organisms to hydrolyze naturally occurring insoluble polymers like cellulose are routinely investigated. In the method used in the demonstration of cellulase, the polymer in the form of Whatmann filter paper cut into thin strips, 4-5 cm long and 0.5 cm wide were immersed in the medium having the following composition.

Solution A

NH_4Cl	: 5.0g
NH_4NO_3	: 1.0g
Na_2SO_4	: 2.0g
K_2HPO_4	: 3.0g
KH_2PO_4	: 1.0g
NaCl	: 15.0g
pH	: 7.0 ± 0.2

Solution B

$MgSO_4 . 7H_2O$	: 0.1g
$MgCl_2 6H_2O$	: 4.0g
Yeast extract	: 0.01g

The pH of this mineral medium is adjusted to 7 by 1N NaOH. Solution A and solution B were sterilized separately at 15 lbs for 15 minutes. They were then cooled to room temperature and mixed. Along with basal medium, culture tubes containing filter paper strips were also sterilized. After mixing the two solutions, the basal medium was transferred into 3-4 ml aliquots aseptically into the tubes containing filter paper strips.

The test organisms were inoculated and incubated at 28±1°C for 3-7 days or until growth appeared in the medium (seen as turbidity against an uninoculated control). Turbidity or growth indicates that the test organisms were able to utilize cellulose as sole source of carbon and hydrolyze it by producing cellulase.

iii) Lipase activity

Lipase activity of the selected antagonistic isolates was carried out as mentioned earlier in the chapter 2.

iv) Gelatinase activity

Gelatinase activity of the selected antagonistic isolates was carried out as mentioned earlier in the chapter 2.

v) Chitinase activity

Chitinase activity of the selected antagonistic isolates was carried out as mentioned earlier in the chapter 2.

4.2.12 *In vivo* evaluation

The effect of *Pseudomonas* (MCCB103) and *Micrococcus* sp (MCCB104) against vibrios *in vivo* was tested. Three tanks of 1 ton capacity each and with a stocking capacity of one lakh larvae were utilized for the experimental purpose. One of the tanks was maintained as control and other two as test tanks. Larvae were fed with Artemia, egg custard and commercial feeds according to their stage. *Pseudomonas* sp. and *Micrococcus* sp. were added daily to the rearing water so as to get 10^6 cfu/ml. Addition of cultures in to prawn larval rearing tanks was started from the early mysis stage (3 days post hatching) to postlarval stage. For microbiological analysis, water and larval samples were taken periodically which included total heterotrophic bacterial count and total *Vibrio* count. Sampling was carried out till the larvae reached PL 8 (35 days). Total heterotrophic bacterial count was done using ZoBell's agar plates of respective salinities corresponding to the samples. Total *Vibrio* count was carried out using thiosulphate citrate bile salts sucrose agar plates. Water samples were subjected to chemical analysis, which included ammonia, nitrite, nitrate, pH, salinity, alkalinity and hardness (APHA,

1989). Temperature was recorded daily. The survival rate, per cent conversion of larvae to post-larvae, mean weight and mean length was determined at the end of the experiment. Mean weight and mean length was calculated from 100 animals, which were collected randomly from each experimental tank.

4.3 Results and Discussion

4.3.1 Isolation of heterotrophic bacteria

The primary isolation was a general recovery of heterotrophic bacteria from various samples as given in the Table 1 employing ZoBell's agar following serial dilution and plating technique. Accordingly, 812 heterotrophic bacteria were initially isolated and maintained as stock cultures for screening antagonistic property.

4.3.2 Primary screening

Primary screening for antagonistic property among the 812 heterotrophic bacteria were screened against 9 species of *Vibrio* and an isolate of *Aeromonas* (representatives of 55 target *Vibrio* and 6 *Aeromonas*). The antagonistic property was tested by disc diffusion method and isolates, which gave even faint halozones, were also segregated. Five antagonistic groups could be obtained during the primary screening for further study.

4.3.3 Secondary screening

In order to confirm the antagonistic potential of the primarily screened organisms, secondary screening of the above 5 antagonistic bacteria against the entire target *Vibrio* (55 isolates, comprising 9 species) and 6 isolates of *Aeromonas* were carried out following disc diffusion method. In this step, only those, which gave very clear and wide halozones, were selected for further experiments. From the above screening, the minimum number of antagonistic bacteria required for the total inhibition of the pathogens was segregated.

The antagonism tests in solid medium by disc diffusion method revealed that all the tested target bacterial isolates were strongly inhibited by the and SAB 270, SAB 274, SAB 301, SAB 303 and SAB 306 isolates (Table 4).

The antagonism tests in solid medium by disc diffusion method revealed that except the two strains of *V. splendidus* II, all others were inhibited largely by isolates SAB 270, 274, 301, 303 (*Micrococcus* sp) and 306 (*Pseudomonas* sp). The *Micrococcus* isolates caused a clearing zone of 15-20 mm with all the target bacterial cultures, whereas the isolate *Pseudomonas* showed inhibition with clearing zone of 11-15 mm (Fig. 1 & 2). *Pseudomonas* was found to be antagonistic to 97% of the total number of pathogenic target isolates and for *Micrococcus* it was 93% (Table 5).

4.3.4 Time course of growth and production of the antagonistic substance

Figure 3 shows that the *Micrococcus* MCCB104 entered the log phase within 6 h and reached stationary phase by 42 h. The growth declined at about 48 h of post-inoculation. The culture started producing the antagonistic substance after 12-15 hours of inoculation by which it had entered the log phase of growth. Production of the antagonistic substance peaked at the stationary phase and the maximum activity was observed at 42 h of growth.

Pseudomonas MCCB103 entered log phase after 6 h and reached stationary phase by 60 h (Fig. 4). The growth declined at about 120 h of post-inoculation. The culture started producing the antagonistic substance after 30-33 h of inoculation by which it had entered the late log phase of growth. Production of the antagonistic compound peaked at the stationary phase and the maximum activity was observed during 60 to 84 h of growth.

4.3.5 Tolerance of sodium chloride concentrations, pH and temperature

The effect of pH, temperature and sodium chloride on growth of *Pseudomonas* MCCB103 is presented in Table 6. Comparatively higher growth was observed between 0.5% to 2.5% NaCl concentration, at pH 6 to 8, and at 35°C and 40°C.

The effect of pH, temperature and sodium chloride on the growth of *Micrococcus* MCCB104 is presented in Table 7. pH 7, 8 and 9 were found to be the optima with corresponding zone of inhibition of 15, 16 and 15 mm diameter, respectively. There was no growth at pH 4 and 5. Growth was maximum at 35°C and 40°C with 16 mm diameter zone of inhibition. There was no growth at 4°C and 45°C. Maximum growth was observed in the medium containing 0.5%, 1% and 1.5% NaCl with 16 mm diameter zone of inhibition. However, the growth was minimal at 3%, and 3.5% NaCl with no antagonistic activity.

4.3.6 Co-culture experiments

Growth of *V. alginolyticus* was inhibited by *Pseudomonas* MCCB103 inoculated at an initial count of 10^5 to 10^7 cfu/ml (Fig. 5). Lower counts of *Pseudomonas* MCCB103 (10^5 cfu/ml and 10^6 cfu/ml) allowed initial growth of *V. alginolyticus* for 24 h. Meanwhile, higher inoculum size (10^7 cfu/ml) of *Pseudomonas* MCCB103 did not allow *V. alginolyticus* to multiply, instead reduction in its viable cell count was observed culminating in total loss of viability within 92 h. The above results indicated that, when the count of *Pseudomonas* MCCB103 was high enough, the growth of *V. alginolyticus* could be controlled under *in vitro* conditions. Growth of *Pseudomonas* MCCB103 was not affected by coculturing with *V. alginolyticus* as it remained at about $1x10^8$ cfu/ml throughout the experimental period.

Growth of *V. alginolyticus* MCCB112 was inhibited by *Micrococcus* MCCB104 inoculated at an initial level of 10^5 to 10^7 cfu/ml (Fig. 6). All counts of the strain MCCB104 in the co-culture allowed initial growth of *V. alginolyticus*, but never attained the cell count of the control. Comparatively, the flasks inoculated with the highest count (10^7 cfu/ml) of *Micrococcus* MCCB104 showed the lowest count of *V. alginolyticus* (approx 10^4 cfu/ml).

4.3.7 Preliminary characterization of the antagonistic substance

The activity of the cell-free culture supernatant of *Pseudomonas* MCCB103 remained relatively stable after the treatments with α-chymotrypsin, trypsin, proteinase K, pronase E, lysozyme, lipase, catalase and α-amylase, as the zones of inhibition (15 mm) obtained were similar to that of control (untreated cell free culture supernatant). In the same way, the antagonistic activity observed with the cell-free culture supernatant, neutralized to pH 7.0, was similar to that of untreated preparation. When autoclaved for 15 min at 121°C, the zone of inhibition was decreased to 9 mm compared to control (15 mm). However, at 80°C and 100°C there was no effect in the inhibitory property and the zone of inhibition was similar to control (Table 8 and 9).

The activity of the cell-free culture supernatant of *Micrococcus* MCCB104 remained relatively stable after the treatments with α-chymotrypsin, trypsin, proteinase K, pronase E, lysozyme, lipase, catalase and α-amylase, as the zones of inhibition (16 mm) obtained were similar to that of control (untreated cell free culture supernatant). Similar antagonistic activity was observed when the cell-free culture supernatant was neutralized to pH 7.0. Study of the heat stability of the antagonistic substance showed that even when autoclaved at 121°C for 15 min, there was no loss of activity (Table 8 and 9).

4.3.8 Pathogenicity on *M. rosenbergii* larvae

As shown in Table 10, the survival of prawn post-larvae was not affected by challenging with *Pseudomonas* MCCB103 and *Micrococcus* MCCB104.

4.3.9 LD_{50} of *Pseudomonas* MCCB103 and *Micrococcus* MCCB104 in mice

LD_{50}, calculated following Reed and Muench (1938) was 10^9 cells for *Pseudomonas* MCCB103 and $>10^{11}$ cells for *Micrococcus* MCCB104.

4.3.10 Hydrolytic potential

The hydrolytic properties such as the production of lipase, gelatinase, amylase, cellulase and chitinase were carried out in order to find the maximum potential use of the above selected antagonistic cultures. Lipase, protease, amylase and chitinase activity was found by presence of clearing zones around the colony growth and the cellulase production was observed by the presence of turbidity in the broth. The results are tabulated as shown in Table 11. All the *Micrococcus* isolates showed gelatinase, amylase and cellulase activity but did not exhibit lipase and chitinase activity. *Pseudomonas* isolate showed lipase, gelatinase and cellulase activity whereas amylase and chitinase activity was absent.

4.3.11 *In vivo* evaluation

The effect of *Pseudomonas* and *Micrococcus* sp. against vibrios *in vivo* was tested by adding culture at 10^5 to 10^6 cfu/ml daily in to prawn larval rearing tank starting from the early mysis stage (3 days post hatching) to postlarval stage. The total *Vibrio* counts in rearing water and of the larvae determined subsequently which received the inoculum was significantly lesser ($p < 0.05$) than that of the control (Table 12 and Table 13). Although the survival rate was comparatively lesser than the control, the treatment resulted in increase in the per cent conversion ratio of larvae and increased the mean

weight and mean length (Table 14). The chemical and physical parameters such as ammonia, nitrite, nitrate, alkalinity, hardness, salinity, pH and temperature ranges recorded during the experimental period are given in Table 15.

The present study showed that a wide range of *Vibrio* and *Aeromonas* associated with *M. rosenbergii* larval rearing systems was inhibited by *Pseudomonas* MCCB 103 and *Micrococcus* MCCB104. Results of the antagonism assay by disc diffusion method with cell-free culture supernatant suggest it an extracellular. The concept of control of pathogens in shellfish farming by non-pathogenic bacterial strains is now gaining importance as a sustainable, cheaper and more effective replacement of antibiotics and their use is on the rise (Rengpipat *et al.* 2000; Verschuere *et al.* 2000; Gomez-Gil *et al.* 2002). According to Havenaar and HiusIn'tVeld (1992), the term 'probiotic' has been defined as a mono- or mixed culture of live microorganisms that when applied to animal, affect beneficially the host by improving the quality of the indigenous microflora in favour of the animal. Antagonistic activity of *Pseudomonas* against bacterial pathogens such as *Salmonella*, *Staphylococcus aureus* and shrimp pathogenic vibrios has been reported (Troller and Frazier, 1963; Oblinger and Kreft, 1990; Chythanya *et al.* 2002). *Micrococcus* spp. are typical components of the heterotrophic bacterial microflora of various environments and have been recovered from soil (Rahman *et al.* 2002), seawater (Tanaka *et al.* 2001), marine sediments (Zhong *et al.* 2002) and prawn rearing water (Phatarpekar *et al.* 2002). Strains of *Micrococcus* spp. have been generally used as starter cultures in fermented beef and pork sausages (Bacus, 1984) as these can prevent the growth of pathogenic microorganisms by organic acid production (Schillinger and Lucke, 1990). Irianto and Austin (2002), reported that an isolate of an unidentified Gram-positive coccus had potential in combating *Aeromonas salmonicida* infections in rainbow trout, *Oncorhynchus mykiss* (Walbaum). It is noteworthy to highlight that many of the *Vibrio* species tested here are reported pathogens in aquaculture systems (Sinderman, 1990, Austin and Austin, 1993).

Co-culture experiments showed that higher level of *Pseudomonas* MCCB103 and *Micrococcus* MCCB104 was required for effective inhibition of *V. alginolyticus* and the

degree of inhibition increased with the increase in the level of the antagonist. Accordingly, 10^7 cfu/ml of *Pseudomonas* MCCB103 was able to eliminate the pathogen within three days whereas 10^6 cfu/ml of *Pseudomonas* MCCB103 required six days for the same. Co-culture experiments showed that the isolate *Micrococcus* MCCB104 inhibited the growth of *V. alginolyticus* when the initial count of the antagonist was 100 to 1000 times greater than that of the prawn pathogen. It implies that for the required efficacy the cultures would have to be supplied in sufficient quantity and also on regular basis.

Antagonistic substance production pattern of *Pseudomonas* MCCB103 in ZoBell's marine medium showed that it increased in the late stationary phase. Appearance of the antivibrio activity in the medium when the cells reaching stationary phase of growth and the maximum activity at late stationary phase suggested the antibacterial factor as a secondary metabolite. Therefore, to get the maximum quantity of the antagonistic substance recovered, the ideal harvest time would be between 60 to 84 h of growth. Growth of *Pseudomonas* MCCB103 in various salinities indicated that the strain could be a suitable candidate probiotic for both non-penaeids and penaeids systems. Members of the genus *Pseudomonas* are known to produce a wide range of secondary metabolites inhibiting several bacterial pathogens (Budzikiewicz, 1993; Arunkumar *et al.* 1997; Chythanya *et al.* 2002). Many of the *Vibrio* and *Aeromonas* isolates subjected here for antagonistic assay had been reported as pathogens in fish and shellfish culture systems (Sinderman, 1990; Austin and Austin, 1993; Lightner, 1996). Inhibitory activity of the cell free supernatant of *Pseudomonas* MCCB103 on the above target bacterial isolates indicates that the inhibitory component is extracellular. Earlier studies in this direction have implicated siderophoric bacteriostatic substances as being produced by *Pseudomonas* (Gram, 1993; Guerinot, 1994). However, preliminary characterization of the inhibitory component in the cell free supernatant of *Pseudomonas* MCCB103 suggested that it was not a protein, lipid or carbohydrate, and did not contain hydrogen peroxide or any compound of alkaline or acidic in nature. It has been reported that pseudomonads do produce antibiotics, pyocyanin and also cyanides (Gurusiddaiah *et al.* 1986; Raaijmakers *et al.* 1997; Chythanya *et al.* 2002). In this context, works are in

progress in our laboratory to purify and characterize the antagonistic component of the extracellular product involved in the inhibitory action. Pridmore *et al.* (1996), reported that variacin, a bacteriocin produced by *Micrococcus varians*, inhibited other gram-positive bacteria but not gram-negative ones. El-Shafei (1997), observed that the introduction of *Micrococcus* to fungal cultures resulted in lysis and inhibition of fungal growth and attributed this to the production of mycolytic enzymes. Preliminary characterization of the antagonistic substance in the cell-free culture supernatant of *Micrococcus* MCCB104 suggested that it was a non-proteinaceous compound.

Stability of *Pseudomonas* MCCB103 cell free supernatant at high temperatures suggests that the antagonistic principle in it is thermostable. Chythanya *et al.* (2002), observed that the antibacterial activity of cell free supernatant of *Pseudomonas* I-2 isolate was not affected at 100°C. Results in our study also indicated that the antagonistic principle of *Pseudomonas* MCCB103 could even withstand 100°C for 30 min. Moreover, the compound of *Micrococcus* MCCB104 was found to be highly heat-stable surviving autoclaving. This thermostability should prove to be of greater use if the compound is incorporated into feed, where exposure to high temperature is unavoidable.

In order to consider a bacterium as a candidate probiotic for application in the culture system, it should be evaluated for pathogenicity in the target-cultured species (Verschuere *et al.* 2000). This study has clearly shown that *Pseudomonas* MCCB103 and *Micrococcus* MCCB104 are non-pathogen to prawn larvae, even at 10^8 cfu/ml. Moreover, *Micrococcus* species have not been recognized and reported elsewhere as prawn pathogens. As mandatory, pathogenicity of the organisms towards the mammalian system was also considered to ensure safety in handling. The high LD_{50} value in mice indicated that the organism is safe to handle as a probiotic.

Precisely, the study suggests that *Pseudomonas* MCCB103 and *Micrococcus* MCCB104 may possibly be used as antagonistic probiotics in prawn larval rearing systems for the biological control of *Vibrio* and *Aeromonas*.

Table 1. Heterotrophic bacterial isolates from various environments as the source for antagonistic bacteria.

Se. No.	Code No.	Source of isolates	Number of isolates
1	SAB 1 – SAB 190 SAB 201 – SAB 210	Pond sediments from Cherthala	200
2	SAB 191 – SAB 200 SAB 211 – SAB 216	Pond sediments from Kumbalangi	16
3	SAB 217 – SAB 290	Seawater from hatcheries	73
4	SAB 291 – SAB 298	Surface swab of prawn	8
5	SAB 299 – SAB 318	Foregut of prawns	20
6	SAB 319 – SAB 340	Midgut of prawns	22
7	SAB 341 – SAB 359	Seawater from hatcheries	19
8	SAB 360 – SAB 421	Healthy larvae of prawns	62
9	DOD 1 – DOD 90	Seawater offshore	90
10	ANT 1 – ANT 80	Intestines of Charangids, Tuna, Synagris, Sardinella longiceps, Etroplus surratiensis, Tilapia, Etroplus maculatus, Nemipterus sp, Mugil sp	80
11	SED 1 – SED 86	Pond sediments from Maradu	86
12	SED 87 – SED 93	Pond sediments from Chathamma	7
13	SED 94 – SED 222	Pond sediments from Panangad	129
		TOTAL ISOLATES	812

Table 2. Biochemical characteristics of the antagonistic *Pseudomonas* isolate

Property	
Gram's Stain	– rods
Marine oxidation fermentation test	Oxidative
Kovac's Oxidase test	+
Arginine dihydrolase	+
Lysine decorboxylase	–
Ornithine decorboxylase	–
Denitrification	+
Utilization of,	
Glucose	+
Trehalose	–
Sucrose	–
Starch hydrolysis	–
Gelatin hydrolysis	+
Fluorescent pigment	+
Growth at 41^{0}C	+
Growth at 4^{0}C	–
Growth in *Pseudomonas* Isolation Agar	Circular, convex, green
Acid production from,	
D-Ribose	+
D-Xylose	+
Galactose	+
L+ Arabinose	+
Mannose	+
D-Sorbitol	–
Rhamnose	–
Lactose	–
Sucrose	–
Trehalose	–
Adonitol	–
Cellobiose	–

Table 3. Biochemical properties of the antagonistic *Micrococcus* isolates

Property			
Pigmentation	Y	Sensitivity to:	
Gram staining	+		
Morphology	S	Furozolidone	−
Growth at pH 6	+	Novobiocin	+
Growth at 37°C	+	Bacitracin	+
Growth in 7.5% NaCl	+	Ampicillin	+
Motility	−	Streptomycin	+
Yellow-brown pigment	−	Rifampicin	+
Glucose fermentation	−	Neomycin	+
Glucose acid	+	Erythromycin	+
Mannose acid	+	Kanamycin	+
Kovac's Oxidase	+	Chloramphenicol	+
Catalase	+	Ciprofloxacin	+
Arginine dihydrolase	−	Oxytetracycline	+
Citrate utilization	+	Nitrofurantoin	+
Nitrate reduction	+	O/129	+
Indole	−		
		Hydrolysis of:	
Utilization of sole C-source:			
		Tributyrin	−
d-mannose	+	Gelatin	+
Meso inositol	−	Starch	+
d-trehalose	−	Cellulose	+
d-xylose	+	Chitin	−
d-mannitol	+		
Adonitol	−		
Sucrose	+		
Arabinose	+		
Raffinose	+		
d-cellobiose	−		
Galactose	−		

+, Positive; −, negative; Y, yellow; S, spherical

Table 4. Selected antagonistic bacteria for the target vibrios and *Aeromonas* sp.

Se. No.	Code	Antagonistic bacterial isolates (Diameter of halozone in mm)					Genus/ Species
		SAB 270	SAB 274	SAB 301	SAB 303	SAB 306	
1	MRCS 11	22	23	23	23	18	*V. cholerae*
2	MRCS 12	20	20	21	19	16	
3	MRCS 13	20	20	20	21	15	
4	MRCS 16	23	24	23	24	18	
5	MRCS 17	22	22	22	22	16	
6	MRCS 19	25	25	25	25	19	
7	MRCS 20	-	-	-	-	12	
8	MRCS 21	20	21	19	21	15	
9	MRCS 23	-	-	-	-	16	
10	MRCS 35	22	22	20	22	17	
11	MRCS 37	20	21	20	19	16	
12	MRCS 39	20	20	19	21	16	
13	MRCS 15	20	19	20	20	15	*V. mediterranei*
14	MRCS 18	22	19	22	19	23	
15	MRCS 22	26	24	26	23	19	
16	MRCS 32	22	22	22	22	16	
17	MRCS 34	22	21	22	22	16	
18	MRCS 36	16	16	15	15	14	
19	MRQL 27	15	14	15	15	13	

Contd.....

Contd.....

Se. No.	Code	Antagonistic bacterial isolates (Diameter of halozone in mm)					Genus/ Species
		SAB 270	SAB 274	SAB 301	SAB 303	SAB 306	
20	MRQL 5	21	20	20	21	17	*V. vulnificus*
21	MRQL 9	21	20	20	21	17	
22	MRQL 10	21	21	22	20	17	
23	MRQL 19	22	20	22	22	16	
24	MRQL 20	21	21	21	21	17	
25	MRQL 23	21	21	21	21	16	
26	MRQL 33	21	20	20	21	19	
27	MRQL 36	20	21	20	19	17	
28	MRCS 24	18	19	17	16	15	*V. nereis*
29	MRCS 28	20	21	19	20	19	
30	MRCS 29	17	15	17	16	17	
31	MRCS 30	19	20	18	17	13	
32	MRCS31	19	20	18	19	13	
33	MRCS 33	20	20	20	20	16	
34	MRQL 13	18	18	18	18	15	
35	MRQL 29	18	17	18	16	13	
36	MRQL 32	17	16	15	17	16	
37	MRQL 34	24	23	23	24	13	
38	MRQL 35	18	18	18	18	15	
39	MRCS 1	20	20	20	20	14	*V. parahaemolyticus*
40	MRCS 6	23	21	22	20	18	
41	MRCS 8	21	21	20	21	16	
42	MRCS 9	22	22	21	21	14	

Contd.....

Contd.....

Se. No.	Code	Antagonistic bacterial isolates (Diameter of halozone in mm)					Genus/ Species
		SAB 270	SAB 274	SAB 301	SAB 303	SAB 306	
43	MRCS 3	-	-	-	-	-	*V. splendidus II*
44	MRCS 5	-	-	-	-	-	
45	MRCS 4	16	17	18	17	16	*V. proteolyticus*
46	MRCS 10	18	19	19	19	19	
47	MRCS 26	23	23	22	23	20	*V. fluvialis*
48	MRNL 1	21	20	22	21	12	*V. alginolyticus*
49	MRNL 2	20	21	20	19	19	
50	MRNL 3	22	21	22	22	18	
51	MRNL 4	22	22	20	22	12	
52	MRNL 5	21	20	20	21	13	
53	MRNL 6	22	22	22	22	14	
54	MRNL 7	20	21	21	22	13	
55	MRNL 8	21	20	22	21	12	
56	MRCS 2	17	16	15	16	18	*Aeromonas* sp.
57	MRCS 7	25	24	24	25	18	
58	MRNL 9	22	23	21	22	16	
59	MRNL 10	22	20	21	21	19	
60	MRNL 16	24	25	24	25	19	
61	MRNL 17	28	26	25	25	17	

Table 5. Percentage of target pathogens inhibited by the selected antagonistic bacterial isolates

Antagonistic bacteria	Genus	Number of target strains inhibited	% of target strains inhibited
SAB 270	*Micrococcus*	57	93.4
SAB 274	*Micrococcus*	57	93.4
SAB 301	*Micrococcus*	57	93.4
SAB 303	*Micrococcus*	57	93.4
SAB 306	*Pseudomonas*	59	96.7

Table 6. Effect of pH, temperature and salt concentration on the growth of *Pseudomonas* MCCB 103

Parameter	Growth (24 h)
pH	
4	−
5	−
6	+++
7	+++
8	+++
9	++
10	+
11	+
Temperature of incubation (°C)	
4	−
15	+
20	+
25	++
30	++
35	+++
40	+++
45	++
Salt concentration (%)	
0	++
0.5	+++
1.0	+++
1.5	+++
2.0	+++
2.5	+++
3.0	++
3.5	++

+: Visible growth; ++: moderate growth; +++: excellent growth; −: no growth

Table 7. Effect of pH, temperature and salt concentration on the growth of *Micrococcus* MCCB104

Parameter	Growth
pH	
4	−
5	−
6	+
7	+++
8	+++
9	+++
10	++
11	++
Temperature of incubation (°C)	
4	−
15	+
20	+
25	++
30	++
35	+++
40	+++
45	−
Salt concentration (%)	
0	+
0.5	+++
1.0	+++
1.5	+++
2.0	++
2.5	++
3.0	+
3.5	+

+: Visible growth; ++: moderate growth; +++: excellent growth; −: no growth

Table 8. Action of enzymes on the supernatant of *Pseudomonas* sp. and *Micrococcus* sp.

Se.No.	Enzyme	Concentration of the enzyme (mg/ml)	pH	Incubation temperature (^{0} C)	*Pseudomo-nas* sp.	*Micrococ-cus* sp.
1	Proteinase K	1	7.0	37	-	-
2	α-chymotrypsin	5	7.0	37	-	-
3	Trypsin	2	7.0	37	-	-
4	Lysozyme	1	7.0	25	-	-
5	Lipase	1	7.0	37	-	-
6	α-amylase	1	7.0	25	-	-
7	Pronase E	2	7.0	37	-	-
8	Catalase	1	7.0	37	-	-

Table 9. Heat stability of supernatants of *Pseudomonas* sp and *Micrococcus* sp.

Se. No.	Temperature (°C)	Activity of *Pseudomonas* sp	Activity of *Micrococcus* sp.
1	80	15	16
2	100	15	16
3	121 for 15 min	9	16
4	28 (Control)	15	16

Table 10. Pathogenicity test of *Micrococcus* MCCB104 and *Pseudomonas* MCCB 103 on *Macrobrachium rosenbergii* larvae (PL-1)

Treatment groups	Number of larvae survived (out of 50 numbers) *			
	24 h	48 h	72 h	96 h
Micrococcus (1.6×10^8 cfu/ml)	48±1	45±1	44±1	41±2
Pseudomonas (1.5×10^8 cfu/ml)	48±1	46±1	45±1	42±4
Control	46±2	44±3	41±2	36±6

*n=3

Table 11. Hydrolytic characteristics of the antagonistic bacteria

Code No.	Genera	Lipase	Gelatinase	Amylase	Cellulase
SAB 270	*Micrococcus* sp.	-	+	+	+
SAB 274	*Micrococcus* sp.	-	+	+	+
SAB 301	*Micrococcus* sp.	-	+	+	+
SAB 303	*Micrococcus* sp.	-	+	+	+
SAB 306	*Pseudomonas* sp.	+	+	-	+

Table 12. Anova table showing the level of significance of total *Vibrio* counts in rearing water between the control tank and test tanks added with *Pseudomonas* sp. and *Micrococcus* sp.

Anova: Two-Factor without Replication

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	10.743932	12	0.89533	9.54628	1.9E-06	2.18338
Columns	2.0613591	2	1.03068	10.9894	0.00041	3.40283
Error	2.2509156	24	0.09379			
Total	15.056207	38				

Table 13. Anova table showing the level of significance of the total *Vibrio* counts in larvae between the control tank and test tanks added with *Pseudomonas* sp. and *Micrococcus* sp.

Anova: Two-Factor without Replication

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	41.153569	12	3.42946	39.5175	7.7E-13	2.18338
Columns	1.3111076	2	0.65555	7.5539	0.00285	3.40283
Error	2.0828041	24	0.08678			
Total	44.547478	38				

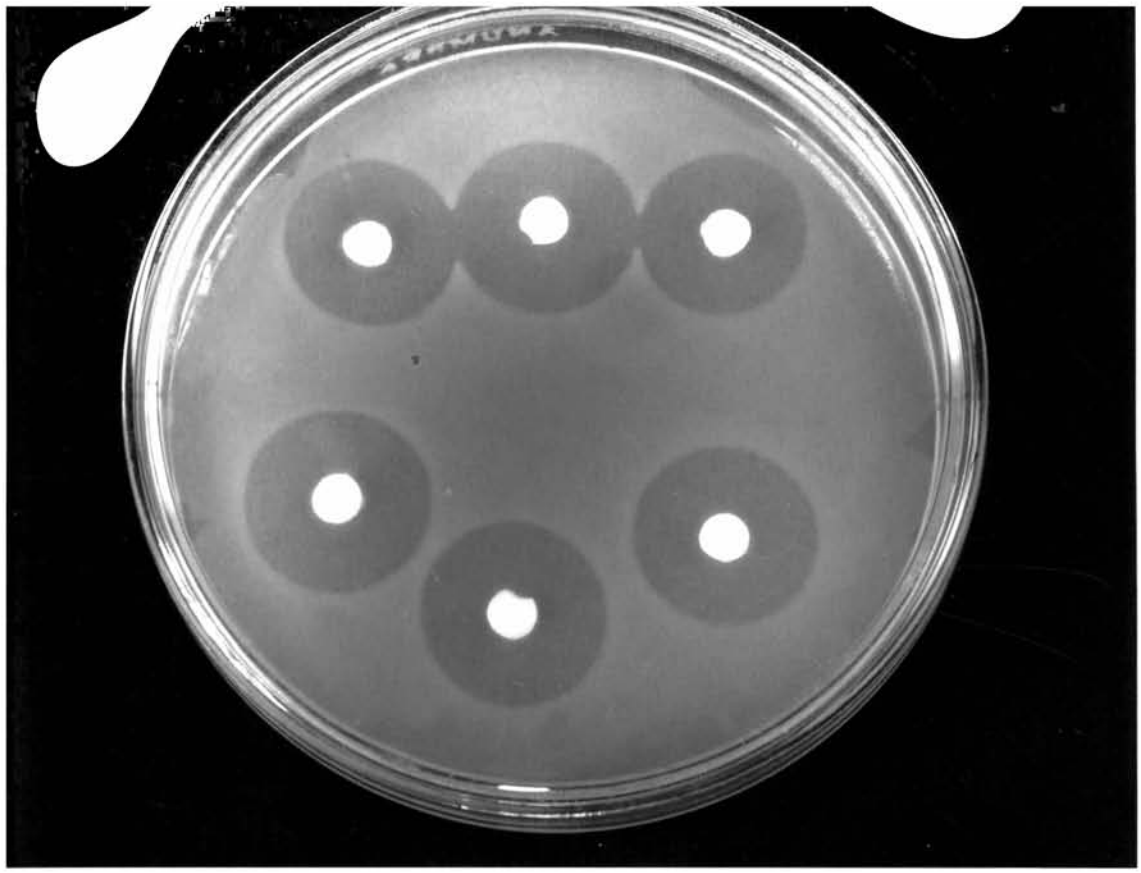
Table 14. Effect of addition of *Pseudomonas* MCCB 103 and *Micrococcus* MCCB104 on survival rate, percent conversion, length and weight of *Macrobrachium rosenbergii* larvae

	Survival rate	Per cent conversion of larvae to post larvae	Length (mm) (mean and standard deviation)	Weight (g) (mean and standard deviation)
Control	60	50	10.2±0.8	0.007±0.002
Test (*Pseudomonas*)	57	63	10.6±1.0	0.009±0.002
Test (*Micrococcus*)	56	59	10.5±0.9	0.009±0.002

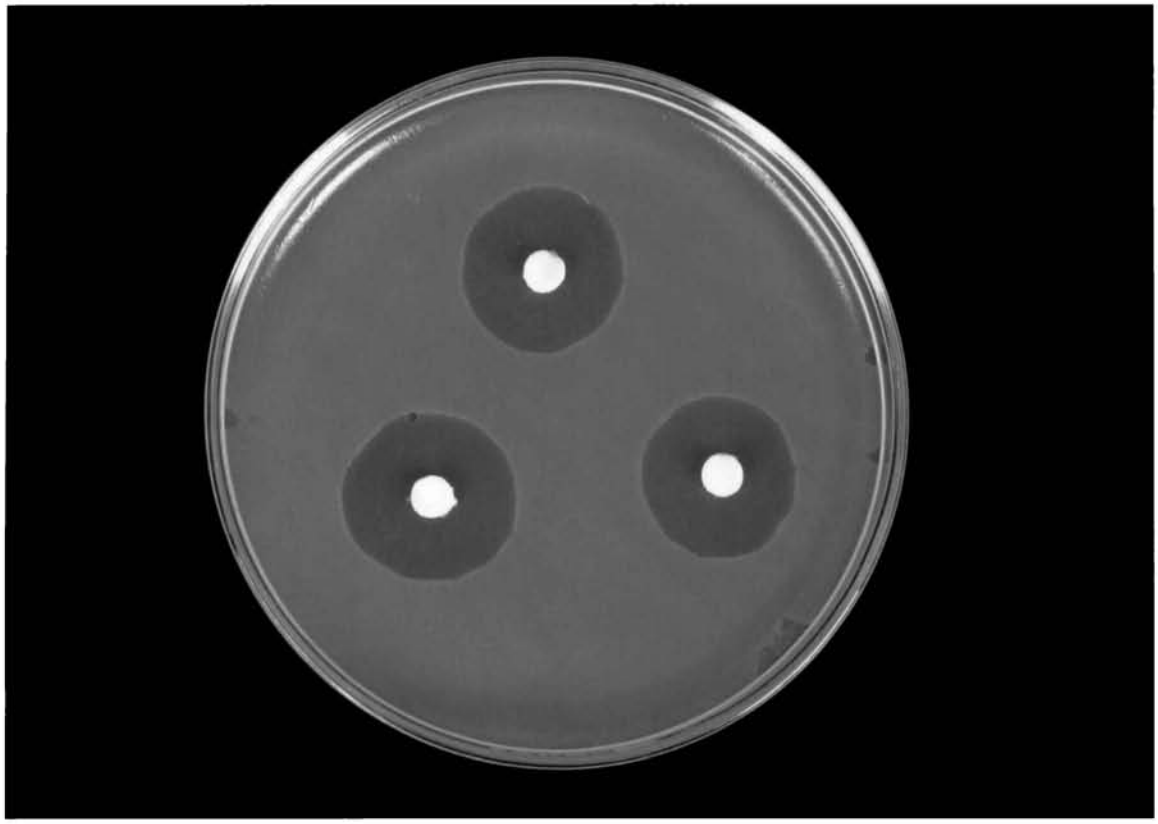
Table 15. Range of physical and chemical parameters recorded during *in vivo* experimentation with *Pseudomonas* and *Micrococcus* isolate in the *M. rosenbergii* larval rearing tanks

Parameter	Control tank	Tank with *Pseudomonas* added	Tank with *Micrococcus* added
Ammonia (ppm)	0.2-2.0	0.5-1.8	0.4-1.9
Nitrite (ppm)	0-1.6	0-1.6	0-0.5
Nitrate (ppm)	0-0.8	0-0.7	0.4-0.8
Alkalinity (mg $CaCO_3$/ L)	15-90	20-90	20-85
Hardness (mg $CaCO_3$/ L)	275-2800	300-2850	325-2950
Salinity (ppt)	1-15	2-15	2-14
pH	6.5-7.5	6.5-7.5	6.5-7.5
Temperature (°C)	26-29	26-29	26-29

Note: Wide range in the parameters such as alkalinity, hardness and salinity was due to the progressive reduction in salinity brought about from 15 to 1 ppt during the larval rearing (Zoea to Post larvae) as the common practice in a commercial hatchery.



1. Demonstration of antagonistic activity of a *Micrococcus* sp. against a *Vibrio* sp.



g. 2. Demonstration of antagonistic activity of *Pseudomonas* sp. against a *Vibrio* sp.

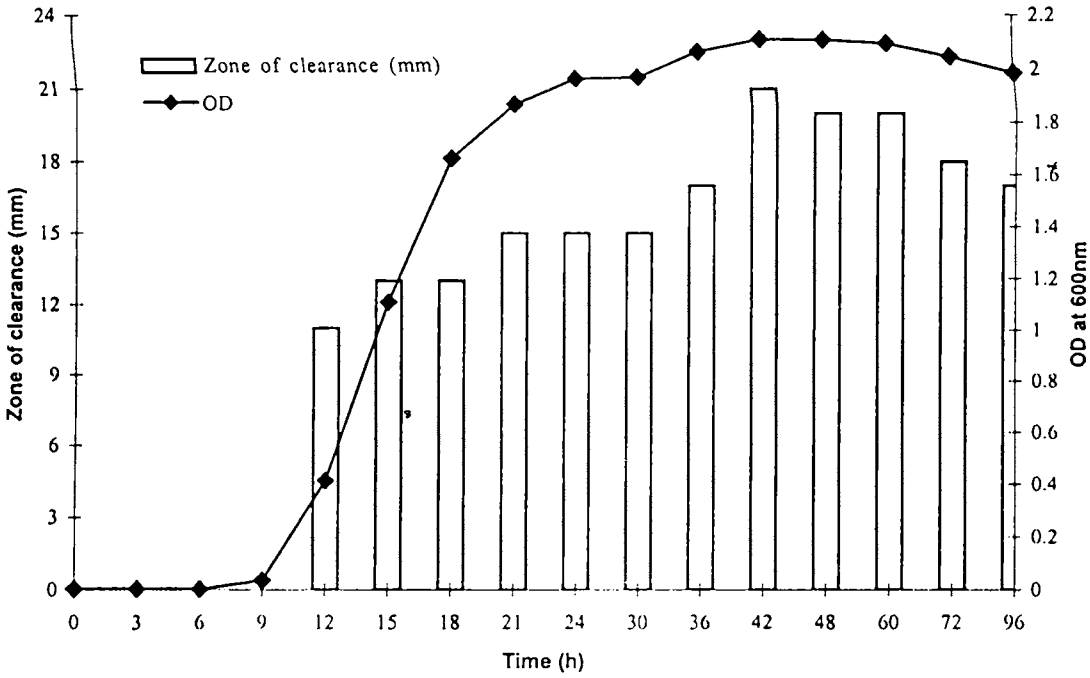


Fig. 3 Growth and antagonistic activity of *Micrococcus* MCCB104 against *V. alginolyticus*

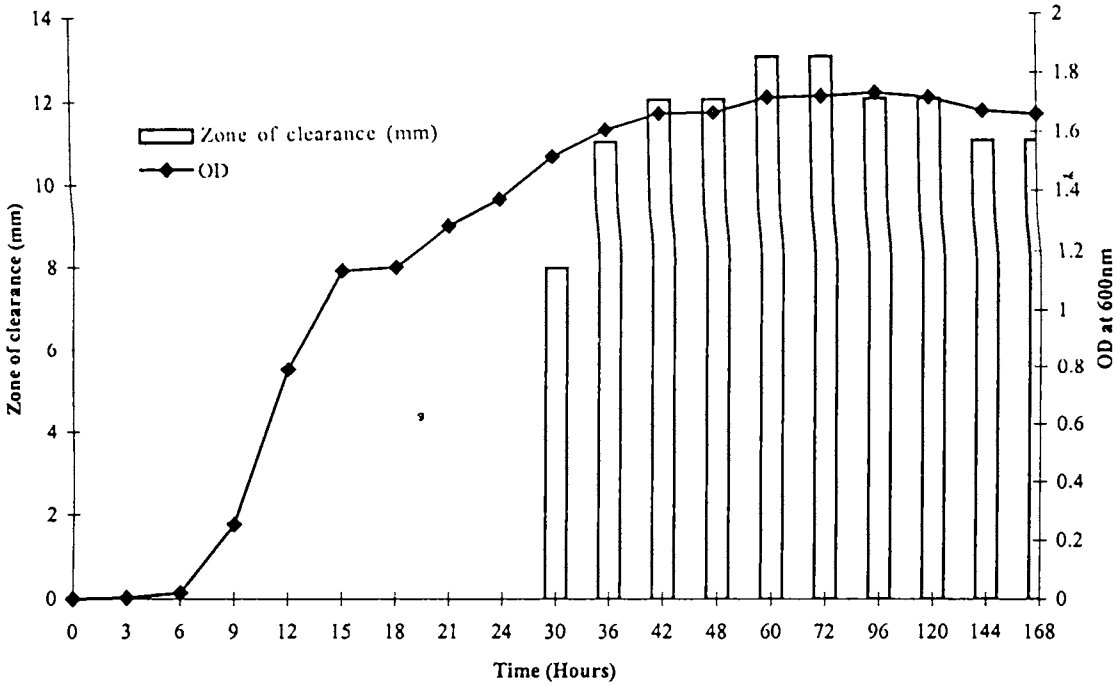


Fig. 4 Growth pattern and antagonistic activity of *Pseudomonas* MCCB103 against *V. alginolyticus*

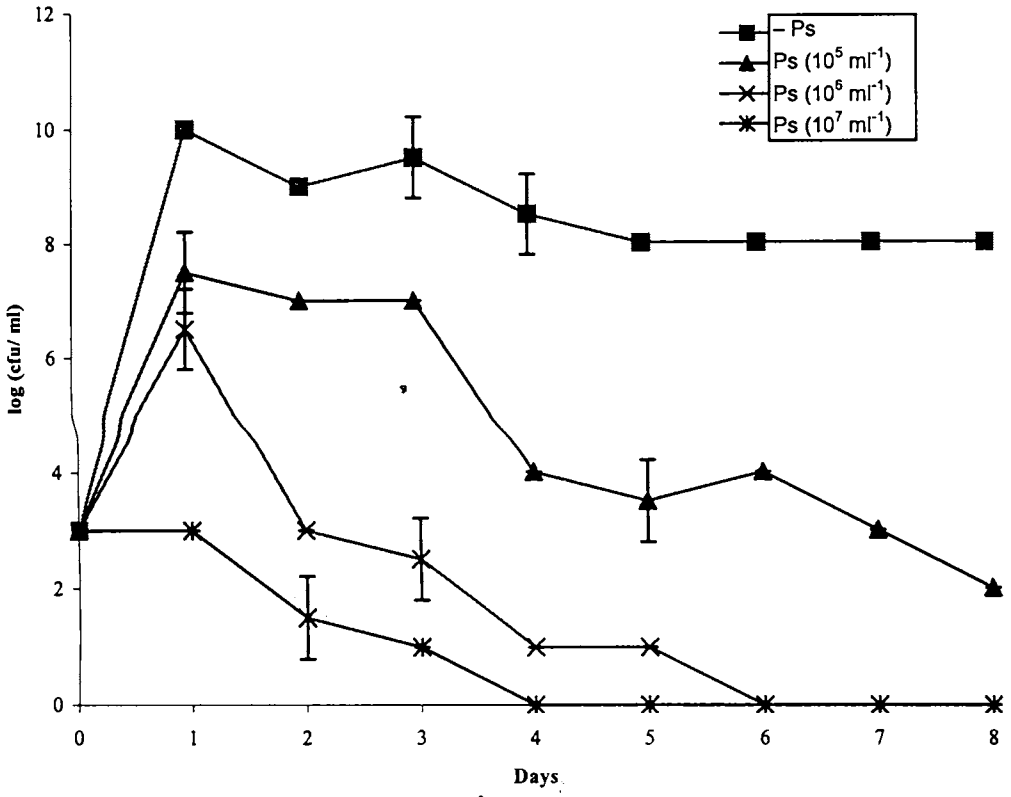


Fig. 5 Growth of *V. alginolyticus* at 28^{0}C in ZoBell's broth (15 ppt) with and without *Pseudomonas* MCCB103 at different initial cell densities

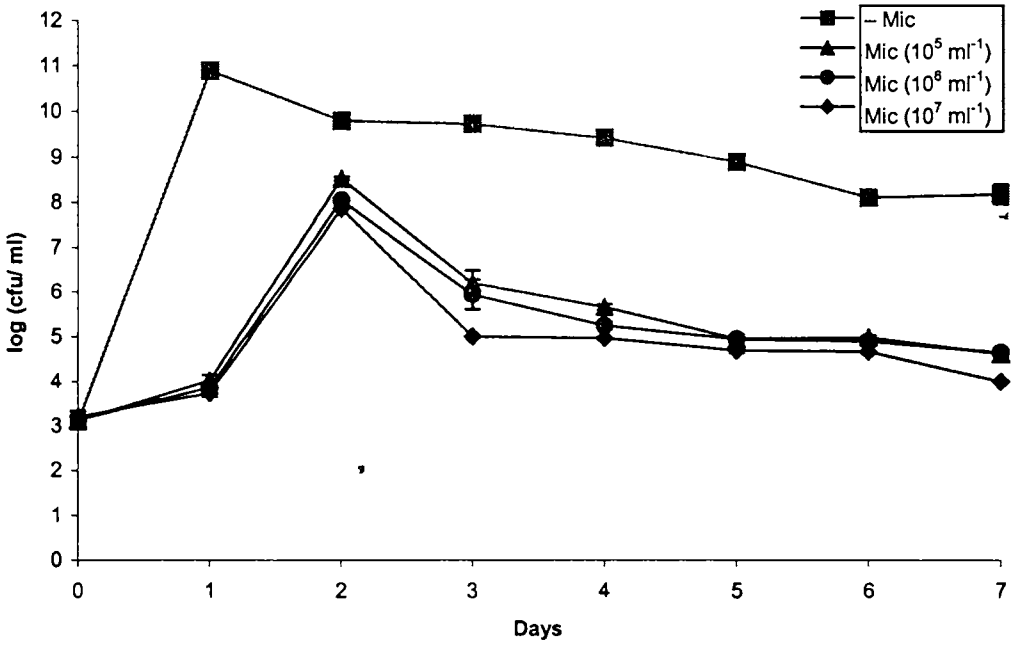


Fig. 6 Growth of *V. alginolyticus* at 28°C in ZoBell's broth (15ppt) with and without *Micrococcus* MCCB104 at different initial cell densities

CHAPTER - 5

CONCLUSION

Macrobrachium rosenbergii (de Man) is native to Thailand and other Southeast Asian countries including India and has been a prominent fishery in these regions. However, as a result of over fishing and destruction of habitat and spawning grounds, the landings have been dwindling rapidly over the years and the species is now a luxury food item; production no longer meets consumer demand. Meanwhile, as a matter of fact, hatchery production of postlarvae of *M. rosenbergii* could be made a reality and it initiated augmented efforts for producing cultured prawn. However, inspite of best of the efforts made, consistent production of sufficient quantity of healthy prawn seed at the right time as per the requirements of country's grow out systems could not be achieved with sustainability. This is still a concern of aquaculturists not only of India but the world over. This instability in the production process is mainly due to diseases and the consequent mass mortality of larvae at different stages; among which vibriosis is the most prominent bacterial disease. It is disappointing to note that despite two decades of research, a viable solution to the global problem has not yet been evolved and the situation continues to be serious demanding much more concerted efforts as quickly as possible. It has been pointed out that lack of adequate information in this systemic infection caused by several species of *Vibrio* is one of the main reasons for the above situation. Nevertheless, vibriosis remains still the major disease in hatcheries, and prompt, specific and rapid detection and identification of the pathogens becomes an absolute essentiality. It has to be borne in mind that conventional microbiological and biochemical analyses need three working days and the information obtained after such a prolonged period is of no use to protect the stock. This invariably makes the rapid detection methods an important requirement. At disease management level, to control vibrios in hatcheries, so far prophylactic and therapeutic use of antibiotics has been the choice especially in commercial establishments. Unequivocally, this practice led to the development of resistance in the pathogens with a possible spread in the environment.

This situation demands an alternate management measure, which includes the introduction of selected bacterial cultures/ products as probiotics with antagonistic properties. The present work was undertaken with the realizations that to stabilize the production process of commercial hatcheries an appropriate, comprehensive and fool proof technology is required primarily for the rapid detection of *Vibrio* and subsequently for its management. Results obtained and conclusions made out of this endeavour are summarized as follows:

- Nine species of *Vibrio* such as *V. cholerae*, *V. nereis*, *V. vulnificus*, *V. alginolyticus*, *V. mediterranei*, *V. parahaemolyticus*, *V. splendidus* II, *V. proteolyticus*, *V. fluvialis* and *Aeromonas* sp. have been found to be associated with larvae of *M. rosenbergii* in hatchery.

- Haemolytic assay of the *Vibrio* and *Aeromonas* on prawn blood agar showed that all isolates of *V. alginolyticus* and *Aeromonas* sp., from moribund, necrotized larvae were haemolytic and the isolates of *V. cholerae*, *V. splendidus II*, *V. proteolyticus* and *V. fluvialis* from the larvae obtained from apparently healthy larval rearing systems were non-haemolytic.

- Hydrolytic enzymes such as lipase, chitinase and gelatinase were widespread amongst the *Vibrio* and *Aeromonas* isolates.

- Dominance of *V. alginolyticus* among the isolates from necrotic larvae and the failure in isolating them from rearing water strongly suggest that they infect larvae and multiply in the larval body and cause mortality in the hatchery. This was also supported by a pathogenicity test where *M. rosenbergii* larvae when challenged with a representative isolate of *V. alginolyticus* at 10^6, 10^7 and 10^8 cfu/ml, mortalities of 80, 87 and 100 per cent respectively were observed within 96 h. This observation suggested that the isolate *V. alginolyticus* was a pathogen to the larvae of *M. rosenbergii*.

Although variations in the antibiotic resistance patterns could be observed among the bacterial isolates, practically all of them proved to be resistant to erythromycin, the antibiotic frequently employed in hatcheries as a prophylactic agent. The incidence of bacterial resistance to oxytetracycline was comparatively higher (40%) followed by ampicillin (24%) and streptomycin (22%). It has to be pointed out that oxytetracycline is the next widely used antibiotic in hatchery.

In order to meet the requirements of rapid detection of *V. alginolyticus* in the larval rearing systems of *M. rosenbergii*, an indirect fluorescent antibody technique (IFAT) based on polyclonal antibodies was developed. Selection of the isolate of *V. alginolyticus* (MRNL 3) was based on the fact that the above isolate had caused severe larval mortality in the pathogenicity test.

The homologous agglutination titre of the antiserum raised against *V. alginolyticus* in rabbits was 4096, determined by the slide agglutination method, where as the titre of affinity- purified antiserum was only 32.

The undiluted antiserum reacted with all isolates of *V. alginolyticus* and *V. parahaemolyticus* whereas the diluted antiserum (1:100) did not react with *V. parahaemolyticus* and also did not cross-react with *Aeromonas* sp, *Pseudomonas* sp, *E. coli*, *Micrococcus* sp, *Bacillus* sp and *Salmonella typhi*.

Un-purified and purified antiserum, diluted to 1:128 and 1:32 respectively with PBS was found to give good fluorescence in IFAT when compared to other dilutions. The IFAT developed was found to be very selective in detecting *V. alginolyticus* alone in a batch of vibrios tested.

For developing antagonistic probiotic bacterial preparation a large collection of heterotrophic bacteria from different environments was screened against *Vibrio* spp. and *Aeromonas* sp. isolated from prawn larval rearing systems. As a result five isolates of antagonistic bacteria, which could inhibit the target pathogens, were segregated and four of them were identified as *Micrococcus* sp and one a

Pseudomonas sp. However, an appropriate antagonistic bacterium could not be isolated for two isolates of *V. splendidus* II.

All the isolates of *Micrococcus* exhibited gelatinase, amylase and cellulase activity but no lipase and chitinase production. The antagonistic *Pseudomonas* was lipase, gelatinase and cellulase producer having no potential for amylase and chitinase activity.

Co-culture experiments showed that the growth of *V. alginolyticus* was inhibited by *Pseudomonas* MCCB103 and *Micrococcus* MCCB104 inoculated at an initial count of 10^5 to 10^7 cfu/ml.

The preliminary characterization of inhibitory substance after treatments with α-chymotrypsin, trypsin, proteinase K, pronase E, lysozyme, lipase, catalase and α-amylase suggested that it was not a protein, lipid or carbohydrate, and did not contain hydrogen peroxide or any compound of alkaline or acidic in nature. Thermostability tests indicated that they were stable at 100°C.

In vivo effect of *Pseudomonas* MCCB103 and *Micrococcus* MCCB104 on vibrios was tested by adding the culture to prawn larval rearing water. The total *Vibrio* count determined subsequent to the addition of inoculum was significantly lesser ($p < 0.05$) than that of the control and the treatment resulted in increased per cent conversion of larvae to post-larvae, with slightly higher mean weight and mean length.

To sum up, through this work, nine species of *Vibrio* and genus *Aeromonas* associated with *M. rosenbergii* larval rearing systems could be isolated and segregated based on the haemolytic activity and the antibiotic sensitivity profile was determined. Highly specific and stable polyclonal antibodies (PAbs) for use in diagnosis or epidemiological studies could be produced, based on a virulent culture of *V. alginolyticus*. This could possibly replace the conventional biochemical tests for identification. As prophylaxis to vibriosis, four isolates of *Micrococcus* spp. and an isolate of *Pseudomonas* sp. could be obtained which could possibly be used as antagonistic probiotics in the larval rearing system of *M. rosenbergii*.

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