Biocontrol of *Vibrio harveyi* in *Penaeus monodon* Larval Rearing Systems Employing Probiotics and Vibriophages

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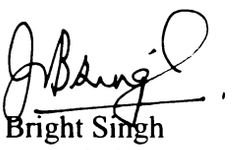
October 2006

Certificate

This is to certify that the research work presented in this thesis entitled "**Biocontrol of *Vibrio harveyi* in *Penaeus monodon* larval rearing systems employing probiotics and vibriophages**" is based on the original work done by Mr. S. Somnath Pai under my guidance, at the National Centre for Aquatic Animal Health, 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.



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Declaration

I hereby do declare that the work presented in this thesis entitled "**Biocontrol of *Vibrio harveyi* in *Penaeus monodon* larval rearing systems employing probiotics and vibriophages**" is based on the original work done by me under the guidance of Dr. I.S. Bright Singh, Professor in Microbiology & Coordinator-NCAAH, National Centre for Aquatic Animal Health, School of Environmental Studies & Cochin University of Science and Technology, Cochin- 682022, 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.

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CHAPTER - 1

Introduction and Literature Review

CHAPTER - 1

Introduction and Literature Review

Aquaculture is a form of agriculture that involves the propagation, cultivation, and marketing of aquatic animals and plants in a controlled environment (Swann 1992). It is a fast growing food sector which now accounts for almost 50% of world's food fish production (FAO 2006). With stagnating/ declining traditional fisheries, aquaculture promises the greatest potential to meet the growing demand of aquatic food. Aquaculture not only provides a sustainable source of aquatic food, but also provides meaningful livelihood to multitudes of poor since it is almost exclusively practiced in peri-urban or rural, remote areas (FAO 2006). Globally, penaeid shrimp culture ranks sixth in terms of quantity and second in terms of value amongst all taxonomic groups of aquatic animals cultivated (FAO 2006). The economic profitability of shrimp culture leads many aquarists to risk a substantial investment in it. Breakthroughs in shrimp larviculture during the 1970s ensured abundant and low cost seed enabling intensification of shrimp culture (Fast & Menasveta 2000). In places where warm-water aquaculture was possible black tiger shrimp, *Penaeus monodon* became preferred variety of shrimp cultivar owing to its fast growth, seed availability and importantly due to the high prices it fetches (Pechmanee 1997). This low cost and abundant seed availability of *P. monodon* was enabled through intensive larviculture in hatcheries (Jory 1997). Indian shrimp culture is dominated by *P. monodon* with the West Coast accounting for 70% of the production (Hein 2002). A vast majority of the culture systems in India are of the extensive and traditional type, followed by semi-intensive variety and hatchery produced seed is the main source of fry for stocking the ponds (Hein 2002). Intensive culture, apart from other problems, results in enhanced susceptibility of the cultured species to diseases (Jory 1997), which in fact have become the biggest constraint in shrimp aquaculture (FAO 2003). Penaeid shrimp production of the *P. monodon* and *P. vannamei* variety relies almost exclusively on hatchery produced seed and loss due to diseases has significant impact on not only the profitability of hatcheries, but also on grow-outs by way of increased input costs (FAO 2003). *P. monodon* larvae are susceptible to a host of

diseases, chief among them being vibriosis caused by various *Vibrio* spp., including *V. harveyi* (Lightner 1988, Alapide-Tendencia & Dureza 1997, Aguerre-Guzman et al. 2001, Austin et al. 2005, Alavandi et al. In Press).

1.1 Vibrios in aquatic systems and aquaculture

Vibrio cholerae, the causative agent of cholera, was the first *Vibrio* species to be discovered by the Italian physician Filippo Pacini (1812 to 1883) while studying the outbreaks of this disease in Florence (Thompson et al. 2004b). Although he pointed out rightly of its spread through contaminated water, few believed him since the miasmatic theory of disease was the belief of the day. Later the English physician John Snow was able to prove its spread through contaminated water when he successfully prevented the disease by providing pure tap water in areas where cholera was endemic (Thompson et al. 2004b). The Dutch microbiologist, Martinus Bejerinck reported the first nonpathogenic *Vibrio* species i.e. *V. fischeri*, *V. splendidus* and *Photobacterium phosphoreum* from aquatic environment (Thompson et al. 2004b).

The second edition of Bergey's Manual of Systematic Bacteriology describes members of the *Gammaproteobacteria* family *Vibrionaceae* as gram negative, usually motile rods, mesophilic and chemoorganotrophic, possessing a facultative fermentative metabolism, and are found in aquatic habitats and in association with eukaryotes (Farmer III & Janda 2005). They generally grow on marine agar and the *Vibrio* selective medium thiosulfate-citrate-bile salts-sucrose agar (TCBS) and are mostly oxidase positive. Sixty seven species are included in this genus so far. The Manual lists *Vibrio harveyi* and *V. carchariae* as synonyms following establishment of homogeneity in their 16S rDNA sequences (Gauger & Gomez-Chiarri 2002). Whole genome fingerprinting studies of these two strains employing robust techniques such as amplified fragment length polymorphism (AFLP), DNA:DNA hybridization and ribotyping also failed make distinctions between them (Pedersen et al. 1998). In contrast, the high degree of heterogeneity in their biochemical profiles identifies them as two species thus making this methodology erroneous to identify them (Alsina & Blanch 1994).

Vibrios are abundantly isolated from estuaries, marine waters and sediment and aquaculture settings globally where they occur either as free-living or associated with organisms such as corals (Rosenberg & Ben-Haim 2002), fish (Muroga 2001, Toranzo et al. 2005), molluscs (Torkildsen et al. 2005), seagrass, sponges, shrimp (Gomez Gil et al. 1998, Vandenberghe et al. 2003, Jayaprakash et al. 2006a) and zooplankton (Heidelberg et al. 2002). Some of them like *Photobacterium leiognathi* and *P. phosphoreum* are found in symbiotic relationship with fish and *P. leiognathi*, *V. fischeri* and *V. logei* are symbionts of squid (McFall-Ngai 1999). In their eukaryotic partners, they colonize the light organs and play a role in communication, prey attraction, and predator avoidance (Fidopiastis et al. 1998, McFall-Ngai 1999).

The external surfaces of marine organisms particularly those of zooplankton harbour large numbers of vibrios (Heidelberg et al. 2002). To date all vibrios are chitinolytic utilizing it both as a carbon and nitrogen source (Heidelberg et al. 2002) and they play a significant role in the mineralization of chitin in aquatic systems (Lipp et al. 2002). A symbiotic association between vibrios and zooplankton has not been ruled out (Nishiguchi & Nair 2003). While the zooplankton may feed off the biofilms formed by vibrios on their surfaces(Thompson et al. 2004b), the bacteria in turn are provided with chitin and cryoprotection at lower temperatures (Lipp et al. 2002). Copepods harbor a high density of *Vibrio* in their guts and on their surfaces (Sochard et al. 1979).

Many *Vibrio* species are ubiquitous in aquaculture settings associated with all cultured species (fish, molluscs, crustaceans) (Ramesh et al. 1986, Alapide-Tendencia & Dureza 1997, Verdonck et al. 1997, Vandenberghe et al. 1998, Thompson et al. 2001, Vandenberghe et al. 2003, Jayaprakash et al. 2006a). A major problem in elucidating their diversity has been the difficulties associated with their identification. Classical methods of phenotyping and fatty acid methyl ester (FAME) profiling can differentiate isolates at genus level but profiles are conspicuously similar among different species within the *Vibrio* genera, making it impossible for species delineation (Bertone et al. 1996, Ottaviani et al. 2003, Thompson et al. 2004b). Moreover it is difficult to compare

FAME profiles generated in different labs due to lack of standard methods of strain cultivation and FAME extraction (Bertone et al. 1996). Biolog studies have revealed a high variation amongst vibrios for a large number of key phenotypic tests like arginine dihydrolase, lysine and ornithine decarboxylases, susceptibility to the vibriostatic agent O/129, flagellation, indole production, growth at different salinities and temperatures, and carbon utilization (Vandenberghe et al. 2003) making identification solely based on phenotypic characters near impossible (Thompson et al. 2004b). The 16S rRNA sequence of small subunit of the ribosome has been widely used to classify and reorganize the entire bacterial taxonomic groups (Kita-Tsukamoto et al. 1993, Wiik et al. 1995). However, the 16S rDNA sequences of members of the genus *Vibrio* are similar at 99.3 % (Kita-Tsukamoto et al. 1993), thereby practically negating its use to delineate species within this genus (Thompson et al. 2005). Currently, the proposed procedure for the identification of vibrios is to make a preliminary grouping into different genera based on the 16S rDNA sequence and phenotypic analyses followed by species delineation using amplified length polymorphic DNA (AFLP), rep-PCR, or *rpo*A, *atp*A, and *rec*A sequences (Ottaviani et al. 2003, Thompson et al. 2004a, Thompson et al. 2004b, Thompson et al. 2005).

Vibrios are amongst the most important bacterial pathogens of aquatic animals in culture (Toranzo et al. 2005). *Listonella* (*Vibrio*) *anguillarum* causes hemorrhagic septicemia in Pacific and Atlantic salmon (*Oncorhynchus* spp. and *Salmo salar*) (Garcia et al. 1997), rainbow trout (*Oncorhynchus mykiss*) (Rasch et al. 2004), turbot (*Scophthalmus maximus*) (Olsson et al. 1998), seabass (*Dicentrarchus labrax*) (Angelidis et al. 2006), seabream (*Sparus aurata*) (Balebona et al. 1998b), striped bass (*Morone saxatilis*) (Lemos et al. 1988), cod (*Gadus morhua*) (Sorensen & Larsen 1986), Japanese and European eel (*Anguilla japonica* and *A. anguilla*) (Rodsaether et al. 1977, Nakai et al. 1987), and ayu (*P. altivelis*) (Kanno et al. 1990). *V. salmonicida* causes "Hitra disease" or cold water vibriosis affecting salmonids and cod cultured in Canada, Norway and United Kingdom (Egidius et al. 1981). As the name of the disease suggests, *V. salmonicida* can grow only at temperatures below 15°C (Colquhoun & Sorum 2001). *V. vulnificus* biotype 2 are pathogenic to eel and occasionally to humans therefore putting aquarists at risk as

well (Amaro & Biosca 1996). Infections and mortality of *Puntazzo puntazzo* caused by *V. vulnificus* biotype 1, *V. splendidus* biovar I, and *V. alginolyticus* have been reported from Greece (Athanassopoulou et al. 1999). Two new psychrotrophic vibrios, *V. viscosus* (reclassified as *Moritella viscosa*) and *V. wodanis* have been associated with "winter ulcer" disease affecting sea farmed Atlantic salmon during winter season (Bruno et al. 1998, Benediktsdottir et al. 2000, Lunder et al. 2000). Cultures of the Iberian toothcarp, *Aphanius iberus* and grouper, *Epinephelus coiides* are afflicted by mortalities due to *V. parahaemolyticus* (Yii et al. 1997, Alcaide et al. 1999).

Molluscs of all species harbour a wide array of diverse bacterial taxons and where members of *Vibrionaceae* dominate (Sunen et al. 1995, Pujalte et al. 1999, Hernandez-Zarate & Olmos-Soto 2006). Oysters, mussels, clams are economically important cultured species in many parts of Europe, North and South America, and Japan and vibrios are major pathogens (Le Moullac et al. 2003). Mortalities in the oyster *Crassostrea gigas* due to *V. splendidus* biovar II and *V. tubiashii* have been reported (Gibson et al. 1998, Sugumar et al. 1998). *V. alginolyticus* has been reported to cause mortalities of scallop, *Argopecten purpuratus* and is toxic towards haemocytes of the mussel, *Mytilus edulis* (Riquelme et al. 1996, Lane & Birkbeck 1999). A brown ring disease in clams is caused by *V. tapetis* (Allam et al. 2002, Paillard 2004). Contamination of oysters, mussels and clams with vibrios, such as *V. vulnificus* and *V. parahaemolyticus* carries a potential risk of food poisoning amongst consumers (DePaola et al. 1997). Domestic and industrial sewage pollution of coastal waters furthers the risk of bivalves in these areas being dominated with human pathogens including *V. cholerae*, *Salmonella* sp. etc (Sunen et al. 1995, Dalsgaard 1998, Lozano-Leon et al. 2003).

Vibriosis is a major disease caused by *Vibrio* spp. afflicting all varieties of shrimps in culture at all stages (Lightner 1988, Singh et al. 1989, Singh 1990, Singh et al. 1998, Jayaprakash et al. 2006a). Vibrios are richly isolated from shrimps with diseases such as 'Red Disease Syndrome', 'Luminescent vibriosis', 'Bolitas negricans', 'Summer Syndrome', 'Penaeid bacterial septicemia', 'Red Leg Disease', 'Shell disease', 'Brown spot disease', 'Black spot disease', 'Burned spot disease', and 'Rust disease' (FI-SH

pharma inc website - URL in reference list, Fisheries and Oceans Canada website - URL in reference list.). The species identified from diseased and healthy *P. monodon* samples are *V. aestuarianus*, *V. alginolyticus*, *V. anguillarum*, *V. campbelli*, *V. cholerae*, *V. costicola*, *V. damsela*, *V. fischeri*, *V. fluvialis*, *V. furnissii*, *V. haloplanktis*, *V. harveyi*, *V. hollisae*, *V. ichtyoenterii*, *V. logei*, *V. mediterranei*, *V. metschnikovii*, *V. natriegens*, *V. nigripulchritudo*, *V. parahaemolyticus*, *V. pelagius*, *V. penaeicida*, *V. protelyticus*, *V. splendidus*, *V. tubiashii*, *V. vulnificus* (Lightner 1988, Lavilla-Pitogo et al. 1990, Song et al. 1993, Alapide-Tendencia & Dureza 1997, Goarant et al. 1998, Vandenberghe et al. 1998, Sung et al. 1999, Sung et al. 2001, Goarant et al. In Press).

Pathogenesis of *Vibrio* may be of primary or secondary in nature (Lightner 1992). Detection of vibrios routinely from healthy and diseased shrimp led researchers to categorise them as opportunistic pathogens (Saulnier et al. 2000). Opportunistic vibrios may cause serious problems in shrimp larvae when they are suffering from stress caused by suboptimal or unstable environment, high stocking densities and inadequate management (Sung et al. 2001). The mode of infection of vibrios is hypothesized to be a three step process viz. using chemotactic motility the bacterium penetrates the host tissues, once inside the bacterium deploys its iron sequestering systems (eg. siderophores) to 'steal' iron from the host and finally causes death by damaging host tissues using its extracellular products (eg. hemolysins, proteases) (Larsen et al. 2001).

Vibrio harveyi is a serious pathogen of both vertebrates and invertebrates in the marine environment and culture systems. Amongst fishes it is pathogenic to cobia fish (*Rachycentron canadum* L.), grouper (*Epinephelus tauvina*), summer flounder (*Paralichthys dentatus*), salmon (*Salmo salar*), seahorse (*Hippocampus* sp.), gilt-head sea bream (*Sparus aurata* L.), sand bar shark (*Carcharhinus plumbeus*), silvery black porgy (*Acanthopagrus cuvieri*), snook (*Centropornus undecimalis*), and trout (*Oncorynchus mykiss*) (Grimes et al. 1984, Kraxberger-Beatty et al. 1990, Saeed 1995, Balebona et al. 1998b, Zhang & Austin 2000, Alcaide et al. 2001, Liu et al. 2004, Gauger et al. 2006). Amongst crustaceans, mass mortalities amongst zoeal larvae of the swimming crab (*Portunus trituberculatus*) have been attributed to this bacterium (Ishimaru & Muroga

1997). At the Fisheries College and Research Institute, Tuticorin, India, a *V. harveyi* –like bacterium was isolated from lesions on the exoskeleton of an Indian spiny lobster (*Panulirus homarus*), but mortalities in the population were < 10% (Abraham et al. 1996). Similarly, the larval stages (phyllosoma) of the rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) suffer mortalities due to infection by *V. harveyi* (Diggles et al. 2000).

Penaeid shrimp culture in tropical and sub-tropical countries suffers vastly due to mortalities caused by *V. harveyi* (Austin & Zhang 2006). The larval stages have enhanced susceptibility compared to adults and 100% mortality can happen overnight giving little time for medication (Soto-Rodriguez et al. 2003). Mortalities of penaeid larvae and adult have been reported from Ecuador (Robertson et al. 1998), Indonesia (Hisbi et al. 2000), Mexico (Roque et al. 2001), Philippines (Lavilla-Pitogo et al. 1990), Taiwan (Liu et al. 1996b), Thailand (Thaithongnum et al. In Press), and Venezuela (Alvarez et al. 1998). Mass mortalities due to *V. harveyi* are also rampant in Indian penaeid aquaculture in all farming regions (Abraham et al. 1997, Abraham & Palaniappan 2004, Alavandi et al. In Press). In fact Indian coastal waters and estuaries harbor high densities of this organism (Nair et al. 1979, Chari & Dubey 2006).

The virulence factors of *V. harveyi* have been a subject intense study and over the years many virulence factors have been identified. The extracellular products (ECPs) of *V. harveyi* have been reported to contain hemolysins and a variety of proteases and other hydrolytic enzymes (Zhang & Austin 2005). Gelatinase, lipase, phospholipase, siderophores, cystein protease, metalloprotease, hemolysins are the key ECPs detected and studied so far (Liu et al. 1997, Lee et al. 1999, Liu & Lee 1999). Except for gelatinase, lipase and phospholipase, all other compounds were found toxic to *P. monodon* adult or larvae in laboratory experiments (Liu et al. 1996a, Soto-Rodriguez et al. 2003). The transmembrane transcriptional regulator, *tox*R which coordinates the regulation of virulence gene expression in addition to transcription of genes coding for outer membrane porins in *V. cholerae* is also present in *V. harveyi* and could mediate the expression of virulence genes (Conejero & Hedreyda 2003). Some strains of *V. harveyi*

could be virulent if they play host to the lysogenic bacteriophage (Ruangpan et al. 1999). A lysogenic phage VHML has been found to be associated with some virulent strains of *V. harveyi* isolated from *P. monodon* culture systems (Oakey & Owens 2000). Sequence analysis of the genome of this phage has the presence of a number transcriptional regulators and a putative N6-Dam (DNA adenine methyltransferase) protein in ORF 17 (Oakey et al. 2002). This translated sequence of this ORF also has a region that codes for a protein with a site similar to the active site for ADP-ribosylating toxin (Oakey et al. 2002). Methyltransferases have been found necessary for the viability and virulence in *Yersinia pseudotuberculosis* and *V. cholerae* (Julio et al. 2001). Recently, a bacteriophage (VHS1) belonging to the family Siphoviridae was found to enhance virulence of *V. harveyi* (Pasharawipas et al. 2005). The phage however was exhibiting a hitherto unreported phenomenon which the authors explained as pseudolysogeny i.e. the phage DNA was not getting integrated with the hosts' but caused modifications which made the phage refractory strains to be more virulent (Khemayan et al. 2006).

Use of antibiotics has been the method of choice amongst shrimp culturists to protect their crop from luminous disease. Early studies pertaining to the control of bacterial pathogens in shrimp aquaculture were directed towards the selection of appropriate antibiotics (Baticados et al. 1990, Hameed & Rao 1994). Antibiotics used in aquaculture are amoxicillin, benzylpenicillin, co-trimazine, enrofloxacin, florfenicol, flumequine, oxolinic acid, oxytetracycline, sarafloxacin, trimethoprim sulphadiazine (Alderman & Hastings 1998, Roque et al. 2001). However, the use of antimicrobials has been largely prophylactic in aquaculture (Baticados & Paclibare 1994, Cabello 2006) their growing use has been a cause of concern (Teuber 2001). In fact use of antimicrobials to save the animals once infection sets in is believed to be futile since infected animals stop feeding (Smith et al. 1994). In *V. harveyi* infection experiments conducted with *Artemia franciscana* nauplii, prophylactic as well as therapeutic enrofloxacin administration were able to reverse the course of infection even in applications made 24 hours after infection (Roque & Gomez-Gil 2003). In contrast, medication was of little use of when shrimp larvae were infected with *V. harveyi* since mortalities were 100% overnight (Prayitno & Latchford 1995). It has been reported that most hatchery operators and shrimp farmers

use a battery of 7-10 antibiotics sequentially irrespective of scientific considerations (Graslund & Bengtsson 2001, Graslund et al. 2003). In a survey conducted in Thailand, it was found most farmers had little knowledge of susceptibility patterns, minimum inhibitory concentrations let alone environmental hazards and human health risks (Graslund et al. 2003). Antibiotic use against bacterial infections must be carried out only after careful analysis of the LC50 and MIC values of the antibiotics. This is necessary to screen out antibiotics which have higher MIC values than LC50 (Soto-Rodriguez et al. 2006a). Unsurprisingly, such haphazard use of antibiotics has resulted in the emergence of *V. harveyi* and other *Vibrio* spp. strains with multiple antibiotic resistance (Holmstrom et al. 2003).

Multiple antibiotic resistant (MAR) *V. harveyi* has been isolated from penaeid culture systems (Karunasagar et al. 1994, Abraham et al. 1997, Roque et al. 2001). *V. harveyi* isolated from Mexican shrimp farms were resistant to 70% of the antibiotics tested (Roque et al. 2001). Sixty percent of *Vibrio* isolated from *Artemia* nauplii reared in a penaeid hatchery in India were resistant to erythromycin, nitrofurazone and oxytetracycline (Hameed & Balasubramanian 2000). In both these studies higher resistance amongst isolates was observed toward antibiotics used in human medicine than in aquaculture. High levels of resistance to oxolinic acid and oxytetracycline amongst *V. harveyi* isolates from shrimp farms in Philippines (Tendencia & de la Pena 2001). The transposon Tn*1721* carrying *tet*A, *tet*R genes and novel β-lactamases, antibiotic resistance determinants that confer resistance to tetracyclines and β-lactams, have been detected in *V. harveyi*, thus explaining their high resistance to these antibiotics (Teo et al. 2000, Teo et al. 2002). Multiple antibiotic resistances have also been linked to enhanced virulence of *V. harveyi* since, MAR strains are also associated with mass mortalities of penaeid larvae (Karunasagar et al. 1994, Abraham et al. 1997).

The large scale resistance to antibiotics observed in bacteria isolated from aquaculture systems coupled with human and environmental concerns pertaining to the spread of resistance to human pathogens and persistence of residues in tissues has invited legislations restricting their use in many countries (Holmstrom et al. 2003). The European

Union, United States of America and Japan importers of shrimp being produced in tropical countries have banned the presence of certain antibiotics and set maximum residual limits (MRL) for others in the meat of shrimp being imported (Alderman & Hastings 1998, Pakshirajan 2002). Following many antibiotics have been banned from use in shrimp culture in India too (Pakshirajan 2002). This has necessitated a search for alternative treatment and management strategies to manage the occurrence and spread of *V. harveyi* in shrimp culture systems in hatcheries as well as farms. Such strategies include the use of immunostimulants, vaccines, probiotics and phage therapy. These however are in their infancy in aquaculture and it may be some more time before they become the norm. Adoption of immunostimulants, vaccines and probiotics coupled with good water quality management strategies to manage diseases of animals in culture for more than a decade now in Norway has yielded encouraging results (Grugel & Wallmann 2004). Practice of polyculture where seabass, snapper, grouper, milk fish, and tilapia are reared along with *P. monodon* resulted in reduction in *V. harveyi* counts (Tendencia & de la Pena 2003, Tendencia et al. 2006a, Tendencia et al. 2006b). The exact nature and mechanism of inhibition could not be elucidated (Tendencia et al. 2004).

1.2 Probiotics in aquaculture

Encountering antimicrobial resistant and difficult to treat bacterial pathogens in shrimp larviculture and grow outs prompted studies in evolving strategies to manipulate and control the microbial environment. One of the strategies currently gaining confidence in the industry was to use beneficial or probiotic bacteria in prophylactic and therapeutic treatment of diseases and have been reviewed extensively in recent times (Fuller 1989, Ringo & Gatesoupe 1998, Gatesoupe 1999, Gomez-Gil et al. 2000, Verschuere et al. 2000b, Irianto & Austin 2002a, Ouwehand et al. 2002, Isolauri et al. 2004, Balcazar et al. 2006, Vine et al. 2006, Farzanfar In Press).

Probiotic bacteria were defined as a "live microbial feed supplement which beneficially affects host animal by improving its intestinal microbial balance" by Fuller (1989). Another definition proposed by Salminen et al. (1999) states "Probiotics are microbial

cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host". This definition includes non-viable cells also since they also have been shown to elicit beneficial effects, but does not include metabolites. Gatesoupe (1999) proposed the definition "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health". All these definitions have a bias towards the gastrointestinal tract as the site of probiotic delivery and activity. In aquatic systems, ambient microbial flora plays a significant role in the microbial population within the animals including the gastrointestinal tract. The flora associated with larvae is not very stable and is influenced by the bacterial flora of the administered food and by the environment (Vandenberghe et al. 1998). Therefore manipulation of the microbial community of the environment could induce positive impact on the health survival of aquatic animals. Taking this into consideration Verschuere et al. (2000b) proposed the definition "a probiotic is defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" to include all probiotics that beneficially modify the community within the host and outside it. The definition does not include microbial cells which are used as single cell proteins and those that do not interact with the host or other bacteria in the environment.

Microbial interactions play a key role in the well being of aquatic animals. Aquaculture practices such as discontinuous culture cycles, disinfection or cleaning of tanks prior to stocking, sudden nutrient fluctuations do not allow establishment of stable microbial communities (Skjermo & Vadstein 1999). Both deterministic and stochastic factors influence the establishment of microbial communities in aquaculture environment. Deterministic factors have a well defined dose-response relationship and include salinity, temperature, oxygen concentration, and quality and quantity of feed. On the other hand stochastic factors i.e. the microorganisms present in the system do not have a dose-response relationship. We can only obtain a probability range of values for any given value of the stochastic factor, since chance favours organisms which are in the right place

at the right time to enter a habitat and to proliferate if conditions are suitable (Verschuere et al. 1997, Verschuere et al. 2000b). Dominance of r-strategists in the larval rearing environment has been observed to positively correlate with rearing success (Verschuere et al. 1997).

The fact that both environmental conditions and chance influence microbial communities opens up possibilities of using probiotics as biological conditioners of rearing water. Instead of allowing spontaneous colonization of the rearing water by accidentally present bacteria, preemptive colonization, the water could be preemptively colonized by the addition of probiotic bacteria, since it has been recognized that preemptive colonization may extend the rein of pioneer organisms (Atlas & Bartha 1997). This hypothesis has been strengthened when PCR-DGGE profiles of bacteria isolated from water, egg, feed and juvenile gastrointestinal tract revealed identical bands pertaining to *Pseudomonas* and *Aeromonas* in influent water, egg and juvenile gastrointestinal samples (Romero & Navarette 2006). This suggests a stable microbiota is established after the first feeding stages and its composition could be derived from water and egg biota (Romero & Navarette 2006). It has been shown that preemptive treatment of *Artemia* juveniles with nine probiotic strains protected them from a virulent strain of *V. proteolyticus* (Verschuere et al. 1999, Verschuere et al. 2000a). Although lactic acid bacteria are not natural intestinal flora of fish, they have been shown to colonize the intestines of Atlantic cod and salmon, rainbow trout, and other fishes (Ringo & Gatesoupe 1998).

The probiotic properties of lactic acid bacteria, *Bacillus* sp., *Pseudomonas* sp., in protecting fish and shellfish from pathogens were studied during the early periods of research in this area (Gatesoupe 1991, Smith & Davey 1993, Gatesoupe 1994, Moriarty 1998). The genera identified as potential probiotics has however expanded over the years to include species such as *Aeromonas hydrophila*, *A. media*, *Carnobacterium*, *Clostridium butyricum*, *Debaryomyces hansenii*, *Micrococcus*, *Pseudomonas aeruginosa*, *P. fluorescens*, *Roseobacter*, *Saccharomyces boulardii*, *S. cerevisiae*, *Streptococcus*, *Teraselmis suecica*, *Vibrio alginolyticus*, *V. fluvialis*, *Weissella*, (Irianto & Austin 2002a, Patra & Mohamed 2003, Jayaprakash et al. 2005, Planas et al. 2006, Zeng-fu et al. 2006).

In Philippines, "Green water" culture has been reported to significantly reduce *Vibrio* populations particularly that of *V. harveyi* in shrimp culture (Tendencia & de la Pena 2003, Lio-Po et al. 2005).

Although *Bacillus* spp. are frequently isolated from intestines of aquatic animals, they are presumed to be of telluric origin (Nicolas et al. 2004). Nevertheless many members of this genus are widely used as probiotics in aquaculture. Presence of cellulase producing *Bacillus* sp in intestines of fish enables them to digest plant cellulose since fish do not produce the enzyme (Saha et al. 2006). An improvement in survival of trout was observed by means of enhanced adhesion in the intestines by *B. animalis* (Ibrahim et al. 2004). Probiotic activity of *Bacillus* sp. has been shown to improve survival of penaeid adults in ponds and larvae in hatchery. Some of the *Bacillus* used in these studies exhibited antagonism towards *V. harveyi* and improved survival of *P. monodon* in grow outs (Moriarty 1998). Use of the commercial probiotic 'Protexin Aquatech' which contains a mixture of *B. circulans*, *B. laterosporus*, *B. licheniformis*, *B. polymyxa*, and *B. subtilis* in *Fenneropenaeus indicus* culture beginning from naupliar stages in hatchery to adults in grow outs improved growth and survival (Ziaei-Nejad et al. 2006). In this study, the specific enzymatic activities of amylase, protease and lipase were significantly higher in shrimp that received the probiotic either through feed or water. In challenge tests conducted with *V. harveyi* on pond reared *P. monodon* following prior feeding with the probiont *Bacillus* S11 significantly enhanced growth, immunity and survival (Rengpipat et al. 1998, Rengpipat et al. 2000, Rengpipat et al. 2003). A *B. subitilis* strain BT23 protected *P. monodon* from *V. harveyi* (Vaseeharan & Ramasamy 2003). Oral administration of *Bacillus* sp. through feed improved digestibility in *Litopenaues vannamei* and better growth and survival were achieved (Lin et al. 2004).

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 (Mazzola et al. 1992), hydrogen cyanide (Westerdahl et al. 1991), or iron-chelating siderophores (Loper & Buyer 1991). Fluorescent pseudomonads produce phenazine and other antimicrobial compounds which

have broad spectrum activity against many phytopathogenic fungi and eubacteria (Mavrodi et al. 2006). Pseudomonads have also been documented as the dominant flora in the eggs and larvae of shrimps which successfully completed larval cycles in hatchery systems (Singh et al. 1989, Smith & Davey 1993). As with their terrestrial counterparts, aquatic pseudomonads are often antagonistic against other microorganisms (Lemos et al. 1985), including fish pathogenic bacteria (Gram 1993, Smith & Davey 1993) and fungi (Bly et al. 1997). Gram et al. (1999a), demonstrated the protection of rainbow trout administered with *Pseudomonas fluorescens* AH2 when challenged with *Vibrio anguillarum*. Studies by Smith & Davey (1993) demonstrated that bathing of Atlantic salmon in a suspension of *P. fluorescens* reduced subsequent mortality from stress-induced furunculosis. *P. aeruginosa* was found to inhibit shrimp pathogenic *V. harveyi*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis* and *Aeromonas* sp. (Toranzo & Torres 1996, Chythanya et al. 2002, Vijayan et al. 2006). Their studies assigned their antimicrobial properties to production of iron-chelating siderophores and pyocyanin.

Among antagonistic bacterial cultures known to be useful in aquaculture, *Micrococcus* has generated interest only recently, even though its association with fish aquatic animals in culture has been documented by Austin & Allen (1982) and Prieto et al. (1987) in dehydrated *Artemia* cysts, cyst-hatching water, and *Artemia salina*. Lalitha & Surendran (2004) reported *Micrococcus* to be a normal flora in the environment of the farmed freshwater prawn *Macrobrachium rosenbergii*. An antagonistic Gram-positive coccus, A1-6 was isolated from the intestines of healthy rainbow trout. The strain could confer protection to the fish in challenge tests conducted with *Aeromonas salmonicida* (Irianto & Austin 2002b). Jayaprakash et al. (2005) reported antagonistic activity of a marine *Micrococcus* strain MCCB104 against a wide range of vibrios isolated from *M. rosenbergii* culture systems.

A few vibrios and aeromonads have also been shown to exhibit probiotic activity. *Vibrio alginolyticus* has been used in Ecuadorian shrimp farms as a probiotic and has been shown to control diseases caused by *A. salmonicida*, *V. anguillarum* and *V. ordalli* (Austin et al. 1995). The potential probiont *V. alginolyticus* C7b, grew well in coculture

with the microalga *Chaetoceros muelleri* (Gomez-Gil et al. 2002). A strain of this bacterium was also shown to antagonise the growth of *V. harveyi* in vitro (Ruangpan et al. 1998). *V. fluvialis* too was shown to have probiotic activity in rainbow trout (Irianto & Austin 2002b). A *V. harveyi* strain VIB571 was found to produce a proteinaceous 32 kDa bacteriocin-like inhibitory substance (BLIS) which inhibited *V. fischeri*, *V. gazogenes* and *V. parahaemolyticus* (Prasad et al. 2005). Gibson et al. (1998) observed the probiotic effects of *A. media* in the Pacific oyster, *Crassostrea gigas* when challenged with *V. tubiashii*. These studies notwithstanding, *V. alginolyticus* is a pathogen of shrimp (Lee et al. 1996, Jayaprakash et al. 2006b), clam (Gomez-Leon et al. 2005), seabream (Balebona et al. 1998a). It was shown that *V. alginolyticus* is responsible for the production of tetrodotoxin in the intestines of fish *Fugu vermicularis vermicularis* (Noguchi et al. 1987). Its pathogenic profile made it the target for screening potential probiotics in other studies (Rico-Mora et al. 1998, Villamil et al. 2003). Therefore use of such strains as probiotics must be cautiously approached.

Selection of an appropriate probiont to achieve specific targets is crucial to obtain successful disease prevention or therapy (Gomez-Gil et al. 2000, Verschuere et al. 2000b). Broadly the selection criteria should consist of

(i) acquiring strains with antagonistic properties against aquatic animal pathogens and/or colonization abilities in the in the intestine or external surfaces of aquatic animals,

(ii) taxonomic identification and collection of background information on the strains,

(iii) in vitro ability of the putative probionts to inhibit and/or outcompete pathogens,

(iv) no pathogenicity / toxicity of selected probionts to any morphogenetic stage of host animals,

(v) in vivo ability of the probiotics to confer protection from disease due to the pathogen,

(vi) non-toxicity of the putative probiotic to non target species, other animals in the environment, and humans,

(vii) an economic cost-benefit analysis.

Screening for antagonism in environmental bacteria against pathogens by in vitro plate assays has been widely used (Verschuere et al. 2000b, Hjelm et al. 2004a). However, currently selection based on properties such as adhesion and colonization to intestine, skin and other surfaces and growth parameters (competition for nutrients) are gaining importance (Vine et al. 2004b). The hypothesis is that preemptive colonization of the intestine and other portals of entry of pathogens by autochthonous bacteria with/ without antagonism but with better adhesion, colonization, and growth characteristics compared to pathogens can prevent pathogen invasions and improve survivals (Irianto & Austin 2002b, Hjelm et al. 2004b, Vine et al. 2004a). Intestines of healthy aquatic animals harbor bacteria such as *Acinetobacter*, *Bacillus*, *Carnobacterium*, *Pseudomonas*, *Roseobacter*, *Shewanella*, and a few species of enterobacteriaceae (Spanggaard et al. 2001, Hjelm et al. 2004b). The dominance of members of Vibrioneaceae is observed in diseased or moribund animals (Singh et al. 1989).

Two major pitfalls of in vitro antagonism based selection of potential probionts are (i) this property may not be elicited under in vivo conditions, and (ii) in vitro antagonism of a pathogen by a probiotic strain need not necessarily confer in vivo protection of the cultured fish or shellfish from the pathogen. In fact in vivo antagonism by probiotic bacteria is yet to be demonstrated in any aquatic animal host-pathogen system. In vitro antagonism of the probiont *Psedomonas fluorescens* strain AH2 against *Aermonas salmonicida* did not protect Atlantic salmon, *Salmo salar* from furunculosis (Gram et al. 2001). Bath treatments of trout, *Oncorynchus mykiss* with *Pseudomonas* strain MT5 failed to treat or prevent mortalities due to *Flavobacterium columnare* infection (Suomalainen et al. 2005). Despite the use of specific and highly sensitive molecular detection tools, the authors failed to detect the *Pseudomonas* strain in tissues of the fish at any stage during the 7-day experimental period indicating lack of adhesion and colonization (Suomalainen et al. 2005). Protection by *Bacillus* spp. may not be universal against all pathogens in all fish and shellfish species. The administration of *B. toyoi* through feed did not reduce mortalities from *Edwardsiella tarda* in the European eel

Anguilla anguilla (Chang & Liu 2002). Therefore, as with antibiotics, probiotics also need to be screened against individual host-pathogen systems.

Alavandi et al. (2004) did not obtain immunity enhancement in *P. monodon* administered *Pseudomonas* strain PM11 at 10^3 cells/ml by supplementation of rearing water every 3 and 7 days. They observed their results to be in contrast to that obtained earlier by Rengipat et al. (2000) in experiments where *P. monodon* was fed daily for 90 days a 1:3 (wet weight) of *Bacillus*:Feed mixture. The above studies seem to point out that either for obtainging immunity enhancement, probiotics may need to delivered via feed so that they directly reach the intestine or, it may also be a pointer that *Pseudomonas* PM11 may not be immunostimulatory. Alavandi et al. (2004) contended that delivery of probiotic bacteria along with feed poses the problem of viability loss of bacteria. However experiments conducted by administering dead probiotic bacteria as feed additives to rainbow trout conferred protection when challenged by *A. salmonicida* (Irianto & Austin 2003). Similarly (Taoka et al. 2006) also observed improvement in survival of tilapia, *Oreochromis niloticus* following administration of live and dead cells of *Bacillus subtilis*, *Clostridium butyricum*, *Lactobacillus acidophilus*, and *Saccharomyces cerevisiae* in a mixture. In both the above studies non-specific immune parameters such as phagocytic activity, lysozyme activity, migration of neutrophils, and plasma bactericidal activity were observed to increase. Therefore, viability of probiotic bacteria for host protection may not be an absolute necessity and the definition may need to be modified to include dead bacteria also as probiotics.

1.3 Bacteriophages as therapeutic agents

Bacteriophages are bacterial viruses that invade bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism and cause bacterium to lyse. They are ubiquitous in the aquatic environment but their abundance is subject to seasonal changes (Wommack et al. 1992), physiochemical changes associated with depth (Paul et al. 1991) and along trophic gradients (Weinbauer et al. 1993). It is hypothesized that they may play a major role in the regulation of bacterial populations in the aquatic environments (Suttle 1994).

Phages are classified into 13 families based on morphology, type of nucleic acid, and presence or absence of an envelope or lipid. Out of 5100 bacterial viruses described so far, about 96% are "tailed phages" composed of an icosahedral head and tail and double stranded DNA as the genome (Ackermann 2001). Tailed phages are grouped in the order *Caudovirales* which consists of three families based on their tail features: *Myoviridae* (contractile tail eg. T-even phages, KVP40), *Siphoviridae* (long, non-contractile tail, eg. λ), and *Podoviridae* (extremely short tail, eg. T7) (Maniloff & Ackermann 1998). All other phages which constitute the remaining 4% of the total, are classified into ten families. Most of the therapeutic phages are tailed, although some cubic (eg. ϕX174), and filamentous (eg. Pf3) phages have also been reported for therapeutic uses (Ackermann 2001).

According to their life cycle, phages are divided into two types – lytic and lysogenic. Lytic phages repeat a cycle in which self-proliferation is synchronous with the destruction of bacteria (eg. T-even phages). Lysogenic phages have a 'lysogenic phase' in their lytic cycle during which their genome is integrated into host genome and multiplies cooperatively with the host bacteria without destroying it (eg. λ). Bacteria that harbour such phages are termed as 'lysogens' and they are resistant to infection by phages that are genetically related to the previously lysogenized phage (Matsuzaki et al. 2005). When under environmental pressures like UV radiation, presence of mitomycin C lysogenic phages may excise themselves from the host genome and start their lytic cycle and ultimately kill the host (Oakey & Owens 2000). Some lysogenic phages are known to carry toxin genes (eg. cholera toxin) in their genome (Wagner & Waldor 2002), and for this reason they are thought to be unsuitable for therapeutic uses (Matsuzaki et al. 2005).

The first step of phage infection is adsorption to its receptor, usually a protein or sugar on the bacterial surface. Phages can be specific to their bacterial host (strain) or polyvalent (capable of infecting across bacterial species or genera). Following adsorption, phage DNA is injected into the cytoplasm, replicated, phage proteins synthesized, and multiple copies of DNA taken into the capsid (constructed de novo). Once packaging of DNA is

completed tail is attached (also synthesized de novo) and progeny phages are liberated by the coordinated action two phage encoded proteins, holin and endolysin (lysin) (Young 1992). While holin protein makes holes in the cell membrane, lysin degrades the peptidoglycan in the cell wall (Wang et al. 2000).

The earliest report of bacteriophage like substance dates back to 1896, when Ernst Hankin, a British bacteriologist observed marked antibacterial activity against *Vibrio cholerae* in the waters of Ganga and Yamuna rivers in India by an unidentified substance which could pass through fine porcelain filters and was heat labile (Hankin 1896). Two years later, the Russian bacteriologist Gamaleya observed similar phenomenon while working with *Bacillus subtilis* (Sulakvelidze et al. 2001). Twenty years later, Fredrick Twort, a medical bacteriologist from England reported similar phenomenon and advanced the hypothesis that it might be due to, among other possibilities, a virus (Twort 1915). Two years later, bacteriophages were "officially" discovered by Fredrick d'Herelle, a French-Canadian microbiologist at Institut Pasteur in Paris while working with an outbreak of severe hemorrhagic dysentery among French troops stationed at Maisons-Laffitte in France. He made bacterium-free filtrates of the patients' fecal samples and mixed and incubated them with *Shigella* strains isolated from the patients. When these samples were plated on agar plates d'Herelle observed the appearance of small, clear areas, which he initially called *taches*, then *taches vierges*, and, later, *plaques* (Sulakvelidze et al. 2001). In contrast to Hankin and Twort, d'Herelle had little doubt about the nature of the phenomenon, and he proposed that it was caused by a virus capable of parasitizing bacteria. The name "bacteriophage" was also proposed by d'Herelle from "bacteria" and "phagein" (to eat or devour, in Greek), and was meant to imply that phages "eat" or "devour" bacteria. d'Herelle actively pursued studies of bacteriophages strongly refuting other researchers who thought that it was enzymes and not virus and considered himself to be their discoverer maintaining the phenomenon described by Twort earlier was distinct from his discovery. The first attempt to use bacteriophages therapeutically was by d'Herelle at the Hospital des Enfants-Malades in Paris in 1919 on a 12-year old boy with severe dysentery. The patient's symptoms ceased after a single administration of d'Herelle's antidysentry phage, and the boy fully

recovered within a few days. Since these results were not published immediately, the first reported application of phages to treat infectious diseases of humans came in 1921 from Richard Brynoghe and Joseph Maisin who used bacteriophages to treat staphylococcal skin disease (Sulakvelidze et al. 2001). It is also reported that d' Herelle used various phage preparations to treat thousands of people having cholera and bubonic plague in India. Commercial production of bacteriophage preparations also started around this time from d'Herelle's commercial laboratory in Paris. Very soon several companies were manufacturing phage preparations targeted against staphylococci, streptococci, *Escherichia coli*, and other bacterial pathogens. These preparations consisted of phage lysed, bacteriologically sterile broth cultures of targeted bacteria or the same preparations in a water soluble jelly base. However, the efficacy of phage preparations was controversial and with the discovery of penicillin, and subsequent advent of antibiotics, commercial production of therapeutic phages ceased in most of the Western world. Nevertheless, phage therapy continued to be pursued seriously in Eastern Europe and Soviet Union together with or instead of antibiotics. Much of their results are published in their native language in journals published from those countries, not widely accessible to the international scientific community. However, recent reviews which had access to translated literature account an extremely high success rate (75-100%) being reported in these papers by phage therapy albeit all the studies lacked control groups (Sulakvelidze et al. 2001).

Phage therapy holds a number of advantages over chemotherapy (Matsuzaki et al. 2005).

(i) It is effective against multi drug resistant pathogenic bacteria since the mechanism of bacteriolysis is completely different to that of antibiotics

(ii) High specificity spares non target bacterial populations

(iii) Phage lysins do not affect eukaryotic cells, therefore there are no known side effects

(iv) It can respond rapidly to the appearance of phage resistant mutants since phages themselves are able to mutate

(v) Cost of developing phage systems are cheaper than that of new antibiotics

Interest in phage therapy in the western world was rekindled with the landmark studies on *Escherichia coli* infections in mice and farm animals in 1982 (Smith & Huggins 1982, 1983, 1987, Smith et al. 1987). Thereafter, potential for phage therapy has been explored with members of the genera *Escherichia*, *Stapyhlococcus*, *Salmonella*, *Klebsiella*, *Proteus* and *Pseudomonas* (Barrow & Soothill 1997, Alisky et al. 1998). Cerveny et al. (2002) demonstrated the therapeutic effect of bacteriophages for both localized and systemic infections caused by *Vibrio vulnificus* in mice model. Vancomycin-resistant *Enterococcus faecium* (VRE) is endemic to many hospitals and causes nosocomial infections. Mice intraperitonially (i.p.) infected with 10^9 cfu of VRE could be rescued (100%) by a single i.p. injection of 3×10^8 pfu of phage strain administered 45 min after bacterial challenge. Even treatment was delayed to the point where all animals were moribund 50% survived after one i.p. injection of the phage (Biswas et al. 2002).

Use of phages in food preservation, food safety and preslaughter treatment of meat before it reaches market shelves is gaining prominence (Joerger 2003). Presence of *Listeria monocytogenes* on fresh-cut fruits and vegetables could be substantially reduced by phage application (Leverentz et al. 2003). Phage application substantially brought down the numbers of the common poultry contaminant *Campylobacter jejuni* in chicken (Wagenaar et al. 2005). Phage control of spoilage bacteria like *Pseudomonas* sp. could significantly increase shelf life of raw chilled meats (Greer 2005). Phages have also been shown to control contamination of fruits, meat and vegetables by human pathogenic *Salmonella*, *E. coli* O157:H7 and inhibit phytopathogens like *Erwinia amylovora* and *Xanthomonas campestris* (Schnabel & Jones 2001, Fiorentin et al. 2004, O'Flynn et al. 2004).

However, there are problems associated with phages as therapeutic agents and remain to be solved (Matsuzaki et al. 2005).

(i) Inactivation of administered phages or phage lysins by neutralizing antibodies or allergic reactions.

(ii) Phage resistant mutant bacteria

(iii) Capture and transfer of transcriptional regulators, virulence and antimicrobial genes across species

Mathematical models of phage therapy predict a 'replication' or 'proliferation' threshold (Payne et al. 2000, Payne & Jansen 2001). According to these models, phage:bacterium ratios are crucial to the outcomes of phage therapy as it is a random hit or miss process. In phage-bacterium mixtures some bacteria could be adsorbed by one or multiple phage(s), whereas some could escape phage adsorption altogether. Therefore phage:bacterium ratios could suffer from 'inundation thresholds' or 'failure thresholds' where the former implies substantially higher phage numbers relative to bacterial number and latter implies higher bacterial numbers to phage numbers. At inundation thresholds there may not bacteria left for progeny phage to infect (proliferate) thus affecting phage titres, while in failure thresholds bacterial numbers would far exceed phage numbers and resistant forms could then cause a reduction in phage titres. In order to overcome this, based on 'a priori' probability it has been estimated that a multiplicity of infection (MOI) of 10 i.e. 10 phage particles to one bacterium would be required to achieve a 99.99% probability of every bacterium receiving at least one phage (Payne et al. 2000, Payne & Jansen 2001, Kasman et al. 2002).

Although no side effects have been reported so far in the therapeutic use of phages, antibodies against them have been detected in the blood of animals that received phage therapy (Yoong et al. 2004). To overcome this, an additional selection criterion can be incorporated where phages with low immunogenicity are selected (Merril et al. 1996).

Resistance to phages is often caused by changes in phage-receptor molecules in gram negative bacteria (Matsuzaki et al. 2005). Mutant phages which have altered receptor preferences are usually isolated from the same original phage population (Tanji et al. 2004). Sometimes altered phage-resistant hosts altogether lose their virulence (Park et al. 2000). However, more studies are required in this area especially in Gram-positive bacteria-phage interactions where little is known.

Phage encoded virulence genes and their transduction poses another problem in the selection of phages as therapeutic agents. Virulence factors of pathogens like *Clostridium botulinum*, *Corynebacterium diphtheriae*, *E. coli* O157:H7, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Shigella flexneri*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *V. cholerae* are coded by lysogenic phages which the organisms carry in their genomes (Boyd et al. 2001). Recent studies in *E. coli*, *S.* enterica, *Staphylococcus aureus*, and *V. cholerae* have shed light on bacteriophage-bacteriophage interactions playing a role in the acquisition and expression of virulence genes in these organisms (Boyd & Waldor 1999). Presence of a lysogenic helper phage in these organisms has been found to potentiate virulence genes encoded by the associated phage (Boyd & Waldor 1999). Therefore candidate phages have to be subjected to a rigorous study of the host range, genome profiles and virulence before selection for therapeutic applications.

1.3.1 Bacteriophages in aquatic systems and aquaculture

As mentioned earlier, viruses are abundant in aquatic systems and numbers are estimated to be of the order 10^{10} l^{-1} in marine waters of which bacteriophages are the dominant members (Ackermann 2001). Bacteriophages in aquatic systems are found in water, sediment and associated with animals on surfaces and internal tissues. Their abundances are similar across all geographic and climatic zones (Fuhrman 1999).

Much of the information on phage:host systems from marine ecosystems we know today come from the studies of Moebus between the period late 1970s and 1980s (Moebus 1983, Moebus & Nattkemper 1983, Hermann & Moebus 1987). Just as vibrios have been identified as dominant culturable bacteria in marine ecosystems, their phages (vibriophages) are also abundant (Moebus & Nattkemper 1983). Phages infecting *V. vulnificus* were found abundant in oysters compared to all other marine samples tested (DePaola et al. 1997, DePaola et al. 1998). Oysters were also found to harbour a large number of phages infecting *V. parahaemolyticus* (Comeau et al. 2005). Bacteriophages infecting *Vibrio*, *Alteromonas macleodii* and *Pseudoalteromonas* were isolated from a Pacific Ocean station (Jiang et al. 2003). Phages isolated had similar host range despite

different restriction fragment length polymorphism (RFLP) profiles. It has been observed that vibriophages in marine environment are highly diverse and most identified so far belong to the family Siphoviridae and Myoviridae of the order Caudovirales.

Phages of fish and shrimp pathogenic bacteria, such as *Aeromonas salmonicida*, *A. hydrophila*, *Vibrio fischeri*, *V. parahaemolyticus*, *Edwardsiella tarda* and *Yersinia ruckeri* have been reported (Nakai & Park 2002). Aquaculture settings particularly in hatcheries do not provide a favorable environment for natural phage populations to thrive. This is because treatment of the intake water with chlorine, filtration and UV treatment are destructive to phages (Durán et al. 2003). Interest in phage therapy in aquaculture has been fairly recent and began with the diseases cause by *Lactococcus garvieae* in yellowtail *Seriola quinqueradiata* (Nakai et al. 1999). Fish that received the phage (*Siphovirus*) by i.p. and those that were fed phage-impregnated feed were able to survive challenge with the pathogen in laboratory trails. Bacteriophage control of the fish pathogenic *Pseudomonas plecoglossicida* was achieved with two phages belonging to Myoviridae and *Podoviridae* in both laboratory and field studies (Park et al. 2000, Park & Nakai 2003). Although phage resistant variants emerged in vitro, no such forms were observed from in vivo challenge experiments in the laboratory trial and the field trial. Vinod et al. (2006) reported improved survival of *P. monodon* post larvae reared in water seeded with a *Siphoviridae* bacteriophage that specifically infects *V. harveyi*. Shivu et al. (In Press) characterized six *V. harveyi*-specific phages isolated from coastal aquaculture systems and could classify five to belong to *Siphoviridae* and one to *Myoviridae*. However there is a need to be cautious in selecting therapeutic phages for controlling *V. harveyi* in culture systems. A lysogenic *Myovirus*-like phage (VHML) has been shown to be a cause of pathogenicity of *V. harveyi* although virulence genes of the phage are yet to be characterized (Oakey & Owens 2000). This phage was also reported to modify the phenotypic profile of infected *V. harveyi* strains making them different from non-infected ones (Vidgen et al. 2006). Earlier, Ruangpan et al. (1999) had assigned the toxicity of *V. harveyi* to *P. monodon* to a lysogenic bacteriophage which the bacterial strain was harbouring, but they could not study the phage further due to storage loss. Recently, Pasharawipas et al. (2005) isolated a *V. harveyi* infecting phage belonging to

Siphoviridae. They observed enhanced virulence to *P. monodon* amongst phage-refractory strains and concluded phage mediated changes to be responsible. However, they observed the phage genome was not integrated with the host in lysogens and termed the phenomenon as pseudolysogeny (Khemayan et al. 2006). Therefore it can be seen that bacteriophages offer a viable alternative to antibiotics andother chemotherapeutants, but a lot more in-depth research is required to understand their biology in the ecosystem as well as in host animals.

Objectives of the study

Mortality due to antibiotic resistant *V. harveyi* is a major problem in peaneid larval rearing systems. With the current ban on the usage of antibiotics, there is very little choice for aquarists to prevent/ manage infections by this bacterium in culture systems. Manipulation of rearing environment with probiotic bacteria beneficial to the host is one of the options for achieving improved productivity without the use of chemotherapeutants. Meanwhile it would be worthwhile to explore the possibility of using bacteriophages infecting *V. harveyi* to control its infections in penaeid larval rearing systems.

Therefore the objectives of the study were

1. Isolation and antibiotic sensitivity of *V. harveyi* from *P. monodon* larval rearing systems
2. Evaluation of probiotic bacteria to inhibit the growth of *V. harveyi* in vitro and improve survival of *P. monodon* larvae in vivo
3. Isolation and characterization of bacteriophages infective to *V. harveyi*

CHAPTER - 2

Isolation, Identification and Antibiotic Sensitivity of *Vibrio harveyi* from *Penaeus monodon* Larval Rearing Systems

CHAPTER - 2

Isolation, identification and antibiotic sensitivity of *Vibrio harveyi* from *Penaeus monodon* larval rearing systems

2.1 Introduction

Vibrios are ubiquitous in estuarine and marine environments worldwide, frequently isolated from the surfaces and internal organs of marine plants and animals (Thompson et al. 2004b). Many species of this group have been implicated as primary and/or opportunistic pathogens of fish and shellfish in culture (Lightner 1988, Toranzo et al. 2005). *Vibrio harveyi* has emerged as a principal pathogen of shrimp in culture systems causing mass mortalities (Lavilla-Pitogo et al. 1990, Abraham et al. 1997, Austin & Zhang 2006, Alavandi et al. In Press). Although it can infect and kill all metamorphic stages of shrimp, the early larval stages in hatcheries are increasingly susceptible (Soto-Rodriguez et al. 2006b, Alavandi et al. In Press). Since shrimp larvae in hatcheries are intensively reared with initial stocking densities ranging from 100-150 nauplii l^{-1} (Pechmanee 1997), mass mortalities due to *V. harveyi* cause serious economic loss (Abraham et al. 1997).

Antibiotics are widely used in aquaculture to counter bacterial infections including those caused by *V. harveyi* (Roque & Gomez-Gil 2003, Cabello 2006). Norfloxacin, oxytetracycline, enrofloxacin, ciprofloxacin, chloramphenicol, erythromycin, furazolidone, nifurpirinol, oxolinic acid, ormetoprim, rifampicin, trimethoprim and various sulfonamides are commonly used drugs in aquaculture (Graslund & Bengtsson 2001, Holmstrom et al. 2003). Antibiotic use in aquaculture has been chiefly prophylactic and seldom therapeutic (Cabello 2006) which results in large quantities of antibiotics reaching the environment where bacteria are exposed to them at sub-lethal levels (Le & Munekage 2004). An obvious outcome of such uncontrolled use is that many fish and

shellfish pathogens have developed multiple antibiotic resistance (MAR) (Alcaide et al. 2005, Akinbowale et al. 2006) while the other is the transfer of resistance across genera and to bacteria associated with terrestrial animals and humans including pathogens (Cabello 2006). The latter hypothesis found evidence recently when identical plasmids carrying the tetracycline resistance transposon Tn*1721* was detected in *Aeromonas salmonicida* strains from fish farms in Scotland and Norway and *Escherichia coli* strains from a German hospital (Rhodes et al. 2000). This proved that transfer of resistance factors between bacteria associated with aquaculture and human pathogens was indeed taking place in the aquatic environment (Rhodes et al. 2000).

MAR indexing of bacteria is a useful method to distinguish various sources of anthropogenic influence to identify regions of high antibiotic contamination (Krumperman 1983). It has been shown that *Escherichia coli* has markedly lower MAR index when isolated from places with little or no human interference compared to those constantly under anthropogenic pressures like poultries, dairies and piggeries (Krumperman 1983, Parveen et al. 2006). MAR indexing allows the study of associations of antibiotic use and bacterial resistance to them (Parveen et al. 2006), therefore it would be useful to examine this phenomenon in shrimp hatchery settings. *V. harveyi* isolates showing MAR profiles have been reported from India (Abraham et al. 1997, Chari & Dubey 2006), Thailand (Jiravanichpaisal et al. 1994), Phillipines (Tendencia & de la Pena 2001), Ecuador (Austin & Zhang 2006), Venezuela (Alvarez et al. 1998) and Mexico (Molina-Aja et al. 2002). However, all these studies incorporated only a limited number of antibiotics obscuring the true extent of resistance of *V. harveyi*. A comprehensive surveillance of resistance of *V. harveyi* in shrimp larval hatchery would assist in the selection of the right antibiotic, study the trend of antimicrobial resistance and associations between antimicrobial use and occurrence of antimicrobial resistance (Grugel & Wallmann 2004). In addition, current regulations being enforced by the Aquaculture Authority and Marine Products Export Development Agency, Govt. of India, ban the use of many commonly used antibiotics like chloramphenicol, neomycin, nitrofurantoin and furazolidone in shrimp culture. Therefore, an extensive screening for suitable antibiotics became necessary for sustainable shrimp larval production, a major

economic activity providing livelihood to a large number of people living along the coasts in remote and peri-urban areas in India. In this study we examined the MAR profile of *V. harveyi* isolated from various locations in shrimp hatcheries of Kerala and Andhra Pradesh during the years 2000 and 2001 in an attempt to understand the extent of resistance in *V. harveyi* isolates from shrimp hatcheries.

2.2 Materials and Methods

2.2.1 Bacteria

Luminescent bacteria were isolated during the years 2000 and 2001 from two hatcheries of Kakinada, Andhra Pradesh, and one hatchery in Kochi, Kerala, which reported luminous bacterial disease in their larval tanks. Samples screened for the presence of luminescent bacteria were nauplii larvae which failed to metamorphose, postlarvae and their rearing water where mass mortality occurred, protozoea, mysis and postlarvae which successfully completed larval cycle, tank surfaces, pipelines, raw seawater from intake point, treated seawater, *Artemia* nauplii and rearing water, outdoor algal culture water, crabs used for feeding broodstock and their rearing water, and hatchery waste water outlet (drain-out). Water samples were serially diluted ten-fold and spread plated on ZoBell's Marine Agar 2216E (ZA) plates. Larvae (Mysis and Post larvae – 10 numbers each; Nauplii 100 numbers) were homogenized in 10 ml sterile seawater, serially diluted, and plated on the same medium. Swabs from crab carapace were spread directly on ZA plates. Sand was collected aseptically from around the intake point of seawater of the hatcheries. Ten grams of sand was suspended in 100 ml sterile seawater, serially diluted and spread plated on ZA plates. All plates were incubated at 28°C and observed after 18-24 hours of growth. Luminescent colonies were marked in the dark and streaked on ZA to obtain pure cultures. All isolates are deposited in the Microbial Culture Collection at National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology (CUSAT).

2.2.2 Identification of isolates

Generic level identification of the isolates was carried following Oliver (1982) and demarcated into species following Alsina & Blanch (1994). Briefly, the tests carried out were Gram stain, motility, cytochrome oxidase (Kovac's), oxidation/fermentation of glucose, catalase, sensitivity to O/129, luminescence, production of arginine dihydrolase, ornithine and lysine decarboxylase, growth at 0% and 8% NaCl, Voges-Proskaeur reaction, citrate utilization, production of acid from L-arabinose and D-galactose, production of gelatinase, amylase, chitinase, and lipase.

2.2.2.1 Oxidation/Fermentation of glucose

The media used was Marine Oxidation Fermentation (MOF) Medium (Himedia) as per manufacturer's recommendations. Twenty two grams of the medium and 15 g agar was added to 1000 ml distilled water and sterilized by autoclaving at 121°C for 15 min. To the above basal medium, 1% glucose was added and 4 ml aliquots were transferred into sterile tubes which were then autoclaved at 110°C for 10 min. The tubes were laid out to slants and after drying, the cultures were inoculated by stab and the streaked on the slant. The tubes were incubated at 28±1 °C and results were recorded as follows:

O – oxidation (yellow colouration in the slant alone)
F – fermentative (yellow coloration throughout the tube)
(F) – fermentation with gas production
A – alkaline reaction (no colour change in the tube)

2.2.2.2 Motility

Motility was testing by soft agar and hanging drop method.

<u>In soft agar medium</u>

Yeast extract	1g
Peptone	5g

NaCl	5g
Agar	3g
pH	7.2
20 ppt seawater	1000 mL

Medium was poured in to tubes in 3ml aliquots and autoclaved at 121°C for 15 minutes. Stab incubated in the medium and incubated at 28±1°C for 24-28 hours. A rhizoidal growth from the line of inoculation to peripheral area were observed.

Hanging Drop Method

A loop full of 6-12 hours old culture grown in ZoBell's broth was placed in the centre of a clean cover slip. A drop of Vaseline was placed on the four corners of the cover slip. Then a cavity slide was placed over the drop in such a way that drop comes within the cavity. The slide and the whole preparation inverted quickly, so that the drop of culture was seen hanging from the coverslip. The slide was placed under oil immersion objective and observed for displacement of cells that could be differentiated from Brownian movement.

2.2.2.3 Cytochrome oxidase (Kovac's)

Presence of cytochrome oxidase was detected following (Kovacs 1956). The organisms were freshly grown on nutrient agar. A platinum loop was used to pick a colony and make a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned violet within 10 seconds, indicating the formation of indophenol.

2.2.2.4 Catalase

The principle of this test is that when organisms producing the enzyme catalase are mixed with hydrogen peroxide (H_2O_2) gaseous oxygen is released. An overnight culture on Zobell's agar slants was used for this test. A thick smear of the test organism was made from the culture on a clean glass slide and a drop of hydrogen peroxide (H_2O_2) is placed on it. Immediate formation of gas bubbles indicates the liberation of oxygen was considered as positive reaction.

2.2.2.5 Sensitivity to vibriostat compound O/129 (2,4-diamino-6,7-di iso-propyl pteridine)

Antibiotic assay filter paper disc of 6mm diameter (Whatman No.1) were prepared aseptically to contain 150µg $disc^{-1}$of the vibriostatic agent O/129 (Furniss et al. 1978). The discs were stored at 4°C and used as required. ZoBell's agar plates were swabbed with a suspension of the test bacterial organism to obtain a confluent growth and the discs were placed on it appropriately spaced. Bacterial isolates sensitive to the pteridine compound developed a clearing zone around the disc.

2.2.2.6 Production of arginine dihydrolase

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 due to the production of ornithine, CO_2 and NH_3 from arginine.

Media

Peptone	1g
NaCl	5g
K_2HPO_4	0.3g

Agar	3g
L(+)-arginine hydrochloride	10g
pH	6.7
Distilled water	1000 ml

The solids (except arginine) were dissolved in distilled water; pH adjusted to 6.7 and added the indicator solution (10 ml of 0.2% phenol red). The medium was sterilized at 121°C for 15 minutes and then the amino acid added. pH was readjusted to 6.7 if required, dispensed in 2 ml aliquots in sterile tubes and overlaid with liquid paraffin. Sterilized at 110°C for 10 minutes.

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. The color change from yellowish orange to red being positive.

2.2.2.7 Production of ornithine and lysine decarboxylase

The amino acid decarboxylase tests demonstrate the bacterial decarboxylation of lysine and ornithine, and these tests are of particular use in identifying members of Enterobacteriaceae. In this test, the decarboxylation or the elimination of a molecule of carbondioxide 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-aminoacid (L(+) Lysine dihydrochloride or L(+) Ornithine monohydrochloride, or 2% of the DL form, was incorporated in Falkow's medium (Falkow 1958).

Media

Peptone	5.0
Yeast extract	3.0

Glucose	1.0
Bromocresol purple (0.2% solution)	10 ml
pH	6.7
Distilled water	1000 ml

The solids (except arginine) were dissolved in distilled water; pH adjusted to 6.7 and added the indicator solution. The medium was sterilized at 121°C for 15 minutes and then the amino acid added. pH was readjusted to 6.7 if required, dispensed in 2 ml aliquots in sterile tubes and overlaid with liquid paraffin. Sterilized at 110°C for 10 minutes.

An inoculum from a culture of the test organisms on a solid medium was introduced with a straight inoculating wire through the paraffin. The various controls included a tube containing only the basal medium that was also inoculated. Both were incubated at 28±1° C and examined daily for 4 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 amino acid.

2.2.2.8 Growth at 0% and 8% NaCl

Growth at different concentration of NaCl was tested by inoculating the organism in Tryptone broth at pH 7.2±0.2 containing 0 and 8 % NaCl respectively. Media was dispensed into tubes in 3 ml aliquots and sterilized at 121°C for 15 minutes. Overnight cultures were inoculated and incubated for 24 hours at 28±1°C. Growth was detected visual observance of turbidity in comparison to an un-inoculated control tube of the same NaCl concentration.

2.2.2.9 Voges-Proskaeur reaction

This helps to determine weather the organism ferments carbohydrate with production of non acidic / neutral end products. Glucose is converted to acid which is detected by the formation of Acetoin (2,3 butaniedion acetyl methyl carbinol) when α-naphthol and KOH are added. To a 48 h old culture, 0.5 ml 6% α-naphthol and 0.5ml 16% KOH solution agitated thoroughly and maintained 2 hours. A positive result was indicated by the development of pale pink color. It appears normally with in 5 minutes but may not reach maximum red color intensity for about an hour. Negative tubes were re-examined and confirmed after long period.

2.2.2.10 Citrate utilization

Simmon's citrate agar (Himedia) was used as per manufacturer's recommendations. The medium was melted and dispensed in 4 ml aliquots into tubes and autoclaved at 121°C for 15 minutes and laid out to form slants. They were inoculated with 24-48 h old cultures and incubated at 28±1°C for 24 hours. Utilization of citrate was established if the alkaline color (blue) appeared after 2-3 days of incubation.

2.2.2.11 Production of acid from L-arabinose and D-galactose

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

Solution A

NH_4Cl	5.0
NH_4NO_3	1.0
Na_2SO_4	2.0
K_2HPO_4	3.0
KH_2PO_4	1.0
NaCl	10.0

Solution B

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

Yeast extract was prepared in the form of a 0.1% stock solution in distilled water and added. Agar was used only in case of carbon sources that were insoluble, in which case, plates were made and spot inoculated.

Solutions A and B were sterilized separately at 121°C for 15 minutes, cooled to room temperature and then mixed together (otherwise the basal medium becomes turbid).

All sugars were added to a final concentration of 0.2% (w/v). Other carbon sources that were insoluble, the concentration was made to 0.1% (w/v) and plates were prepared.

All carbon sources were sterilized at 110°C for 10 minutes. The medium containing the carbon sources were dispensed into sterile test tubes in 2 ml aliquots. Control tubes without the carbon source were also maintained for each culture.

The test media were inoculated with an inoculation needle, touching the inoculum to the side of the test tube, incubated for 24-48 hrs at 28±1°C. Following incubation the tubes were observed for turbidity against control. Only in case of negative results, they were incubated further for 7 days.

2.2.2.12 Production of gelatinase, amylase, chitinase, and lipase

Gelatinase

When proteolytic organisms are grown media containing gelatin, it is liquefied and loses the gelling property even when cooled to 4°C.

Media

Peptone	5g
Yeast Extract	1g
Gelatin	20 g
20 ppt sea water	1000 ml
pH	7.5±0.2

The medium was melted, poured into tubes and autoclaved at 121°C for 15 minutes. A 24 h old culture was inoculated and incubated at 28±1°C for 3-5 days. Un-inoculated tubes were maintained as control. After the incubation period the tubes were kept at 4°C for 6 h. The tubes were inverted to observe for liquefaction of gelatin while the control remains solid.

Amylase

The production of amylase was detected by hydrolysis of the starch. by the organism was detected by inoculating a loop full of culture in to starch agar plate.

Media

Peptone	5g
Starch	5g
Beef extract	5g
Agar	20g
Yeast extracts	1g
NaCl	20g
pH	7.5

Gram's Iodine solution

Iodine	1g
KI	2g
Distilled water	100 ml

Medium was autoclaved 121°C for 15 minutes and dispensed in to sterile petri plates and single line streak of the organism was made across the centre of the plate. Following four days of incubation at 28±1°C the plates were flooded with Gram's iodine. Hydrolysis of starch was detected by the formation of clear zone around the colony.

Chitinase

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

Purified colloidal chitin (Lingappa & Lockwood 1961) was made by treating crude chitin alternatively with 1N-NaOH and 1N-HCl several times, and then with ethanol until all foreign material had been removed. The purified chitin was then dissolved in cold con. HCl, filtered through glass wool, precipitated in distilled water and washed several times in distilled water until neutral. This colloidal chitin was then added to the nutrient basal medium.

Media

Peptone	5.0 g
Beef extract	5.0 g
NaCl	5.0 g
Colloidal chitin	5.0 g (or 5% v/v)
Agar	20.0 g
Distilled Water	1000 ml
pH	7.5±0.3

The medium was sterilized at 121°C for 15 minutes and poured into plates. The test cultures were spot inoculated and incubated at 28±1°C for 7 days. Hydrolysis of chitin was represented by a clearing zone around the spots.

Lipase

Media

Peptone	5g
Beef extract	5g
Yeast extract	1g
Tributyrin	1g
Agar	2g
20 ppt sea water	100ml
pH	7.2 ± 0.2

The medium was autoclaved at 121°C for 15 minutes and poured into sterile plates. A 24 h old culture was spot inoculated onto the plates and incubated at 28±1°C for 7 days. A zone of clearance was observed in the plates of positive sample

2.2.3 Antibiotic Sensitivity

Sensitivity of the isolates to 81 antibiotics was tested using commercially available antibiotics discs (Himedia). The antibiotics discs used under each antibiotic class are given in Table 1. ZA plates were swabbed with 0.5 ml overnight grown cultures of *Vibrio harveyi* and the antibiotic discs placed aseptically. A maximum of six discs were placed sufficiently distanced from each other and all cultures were tested twice. The plates were incubated at 28±1°C for 18 hours and the clearing zone formed around the discs recorded using HiAntibiotic ZoneScale (Himedia). Isolates were recorded as resistant if there was no visible zone around the discs. The MAR index (number of antibiotics to which the isolate was resistant / total number of antibiotics tested) was determined for each isolate (Krumperman 1983). The MAR index for each source (aggregate antibiotic resistance score of all isolates from the sample / number of antibiotics × number of isolates from the sample) was also determined (Krumperman 1983).

2.2.4 Statistical analysis

A similarity profile of the isolates based on the antibiotic sensitivity pattern was done using the software PRIMER 5 for Windows (Version 5.2.8). A Bray-Curtis similarity index with square root transformation was opted. Dendrogram was plotted using group average cluster mode for grouping *V. harveyi* isolates with respect to antibiotic sensitivity.

2.3 Results and Discussion

Sixty luminescent bacterial isolates were obtained from larvae and their rearing water where mass mortality occurred and 27 were obtained from the other sources sampled (Table 2). Samples taken from tank surfaces, pipelines, treated seawater, *Artemia* nauplii and their rearing water, and outdoor algal culture water did not yield any luminescent bacterial isolate. All 87 isolates were presumptively identified as *Vibrio harveyi* based on phenotypic characters (Table 3). All isolates produced the hydrolytic enzymes gelatinase, amylase, lipase and chitinase associated with virulence (Austin & Zhang 2006). *V. harveyi* was present in intake seawater but could not be detected in treated seawater. This meant that the treatment procedures such as chlorination, dechlorination, filtration and UV irradiation could effectively eliminate the bacterium. This meant the source of *V. harveyi* into shrimp hatcheries could be the incoming nauplii or biofilms in the tanks and air/water piping systems as recorded by Abraham & Palaniappan (2004) as reservoirs of the species. However in our study we did not obtain *V. harveyi* from the tank surfaces or piping systems altogether. It must be mentioned, that the hatchery operators were following hygienic practices and were extremely vigilant to the presence of luminescence in their systems. The possibility of *V. harveyi* entering into a viable but non-culturable (VBNC) state during the treatment process and its subsequent resuscitation once inside the larvae cannot be ruled out since it has been reported that *V. harveyi* enters into VBNC state under stressed conditions like low nutrient environment (Ramaiah et al. 2002).

Other vibrios like *V. cholerae*, *V. vulnificus*, *V. splendidus* biotype I, *V. parahaemolyticus*, *V. alginolyticus* and *V. shilonii* are also known to enter into such states and remain virulent when exposed to harsh conditions (Colwell et al. 1985, Thompson et al. 2004b, Coutard et al. 2005). Recovery of isolates from water samples collected from hatchery drain out was indicative of ineffective treatment of the wastewater, although operators are known to chlorinate the wastewater prior to release into the environment. Since a majority of the shrimp hatcheries in Andhra Pradesh are located in clusters in close proximity to each other, such laxity can escalate the risk of epidemic outbreaks.

The antibiotic resistance amongst the isolates was extremely high (Table 1). Ninety percent of the isolates were resistant to the tetracycline antibiotics tested. Resistance was also high amongst the isolates to anitibiotics belonging to the classes' quinolones (76%), β-lactams (70%) and macrolides (64%). The least resistance was observed to be against antibiotics of aminoglycosides class (26%). Since the resistance was exceptionally high to the antibiotics, it showed the futility of their application in controlling *V. harveyi* infections in *P. monodon* larval rearing systems. Amongst the antibiotics banned for use in shrimp culture in India (Pakshirajan 2002), resistance was less than 20 % to nitrofurantoin, nitrofurazone and neomycin, while it was more than 80% to floxidine, enrofloxacin, pefloxacin, lomefloxacin, vancomycin, sparfloxacin and nalidixic acid (Table 1). Quinolones/ fluroquinolones and their derivatives such as oxolonic acid and enrofloxacin have been reported as a potent chemotherapeutants against *V. harveyi* (Roque & Gomez-Gil 2003) but in this study the resistance to these antibiotics was extremely high. It was observed that the resistance of the isolates from Andhra Pradesh to all antibiotics tested increased substantially from 39.23 % in the year 2000 to 55.59 % in 2002. Two isolates (LB1 and MCCB 111) obtained during the year 2001 from a sample of rearing water of postlarvae where mass mortality occurred were sensitive to only 10/81 antibiotics. Difference in the resistance profile between isolates of Andhra Pradesh and Kerala were found insignificant. One or more antibiotics across all classes examined in this study are used in shrimp farming at hatchery or farm level (Graslund & Bengtsson 2001, Graslund et al. 2003) and most of this use is prophylactic (Cabello 2006) and against viral infections as well (Holmstrom et al. 2003). Antibiotics being the easiest

solution to bacterial infections, are widely used in shrimp culture (Graslund & Bengtsson 2001, Graslund et al. 2003). Therefore it has been hypothesized that aquaculture activities could be a source of antibiotics in the environment (Le & Munekage 2004). Roque et al. (2001) had previously observed that 70% of the *V. harveyi* isolates from Mexican shrimp farms and hatcheries were resistant to multiple antibiotics. When the relationship between the isolates of different sources or seasons or regions based on their resistance profile was evaluated, a similarity level of 40-90% was observed amongst all isolates and no significant relationship could be discerned (Fig. 1). This clearly showed a highly heterogenous nature in the resistance profile and that the process of resistance acquisition is independent of the use and the consequent presence of the antibiotic in the system.

All isolates had a very high MAR index at an average of 0.63 with little variation between those from different samples (Table 2). This situation poses an additional threat of antimicrobial resistance to be acquired by human pathogenic bacteria in the environment (Cabello 2006). Evidence to this effect was obtained during an outbreak of cholera in Latin America in 1992, where antibiotic resistance in *V. cholerae* was linked to contact with antibiotic resistant bacteria selected through heavy use of antibiotics in Ecuadorian shrimp farms (Angulo et al. 2004). Isolation of identical antibiotic resistance determinants in hospital isolates of *Escherichia coli* and *Aeromonas salmonicida* isolates from fish farms are proposed as proofs for dissemination of resistance determinants genes between fish pathogenic bacteria and human pathogens (Rhodes et al. 2000). This has also formed the basis for the widespread condemnation of antibiotic use in aquaculture (Cabello 2006). Two arguments countering this have been proposed, one, evidence of strains carrying identical antibiotic resistance determinants is purely circumstantial and two, multiple antibiotic resistant human pathogens account for only 4 % of infections worldwide (Wassenaar 2005). Therefore there is a need for more in depth studies on this issue. Recent data however has shown that antibiotic resistance in bacteria (aquatic and human pathogens) declines substantially following complete abolition of their use in countries like Norway where strict regulations and enforcement prevents non-human uses of antibiotics (Grugel & Wallmann 2004). Diseases affecting aquaculture species in Norway are largely controlled by vaccination, use of bioremediators and probiotics and

proper water quality management which implies antibiotic use can be completely avoided if such practices are followed. Therefore it is possible to completely avoid antibiotic use in shrimp farming provided well established biological control measures are available. This study has shown that *V. harveyi* is most susceptible to aminoglycosides group of antibiotics and use others like tetracylines, macrolides, β-lactams and some of the quinolones is futile. It was also observed that the isolates had a high MAR index and antibiotic resistance pattern in isolates is highly heterogenous. This implies there is an urgent need for biological control measures as alternatives for improving sustainability and productivity of shrimps in culture.

Table 1. Resistance of *Vibrio harveyi* to different antibiotics tested

Inhibitors of	Chemical Class	Antibiotic (conc in µg/ disc)	% of *V. harveyi* isolates resistant			
			AP2000 n=35	AP2001 n=37	Kerala 2000 n=15	Total n=87
Cell wall synthesis	β-Lactams	Amoxycillin (10), Ampicillin (10), Methicillin (5), Oxacillin (1), Piperacillin (100), Penicillin G (10)*	100.0	100.0	100.0	100.0
		Carbenicillin (100)	42.9	62.2	46.7	51.7
		Cefachlor (30)	25.7	56.8	13.3	36.8
		Cefadroxil (30)	74.3	89.2	86.7	82.8
		Cefalexin (30)	91.4	94.6	93.3	93.1
		Cefaloridine (10)	97.1	94.6	93.3	95.4
		Cefazolin (30)	91.4	97.3	100.0	94.3
		Cefalothine (30)	51.4	89.2	40.0	65.5
		Cefamandole (30)	34.3	43.2	6.7	33.3
		Cefaperazone (75)	31.4	94.6	20.0	56.3
		Cefaradine (25)	88.6	97.3	100.0	94.3
		Cefoxitin (30)	22.9	86.5	55.6	57.5
		Ceftazidime (30)	42.9	73.0	20.0	51.7
		Ceftizoxime (30)	17.1	18.9	0.0	14.9
		Ceftriaxone (10)	20.0	13.5	33.3	19.5
		Cefuroxime (30)	54.3	54.1	33.3	50.6
		Cephotaxime (10)	17.1	24.3	26.7	21.8
		Cloxacillin (1)	97.1	100.0	100.0	98.9
		Ticarcillin (75)	100.0	100.0	93.3	98.9
		Imipenem (10)	31.4	48.6	20.0	36.8
	Glycopeptides	Vancomycin (5)$^{+}$	100.0	100.0	93.3	98.9
	Polypeptide	Bacitracin (8)*	48.6	21.6	60.0	39.1
Protein synthesis	Aminoglycosides	Spectinomycin (100)	45.7	54.1	40.0	48.3
		Amikacin (10)	31.4	45.9	33.3	37.9
		Framycetin (100)	2.9	43.2	60.0	29.9
		Gentamycin (10)	2.9	16.2	6.7	9.2
		Kanamycin (30)	17.1	24.3	13.3	19.5
		Neomycin (30)$^{+}$	5.7	29.7	13.3	17.2
		Netillin (10)	2.9	18.9	13.3	11.5
		Streptomycin (10)	45.7	51.4	26.7	44.8
		Tobramycin (10)	2.9	27.0	33.3	18.4
	Macrolides	Azithromycin (15)	22.9	43.2	40.0	34.5
		Clarithromycin (15)	54.3	64.9	53.3	58.6
		Erythromycin (10)	65.7	83.8	80.0	75.9
		Tylosine (15)	45.7	100.0	86.7	75.9
		Oleandomycin (15)	71.4	86.5	93.3	81.6
		Roxithromycin (30)	31.4	70.3	46.7	50.6
		Spiramycin (30)	57.1	94.6	80.0	77.0
	Tetracyclines	Doxycycline HCl (10)	100.0	97.3	98.6	98.9
		Chlortetracycline (30)	54.3	73.0	100.0	70.1
		Minocycline (30)	91.4	100.0	100.0	96.6
		Oxytetracycline (30)	77.1	100.0	93.3	89.7
		Tetracycline (10)	94.3	100.0	100.0	97.7

Table 1 contd

	Chloramphenicol	Chloramphenicol (10)$^{+}$	37.1	62.2	13.3	43.7
	Rifamycins	Rifampicin (2)	11.4	54.1	53.3	36.8
	Lincosamides	Clindamycin (10)	80.0	100.0	100.0	92.0
		Lincomycin (2)	94.3	94.6	93.3	94.3
	Steroids	Fusidic acid (10)	91.4	100.0	100.0	96.6
	Nitrofurans	Nitrofurazone (100)$^{+}$	5.7	18.9	20.0	13.8
		Furazolidone (50)$^{+}$	0.0	59.5	60.0	35.6
		Furaxone (100)$^{+}$	2.9	81.1	46.7	43.7
	Heterocyclic compounds	Methenamine mandalate (3)	5.7	24.3	20.0	16.1
Nucleic acid synthesis	Sulfonamides	Trimethoprim (5)	11.4	56.8	46.7	36.8
		Sulfadiazine (100)	85.7	100.0	80.0	90.8
		Sulfafurazole (300)	28.6	62.2	26.7	42.5
		Sulfamethizole (300)	65.7	97.3	86.7	82.8
		Sulfamethoxy-pyridazine (300)	51.4	97.3	93.3	78.2
		Sulfaphenazole (200)	28.6	81.1	60.0	56.3
		Triple Sulphas (300)	11.4	70.3	40.0	41.4
	Quinolones / Fluoroquinolones	Ciprofloxacin (1)$^{+}$	45.7	43.2	53.3	46.0
		Enrofloxacin (5)$^{+}$	85.7	86.5	86.7	86.2
		Floxidine (20)$^{+}$	77.1	89.2	80.0	82.8
		Lomefloxacin (10)$^{+}$	94.3	100.0	100.0	97.7
		Pipemidic acid (20), Nalidixic acid (30)$^{+}$	100.0	100.0	100.0	100.0
		Nitroxoline (30)$^{+}$	11.4	48.6	66.7	36.8
		Norfloxacin (10)$^{+}$	22.9	67.6	33.3	43.7
		Ofloxacin (2)$^{+}$	42.9	83.8	46.7	60.9
		Pefloxacin (5)$^{+}$	80.0	100.0	93.3	90.8
		Sparfloxacin (5)$^{+}$	100.0	100.0	93.3	98.9
	Aminocoumarins	Novobiocin (30)	2.9	70.3	60.0	41.4
	Nitrofurantoin	Nitrofurantoin (100)$^{+}$	5.7	16.2	13.3	11.5
	Metronidazole	Metronidazole (4)$^{+}$	11.4	83.8	80.0	54.0
Cell membrane	Polypeptides	Polymixin B (50)*	60.0	94.6	93.3	80.5
		Colistin (10)	74.3	94.6	100.0	87.4
Metabolism	Fosfomycin	Fosfomycin (50)	37.1	27.0	60.0	36.8

*Concentration per disc in International Units (IU)
$^{+}$Antibiotics banned from use in aquaculture (Pakshirajan 2002)
AP: Andhra Pradesh

Table 2. Multiple antibiotic resistance (MAR) index of *Vibrio harveyi* isolated from various sources in *Penaeus monodon* larval rearing hatcheries

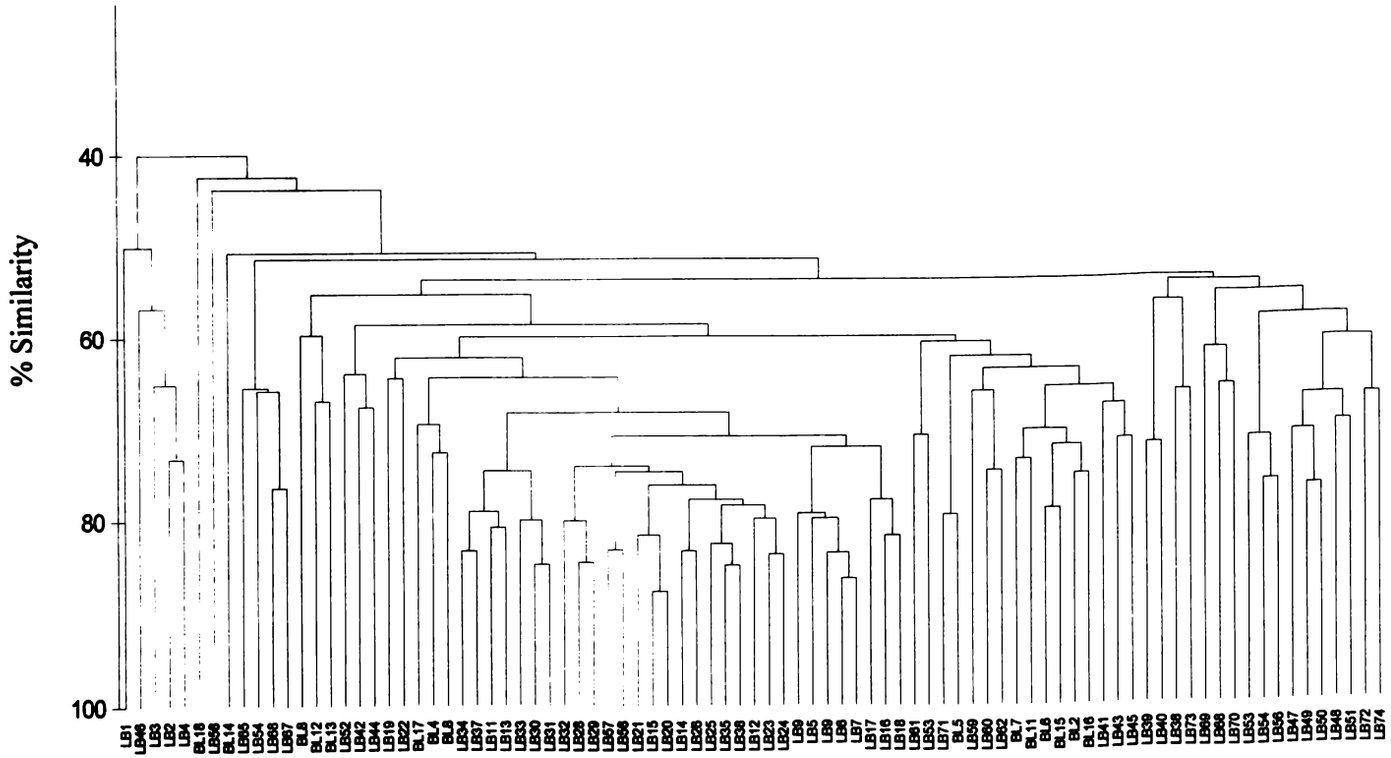
Source	No. of isolates	MAR Index
MPLW	14	0.58
MPL	36	0.54
MNL	10	0.75
PLN, MysN	2	0.66
HDO	6	0.74
RSW-I	8	0.65
RW-C, CC	4	0.76
BS	7	0.77

MPLW: Water from postlarval tank where mortality occurred, MPL: Larvae from postlarval tank where mortality occurred, MNL: Nauplii which failed to metamorphose to protozoea due to luminescent bacteria
PLN: Post larvae which completed the larval cycle, MysN: Mysid larvae which completed the larval cycle
HDO: Water from hatchery drain out, RSW-I: Intake seawater before treatment
RW-C: Water from crab maintenance tanks, CC: Crab carapace, BS: Sand around intake point on the beach

Table 3. Phenotypic characters of *Vibrio harveyi* isolates from various sources in *Penaeus monodon* larval rearing hatcheries

Character	
Micromorphology	Rod
Gram Stain	-
Growth on Thiosulphate Citrate Bile salts Sucrose Agar (TCBS)	Green colonies
Oxidation/ Fermentation of glucose (MOF)	Fermentative
Motility	+
Cytochrome oxidase (Kovac's oxidase)	+
Sensitivity to O/129 (150μg)	+
Luminescence	+
Catalase	+
Arginine dihydrolase production	-
Lysine decarboxylase production	+
Ornithine decarboxylase production	+
Growth in	
0% NaCl	-
8% NaCl	+
Voges-Proskaeur reaction	-
Citrate utilization	+
Production of acid from	
L-Arabinose	-
D-Galactose	-
Production of	
Gelatinase	+
Amylase	+
Chitinase	+
Lipase	+

Fig. 1. Dendrogram of antibiotic resistance profile of *Vibrio harveyi* isolates determined by Bray-Curtis similarity index.Numbers of dendrogram represent the strains of *V.harveyi*



CHAPTER - 3

Evaluation of Probiotic Bacteria to Inhibit the Growth of *Vibrio harveyi* in vitro and Improve Survival of *Penaeus monodon* Larvae in vivo

CHAPTER - 3

Evaluation of probiotic bacteria to inhibit the growth of *Vibrio harveyi* in vitro and improve survival of *Penaeus monodon* larvae in vivo

3.1 Introduction

A major cause of shrimp larval mortality in hatcheries is infection by the Gram-negative bacterium *Vibrio harveyi* commonly known as luminescent bacteria due to the characteristic blue-green light it emits. The organism has been reported as an opportunistic pathogen to both juvenile and adult shrimp during culture where it seriously threatens economic feasibility by causing mass mortalities. Extensive use of antibiotics to control its incidence not only resulted in the emergence of multiple antibiotic resistant strains (Karunasagar et al. 1994, Abraham et al. 1997) but has also caused grave environmental and human health concerns (Holmstrom et al. 2003, Nogueira-Lima et al. 2006) leading to their prohibition.

Such concerns have resulted in the exploration of biological control methods for mitigating the adverse impact of pathogens, one of which includes the use of bacteria live or dead as probiotics (Verschuere et al. 2000b, Irianto & Austin 2003). Probiotic bacteria could prevent the establishment pathogenic bacteria by out-competing them for adhesion and colonization sites in the intestines and other tissues of the animal (Vine et al. 2004a). They could also produce inhibitory substances actively preventing pathogen establishment (Verschuere et al. 2000b). When added to rearing water, they may act as bioremediation agents improving water and sediment quality, augment nutrient cycling in the system and initiate colonization of other beneficial microflora effecting an overall positive impact on growth rates and productivity (Boyd & Massaut 1999, Prabhu et al. 1999). A wide array of both Gram-negative and Gram-positive bacteria

with probiotic potential against a multitude of fish and shellfish pathogens have been reported (Irianto & Austin 2002a, Balcazar et al. 2006).

Fluorescent pseudomonads produce phenazine and other antimicrobial compounds which have broad spectrum activity against many phytopathogenic fungi and eubacteria (Mavrodi et al. 2006). Pseudomonads have also been documented as the dominant flora in the eggs and larvae of shrimps of successfully completed larval cycles in hatchery systems (Singh et al. 1989). Dopazo et al. (1988) demonstrated the antibacterial activity of marine bacteria including *Pseudomonas* sp. against fish pathogens. Their ability to control bacteria causing diseases of aquatic animals in culture and effect improvement in productivity is gaining widespread acceptance. *Pseudomonas fluorescens* AH2 strain was found to accord protection to rainbow trout from *Vibrio anguillarum* (Gram et al. 1999b) while *Pseudomonas aeruginosa* was found to inhibit shrimp pathogenic *V. harveyi*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis* and *Aeromonas* sp. (Chythanya et al. 2002, Vijayan et al. 2006). Their studies assigned the antimicrobial properties of the pseudomonads to production of iron-chelating siderophores and pyocyanin.

Amongst Gram-positive bacteria, *Bacillus* sp. are widely used as probiotics for humans and animals (Gatesoupe 1999, Duc et al. 2004). Although in terrestrial animals they are presumed to be of telluric origin, *Bacillus* sp. are frequently isolated from the intestines of healthy aquatic animals (Gatesoupe 1999). Preemptive use of *Bacillus* sp. in *Penaeus monodon* culture systems has yielded significantly better growth and survival in the presence of pathogenic *V. harveyi* (Moriarty 1998, Rengpipat et al. 2003). Recently, Ziaei-Nejad et al. (2006) obtained improved survival of *Fenneropenaeus indicus* larvae and adult maintained on a mixture of *Bacillus* spp. *Micrococcus* sp. is occasionally isolated from intestines of healthy fish, but is regarded as a transient microflora as it is not detected during all developmental stages (Sugita et al. 2002). A few strains however have been shown to posses antibacterial properties against *V. alginolyticus*, *V. vulnificus*, *Lactococcus garvieae*, and *Pasteurella piscida* (Sugita et al. 2002, Jayaprakash et al. 2005). A Gram-positive coccus A1-6 phenotypically similar to

Micrococcus sp. could protect the fingerlings of rainbow trout from *Aeromonas salmonicida* infection (Irianto & Austin 2002b).

In the present study we evaluated the capability of four microorganisms, *Pseudomonas* MCCB102 and MCCB103, *Bacillus* MCCB101, and *Micrococcus* MCCB104 proposed as potential probiotics (Jayaprakash et al. 2005, Vijayan et al. 2006) to confer protection to *Penaeus monodon* larvae from the shrimp pathogen *Vibrio harveyi*.

3.2 Materials and Methods

3.2.1 Bacteria

Four putative probiotic bacterial isolates, *Pseudomonas* MCCB102 (PS102) (Vijayan et al. 2006), *Pseudomonas* MCCB103 (Jayaprakash 2005), *Micrococcus* MCCB104 (Jayaprakash et al. 2005) and *Bacillus* MCCB101 from the microbial culture collection at National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology (CUSAT) were chosen for testing their probiotic potential against the 87 isolates *Vibrio harveyi* isolated in this study. All isolates were cryopreserved in 20-ppt salinity ZoBell's marine broth 2216E (ZB) with 10% glycerol at -80°C. Working cultures were maintained in 20-ppt ZoBell's marine agar (ZA) slants at 28°C.

3.2.2 Antagonism Assay

Antagonism of the culture as well as cell-free supernatants was tested following Jayaprakash et al. (2005). Briefly, the probiotic bacterial cultures were grown in 20-ppt salinity ZB at 28°C for 5 days. Six mm diameter discs from Whatman No.1 filter papers (stack of 3 filter papers) were prepared, sterilized at 121°C for 15 min and dried. These discs were placed on ZA plates, previously swabbed with 0.5 ml overnight grown culture (1 OD) of the target bacterial isolates. Aliquots (20 µl) of the four

probiotic bacterial cultures were dispensed on to the discs separately in triplicate. Antagonism between the four putative probionts was also tested in the same way. The plates were incubated for 24 h at 28°C and the formation of a zone of clearing around the discs was considered as positive indication of inhibitory activity.

Antagonism of cell free supernatant of the four probiotic bacteria was also tested. The four probiotic cultures were grown in ZB for 5 days on a shaker (120 rpm) at 28°C. Cells were pelleted by centrifugation (10000 x g, 4°C, 10 min), the pH of the supernatant adjusted to 7.0 and then passed through 0.2 µm pore-size cellulose-acetate membrane filter (Sartorius). Inhibitory activity on the target microbial cultures was detected by disc diffusion method as described above and the zone of inhibition around the discs recorded after 24 h using HiAntibiotic ZoneScale (Himedia).

3.2.3 Coculture Experiments

Co-culture experiments with *Pseudomonas* MCCB102 and *V. harveyi* MCCB111 was carried out following the method of Gram et al. (1999b). They were precultured separately in ZB at 28°C on a shaker at 120 rpm overnight. From the above cultures, *V. harveyi* was inoculated in 100 ml ZB to obtain an initial cell count of 10^3 cfu ml^{-1} (approx), whereas the initial levels of *Pseudomonas* MCCB102 in those flasks were 10^4, 10^5, 10^6, 10^7 and 10^8 cfu ml^{-1} respectively. All combinations were maintained in duplicate and were repeated twice. The flasks were incubated at 28°C on a shaker (120 rpm), and samples (1 ml) were withdrawn at 24 h intervals for determination of cell count. Counts of *V. harveyi* were estimated by using H&L medium (Hugh & Leifson 1953). Tubes containing 4 ml of H&L medium were inoculated with 1 ml aliquots of serially ten-fold diluted culture and overlayed with sterile liquid paraffin and incubated at 28°C for 24 h. The fermentative growth of *V. harveyi* caused a change in the pH of the medium. The highest dilution, which showed growth, was used to calculate the count of *V. harveyi* in the sample (1×10^n, where n is the highest dilution which showed fermentative reaction) and expressed as Log_{10} cfu ml^{-1}. The cell count of

Pseudomonas MCCB102 was monitored on *Pseudomonas* isolation agar (PIA) (Himedia) by spread plate method. Coculture experiments with *Pseudomonas* MCCB103 were also carried out similarly.

Experiments with *Micrococcus* MCCB104 and *Bacillus* MCCB101 with *V. harveyi* MCCB111 were carried out individually and in combination at the same initial cell numbers as above. Counts of the pathogen were monitored by withdrawing daily 1 ml samples which were serially diluted 10-fold and 0.2 ml aliquots spread plated on Thiosulphate Citrate Bile salts Sucrose (TCBS) agar (Himedia) and ZA plates. The plates were incubated at 28°C for 24 hours and colonies formed on TCBS were counted and expressed as Log_{10} cfu ml^{-1} of *V. harveyi* in the co-culture. Both the Gram-positive bacteria (*Bacillus* MCCB101 and *Micrococcus* MCCB104) do not grow on TCBS agar and also could be easily differentiated from *V. harveyi* on ZA. *Micrococcus* MCCB 104 formed yellow non-luminescent colonies while those formed by *Bacillus* MCCB 101 apart from being non-luminescent were rough and white.

3.2.4 Effect of the probiotic bacteria to *Peneaus monodon* post larvae

Groups of 2000 *P. monodon* larvae at Protozoea I stage were introduced into six 100-L FRP tanks and reared until metamorphosis to postlarvae 30 at a commercial shrimp hatchery. The effect of the four probiotics *Bacillus* MCCB101, *Micrococcus* MCCB104, *Pseudomonas* MCCB102 and MCCB103 on the health and survival of *Penaeus monodon* larvae was assessed independently. The combined effect of *Bacillus* MCCB101 and *Micrococcus* MCCB104 (designated 'Enterotrophotic') was also assessed. One group was maintained as control without any probiotic exposure. All probiotics were precultured in ZB at 28°C for 5 days and added to the rearing water every two days to obtain a final density of 10^6 cfu ml^{-1}. The larvae were maintained on a diet of *Chaetoceros* sp. (80000-130000 cells ml^{-1}) from protozoea I to mysis III and thereafter on freshly hatched *Artemia* nauplli. Water in the tanks was topped up until conversion to PL-1 following which 25-30% water was exchanged daily.

Physicochemical parameters of the rearing water such as total ammonia, nitrite, pH, salinity and temperature were monitored regularly following standard methods (APHA et al. 1995). The total heterotrophic bacterial population and total vibrio count of the rearing water and larvae were monitored periodically by spread plating 0.2 ml aliquots after 10-fold serial dilution on ZA and TCBS agar. Ten larvae from each group were washed gently in sterile seawater to remove loosely adhering particles. The larvae were lightly dried by blotting them on a sterile filter paper and macerated in 1 ml of sterile seawater. This suspension was serially diluted 10-fold and 0.2 ml aliquots were spread plated on ZA and TCBS in duplicate. The plates were incubated at 28±1 °C for 24-72 hours and those having 30-300 colonies were taken for estimating the bacterial counts which were expressed as cfu ml^{-1} and cfu $larvae^{-1}$ for water and larval samples respectively. From each of the final water and postlarval (PL-30) samples, a representative of 20 colonies were isolated, streaked on ZA to purity and presumptively grouped to genus/ family level following Oliver (1982). The health of the post larvae (PL-30) was assessed (20 numbers from each group) and scored by microscopic observation for features such as muscle opaqueness, deformities, size variation, gut content, colour and condition of the hepatopancreas, epibiont fouling, intestinal persistalsis, and muscle to gut ratio as per the FAO guidelines (FAO 2003). A formalin stress test was also given by subjecting 100 larvae from each group to 100 ppm formalin for 1 h and then monitoring them in normal rearing water for another 3 h. Final survival was taken when the larvae reached PL-30 stage statistical significance was arrived at by using the χ^2 test.

3.2.5 Evaluation of protection accorded by the probiotics to postlarvae of *P. monodon* from *Vibrio harveyi* challenge

Three groups of 30 postlarvae (PL-24) from each of the treatments (probiotic treated and control) above were transferred to plastic containers (3 L capacity). From the control group two sets of 30 PL each were maintained. They were acclimatized for 24 h following which an overnight culture of *V. harveyi* MCCB 111 was added to water to obtain 10^6 cfu ml^{-1} except to that of the negative control group. One group of 3 × 30

from the control was not challenged. An ad-libitum feeding regime with freshly hatched *Artemia* nauplii and probiotic addition as mentioned above was continued. The larvae were observed for 10 days. Assessment of the microbial population of water and larvae was carried out on ZA and TCBS agar periodically as mentioned earlier. After 10 days of observation, a relative percent survival was calculated. Results were statistically analysed using single-factor ANOVA followed by least significant difference (LSD) to compare different treatment groups.

Relative Percentage Survival (RPS)

$$= [1 - (\%\ \text{mortality in the test} \div \%\ \text{mortality in the control})] \times 100$$
$$100$$

3.3 Results

3.3.1 Antagonism Assay

In the antagonism assay, the culture and cell-free supernatants of putative probionts *Pseudomonas* MCCB102 and MCCB103 inhibited all 87 isolates of *Vibrio harveyi* and the *Bacillus* MCCB101. A clear zone of inhibition in the range 12-15 mm was obtained against *V. harveyi* with both the peudomonads. *Micrococcus* MCCB104 inhibited all *V. harveyi* isolates and the two *Pseudomonas* spp. A turbid zone of inhibition in the range of 18-22 mm was obtained against *V. harveyi*. *Micrococcus* MCCB104 did not inhibit *Bacillus* MCCB101. Neither the culture nor the cell-free supernatants of *Bacillus* MCCB101 was inhibitory to any of the isolates tested. This made us to attempt the effect of *Micrococcus* MCCB104 and Bacillus MCCB101 in combination as probionts in *Penaeus monodon* larval rearing.

3.3.2 Coculture Experiments

In the coculture experiments of *Pseudomonas* MCCB102 with *V. harveyi* MCCB111 the latter became completely undetectable in 48 h at initial cell densities greater than

10^5 cfu ml^{-1} (Fig. 2), while similar situation with *Pseudomonas* MCCB103 was obtained only at initial cell densities greater than 10^6 cfu ml^{-1} (Fig. 3). At lower initial cell densities of the two pseudomonads, *V. harveyi* MCCB111 counts could reach up to 10.5 log cfu ml^{-1} in 24 h and remained well detectable even after five days. Both pseudomonads could be recovered in high cell numbers on PIA even after 5 days from all combinations (data not shown), which showed they were unaffected by *V. harveyi* MCCB111. *Bacillus* MCCB101 and *Micrococcus* MCCB104 did not inhibit the growth of *V. harveyi* MCCB111 even when inoculated at 10^8 cfu ml^{-1} initial cell densities (Tables 4 & 5). In coculture experiments of *V. harveyi* MCCB111 with *Micrococcus* MCCB104 and *Bacillus* MCCB101, counts of the *Vibrio* were found to increase and remained unaffected even at 10^8 cfu ml^{-1} initial densities of the probionts. Akin to with the pseudomonads, counts of *Bacillus* MCCB101 and *Micrococcus* MCCB104 were unaffected by *V. harveyi* MCCB111 in the cocultures.

3.3.3 Impact of probiotics on larval health and survival

Improved survival of *P. monodon* post larvae was obtained when their rearing water was supplemented with all the probiotics individually as well as in combination of *Bacillus* MCCB101 and *Micrococcus* MCCB104 (Enterotrophotic) (Table 6). Survival was 70% or more in tanks treated with *Micrococcus* MCCB104, *Bacillus* MCCB101 and Enterotrophotic, while in those that received *Pseudomonas* MCCB102 and MCCB103 it was 66.35 and 58.3 % respectively. The survival obtained with the probiotic treatment groups were significantly higher ($p<0.01$, $\chi^2 = 121.9$) in comparison to the control. Based on χ^2 values, the order of significance was treatment with *Micrococcus* MCCB104 followed by *Bacillus* MCCB101, Enterotrophotic, *Pseudomonas* MCCB102 and lastly *Pseudomonas* MCCB103. When 20 larvae from each group were examined under the microscope all were transparent, with their guts full, dark hepatopancreas, high intestinal peristalsis, no fouling or deformities on the exoskeleton or gills, muscle:gut ratio at the 6^{th} abdominal segment > 3:1, and uniform size (%CV <15%) (Table 7). Total ammonia was consistently higher in all probiotic

treated tanks than the control (Table 8). When 100 post larvae (PL-30) from each treatment were subjected to formalin (100 ppm) stress test for 1 h, there were no mortalities indicating good health of the larvae (Table 9). Total *Vibrio* population was lowest in rearing water and larvae of tanks which were supplemented with *Pseudomonas* MCCB102 and MCCB103. Notably, *Vibrio* population was not detected in the larvae of tanks supplemented with *Pseudomonas* MCCB102 until stage PL-8. *Vibrio* population counts were similar in the control and tanks supplemented with *Bacillus* MCCB101, *Micrococcus* MCCB104 and Enterotrophotic (Table 10). Luminescent bacteria were not detected in any of the treatments and the control at any stage during the experiment. The bacterial diversity in the larvae of all treatment groups and the control was high and presumptive generic level identification revealed the isolates to belong to *Pseudomonas* sp., *Vibrio* sp., *Photobacterium*, *Bacillus* sp., *Planococci* sp., *Micrococcus* sp., *Acenitobacter* sp. (Table 11).

3.3.4 Response of probiotic treated post larvae to challenge with *Vibrio harveyi* MCCB111

When larvae from each of the probiotic treatment and the control were challenged with *V. harveyi* at stage PL-25, 10-day post challenge survival (RPS) was highest in tanks supplemented with *Micrococcus* MCCB104 (86.67) and *Pseudomonas* MCCB102 (80.0) and MCB103 (86.67) (Table 12). An RPS of 66.67 and 36.67 was obtained in tanks supplemented with *Bacillus* MCCB101 and Enterotrophotic respectively. Using single-factor ANOVA followed by LSD the survivals obtained in tanks supplemented with *Bacillus* MCCB101, *Micrococcus* MCCB104, *Pseudomonas* MCCB102 and MCCB103 were observed to be significantly higher to that of control tanks ($p < 0.05$) while Enterotrophotic treatment was found not significant ($p > 0.05$).

V. harveyi counts were estimated from the luminous bacterial counts obtained from total plate count enumeration plates (Table 13). The challenge concentration obtained in the tanks was as fixed at about 10^6 cfu ml^{-1} and at this point they were the only

colonies appearing on TPC plates at the countable (30-300 colonies) higher dilutions. Their numbers declined to about 10% of the colonies appearing on TPC plates by the 5^{th} day and no *V. harveyi* cells were detectable in the samples drawn on 10^{th} day after challenge. Luminous colonies could not be recovered from any of the larval samples. The luminescent bacterial counts in the rearing water detected through TCBS were one log lower than that detected on ZoBell's agar. The total *Vibrio* count was found to decline during the course of the experiment in the probiotic supplemented tanks. Similar to the observations of the previous experiment, total *Vibrio* count was least in tanks supplemented with *Pseudomonas* MCCB102 and MCCB103. *Vibrio* counts in the larvae of control tanks which did not receive any probiotic supplement but challenged with *V. harveyi* MCCB111 was found to increase over the 10-day observation period. Here too the bacterial diversity in the larvae of the final sample was high in all treatment groups and the presumptive generic identification carried out revealed the presence of *Pseudomonas* sp., *Bacillus* sp., *Planococci* sp., *Micrococcus* sp., *Vibrio* sp., and *Photobacterium* sp (Table 14).

3.4 Discussion

The data generated through coculture experiments suggest that pseudomonads at cell densities $>10^5$ cfu ml^{-1} for *Pseudomonas* MCCB102 and $>10^6$ cfu ml^{-1} for *Pseudomonas* MCCB103 were required for elimination of *V. harveyi* from the system suggesting that in vivo studies should be designed accordingly. In contrast, *Bacillus* MCCB101 and *Micrococcus* MCCB104 could not inhibit *V. harveyi* in coculture. This situation was observed more or less in the same order in vivo when introduced into the larval rearing tanks. Clearly *Vibrio* population was found affected by the pseudomonads and not by *Micrococcus* MCCB104, *Bacillus* MCCB101 or the combination of the last two. Enterotrophotic. However larval survival at the end of the experiment was not related to the vibriocidal property of the pseudomonads as the survival in the tanks supplemented with them was 66.35 and 58.3 % (*Pseudomonas* MCCB102 and MCCB103 respectively) while it was more than 70% in tanks

supplemented with *Micrococcus* MCCB104, *Bacillus* MCCB101 and Enterotrophotic. This again suggests a complex relationship between *Vibrio* and probiotic population and larval survival where the relationship is not straightforward.

In the challenge experiment with *V. harveyi* also the total *Vibrio* count was the lowest in tanks treated with *Pseudomonas* MCCB102 and MCCB103. But the post challenge larval survival was not related to this situation as the RPS was highest in tanks supplemented with *Micrococcus* MCCB104 where *V. harveyi* numbers stood higher while with *Pseudomonas* MCCB103, *V. harveyi* depleted rapidly subsequent to administration. These observations suggest once again that survival of larvae is not dependent on *Vibrio* population size alone. The lower *Vibrio* populations in tanks treated with *Pseudomonas* MCCB102 and MCCB103 suggests it could be attributed in some part to the antimicrobial compounds produced by them as was expected from the results of the in vitro disc diffusion assays and coculture. This *Vibrio* inhibition however did not translate into higher larval survival. In contrast survivals were much higher in tanks treated with *Micrococcus* MCCB104 (where only a vibriostatic effect was observed in disc diffusion assay), *Bacillus* MCCB101 (no in vitro antagonism), and Enterotrophotic even at higher *Vibrio* counts. It must be mentioned that challenge experiments with *V. harveyi* and other pathogenic vibrios do not necessary result in the manifestation of symptoms and reproducibility rests on many intrinsic and extrinsic factors which has led to the hypothesis that they could be opportunists (Saulnier et al. 2000, Soto-Rodriguez et al. 2003, Jayaprakash et al. 2006b).

All these observations points to the existence of a 'probiotic effect' exerted on the larvae of *P. monodon* by the four probionts when supplemented to the rearing water where their presence has beneficially impacted larval survival. Verschuere et al. (2000b) in their definition of probiotics have included all microbial adjuncts that exert a beneficial effect on the host by modifying the host-associated or ambient microbial community or ensure improved use of the feed or enhance its nutritional value or enhance the host response towards disease or improve the quality of its ambient

environment. This implies probiotics can favour the host animal in ways other than antagonism of pathogens as *Bacillus* MCCB101 which improved larval survival even though it was not exhibiting in vitro antagonism to *V. harveyi*. Signal antagonists like halogenated furanones from the alga, *Delisea pulchra* were found to interfere with cell-cell communication of *V. harveyi* which was found to reduce virulence to *P. monodon* but did not affect the growth of the bacterium (Manefield et al. 2000).

One of the noteworthy observations made in this study was the good survival and metamorphosis of larva in the control tanks which were not supplemented with probiotics at any point during the experiment. It could be reasoned that the overall generic diversity in the system was higher which might have paved the way for preventing colonization by *V. harveyi* in the larvae when challenged with the pathogen. This could be the reason for not recovering *V. harveyi* from any of the larval samples (including control) following challenge. Singh et al. (1989) have linked higher generic diversity to better survival and results of the present study further corroborate this. It was also observed that *V. harveyi* counts on TCBS were at least 1 log value lower compared to those from ZA plates, therefore the ZA counts were chosen for analysis. Addition of probiotic bacteria could also impact the physico-chemical parameters of the water which can also impact the larvae. Although total ammonia was consistently higher in all probiotic treated tanks compared to that in control, it did not appear to influence the health or survival of the larvae.

In vitro inhibition of vibrios including *V. harveyi* by *Pseudomonas aeruginosa* and *P. fluorescens* is well documented in literature (Dopazo et al. 1988, Toranzo & Torres 1996, Gram et al. 1999b, Chythanya et al. 2002, Vijayan et al. 2006) but in vivo studies are sparse. Gram et al. (1999b) report protection of rainbow trout from mortalities due to *V. anguillarum* when *P. fluorescens* AH2 probiotic was added to the rearing water of the fish. They however do not mention the status of populations of *V. anguillarum* or other *Vibrio* in their in vivo experiments. From the same group there was another report where they could not obtain in vivo protection of salmon from *Aeromonas salmonicida* caused furunculosis with the probiont *P. fluroescens* AH2 even though the latter

inhibited the pathogen in vitro antagonism assays. Protection to rainbow trout from *Flavobacterium columnare* infections also could not be conferred by bath treatments with an antagonistic *Pseudomonas* sp. MT5 (Suomalainen et al. 2005). The authors were also unable to detect the probiont in the fish after treatment using a specific detection length heterogeniety PCR (LH-PCR) assay. In vivo experiments in protecting fish and shellfish from pathogens using *P. aeruginosa* have not come to our attention. Nevertheless they are well documented to accord protection to plants from phytopathogenic fungi (Mavrodi et al. 2006). Additionally, their inhibitory activity against *Staphylococcus* sp. and *Helicobacter pylori* are well documented (Arunkumar et al. 1997, Krausse et al. 2005). In this background, the improved survival compared to the control we obtained with the two *P. aeruginosa* strains, MCCB102 and MCCB103 before and after challenge with *V. harveyi* MCCB111 is significant.

Preemptive manipulation of bacterial flora of shrimp larval rearing environment with probiotic strains with or without antagonism has been known to yield improved survival (Verschuere et al. 2000a). The diversity and composition of the gut microflora of early larval stages of shrimps and fishes reflects that of the ambient microflora (Hansen & Olafsen 1999). Therefore preemptive manipulation of the microflora during the early larval stages is likely to significantly impact survival and health of the larvae not only in hatcheries but also in farming stages. This was demonstrated in the experiments conducted by Ziaei-Nejad et al. (2006) in which they obtained improved feed conversion ratio, specific growth rate and survival of *F. indicus* maintained on a mixture of *Bacillus* spp. from sub-larval stages hatchery to adults in farms. The authors however do not report any antagonistic activity of the *Bacillus* spp. used by them against *Vibrio* spp. in their study nor are they clear on the *Vibrio* populations in the water or shrimp following probiotic administration. They however report enhanced enzyme activity in the gut of shrimp following feeding with *Bacillus* spp. Supplementation of rearing water and feed of *P. monodon* adults by a probiotic strain of *Bacillus* sp. resulted in improved survival and protection from challenge with *V. harveyi* (Rengpipat et al. 1998). Feeding of shrimp with *Bacillus* sp. allowed their domination over *Vibrio* in their intestines while *Vibrio* spp dominated the intestinal

flora amongst control animals. The *Bacillus* MCCB101 strain employed in this study was not antagonistic to *V. harveyi* in vitro, but it could significantly impact survival of *P. monodon* larvae before and after challenge with the pathogen.

Micrococcus as a probiotic for fish and shellfish has only recently been identified (Irianto & Austin 2002b, Jayaprakash et al. 2005). In the study of Irianto & Austin (2002b) protection of rainbow trout from furunculosis could be obtained when *Micrococcus* A1-6 was administered along with feed. In a later study they also showed that inactivated cells of culture A1-6 could also accord protection to rainbow trout from furunculosis (Irianto & Austin 2003). In this study addition of *Micrococcus* MCCB104 to rearing water of *P. monodon* larvae was sufficient to improve survivals before and after challenge. The lack of significance in the survival of larvae after challenge with *Vibrio harveyi* MCCB111 in the tank supplemented with Enterotrophotic cannot be explained at this stage since independently both the probionts that went into the combination had significantly improved survival. In fact the survival in this treatment before challenge was significantly higher than control.

One of the criteria for selecting candidate probionts is that they must be non toxic (Verschuere et al. 2000b). From the higher survival and good health of the larvae observed with all probiotic treatments, it was evident that they did not exhibit any toxicity. Colonization of the intestine and other tissues of marine larvae takes place during the first feeding stages and the diversity of the ambient flora determines the microbial diversity in the animal (Hansen & Olafsen 1999). If opportunists are dominant, in the ambient water, then they will colonize and proliferate. Therefore it has been suggested and experimentally validated that probiotic treatment at early stages significantly impacts survival positively preventing opportunists from making deleterious impact (Ringo & Vadstein 1998, Skjermo & Vadstein 1999). Furthermore experiments conducted by preemptive treatment with selected bacterial strains in gnotobiotic *Artemia* juveniles protected them from mortalities due to *V. proteolyticus* although the probiotic strains did not inhibit the *Vibrio* in in vitro antagonism assays (Verschuere et al. 1999, Verschuere et al. 2000a). Probiotic treatment of *P. monodon*

larvae and post larvae with *Saccharomyces boulardii*, *Lactobacillus plantarum* and *Bacillus subitlis* improved survival even though *Vibrio* populations were always present in the tanks (Anikumari 2005). In this study we have observed that presence of vibrios in the water and larvae (in all treatments including control) did not negatively impact survival. Addition of probiotic *V. pelagius* also did not impact *Vibrio* populations in the larvae of turbot, *Scophthalmus maximus*; both probiotic treated and control had similar TVC counts (Ringo & Vadstein 1998).

Generic diversity index in all the tanks were more or less the same which could have contributed to lower mortalities in the control before and after challenge with *V. harveyi* MCCB111. High generic diversity in the larvae has been linked to better survivals (Singh et al. 1989) and the results of this study further validate the earlier finding. Identification of bacterial diversity using phenotypic characters and fatty acid methyl ester (FAME) analysis are not conclusive and stringer molecular methods may be required (Verschuere et al. 1999).

Fate of probiotics in aquaculture and mechanism of their action are much needed information that would go a long way in optimizing their use. In the present study we looked for the characteristic colonies of our probiotic strains on the enumeration plates, but they could never be detected. We attempted to recover the pseudomonads by plating samples on PIA and noticed counts rapidly tapering off over 2-3 days (data not shown). It has been reported that *V. pelagius*, a probiotic strain applied to the water also could not be detected in rearing water or gut of turbot after 7 days post application (Ringo & Vadstein 1998). In other studies in fish and shellfish, the applied probiotics could seldom be recovered (Irianto & Austin 2002b). Therefore, fate of probiotics in aquaculture (in larviculture as well as in farms) needs further in-depth study using specific detection techniques for tangible explanations. The present study proposes the four strains *Bacillus* MCCB101, *Pseudomonas* MCCB102 and MCCB103, and *Micrococcus* MCCB104 as potential probionts which can accord protection to larvae and post larvae of *P. monodon* from vibriosis in general and *V. harveyi* in particular. The in vivo trials in this study were conducted in simulated hatchery mesocosms in 100 l

. FRP tanks at a commercial shrimp hatchery. The strengths of these probionts will emerge when they are tested on a pilot scale in larger capacity commercial production tanks.

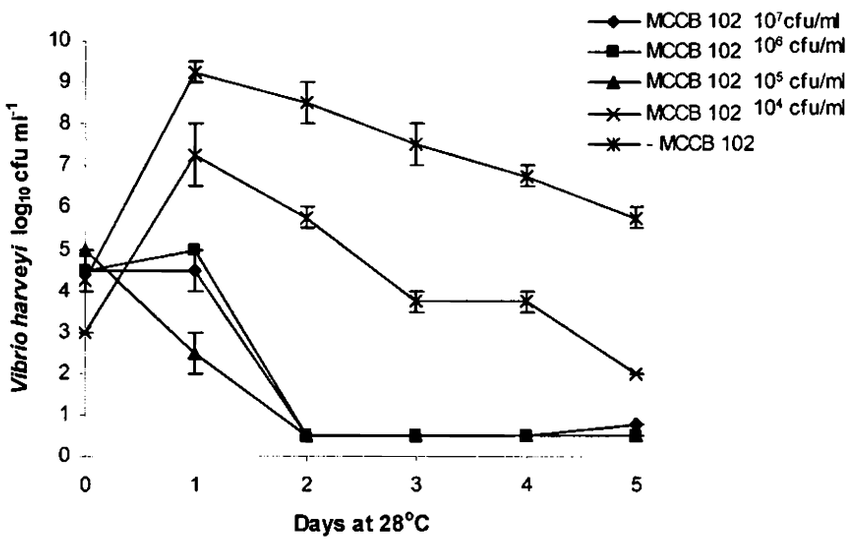


Fig. 2. Growth of *Vibrio harveyi* at different cell densities of *Pseudomonas* MCCB102 in coculture

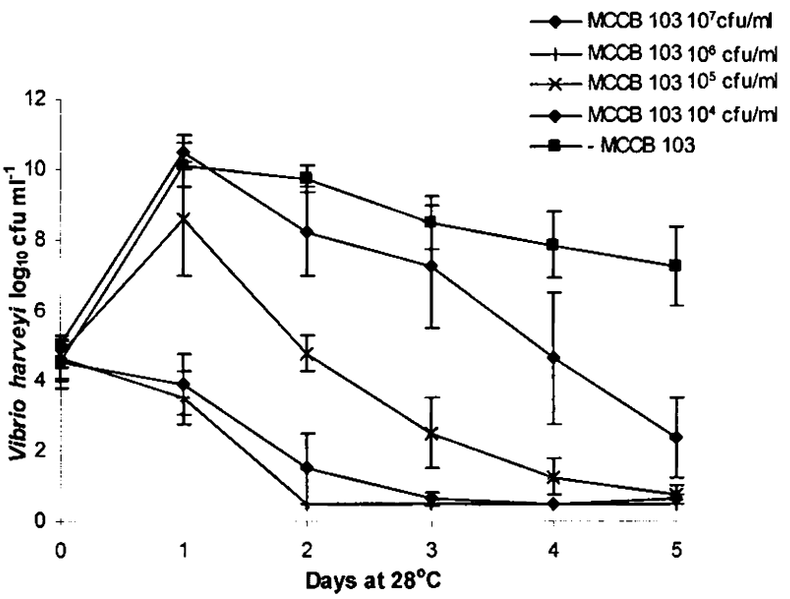


Fig. 3. Growth of *Vibrio harveyi* at different cell densities of *Pseudomonas* MCCB103 in coculture

Table 4. Growth of *Vibrio harveyi* over 5 days at different cell densities of *Bacillus* MCCB101

Bacillus MCCB101 initial cell density	*Vibrio harveyi* LB 3 counts (Mean Log_{10} cfu ml^{-1})		
	0 h	24 h	120 h
10^8 cfu ml^{-1}	3.91 ±0.03	8.77 ±0.01	8.57 ±0.03
10^7 cfu ml^{-1}	3.87 ±0.04	9.71 ±0.02	9.15 ±0.05
10^6 cfu ml^{-1}	3.93 ±0.01	9.61 ±0.06	9.30 ±0.06
10^5 cfu ml^{-1}	3.99 ±0.09	9.72 ±0.01	9.21 ±0.12
10^4 cfu ml^{-1}	4.04 ±0.09	9.74 ±0.03	9.37 ±0.03
No MCCB101	3.97 ±0.02	9.80 ±0.01	9.10 ±0.15

± Standard Error

Table 5. Growth of *Vibrio harveyi* over 5 days at different cell densities of *Micrococcus* MCCB104

Micrococcus MCCB 104 initial cell density	*Vibrio harveyi* LB 3 counts (Mean Log_{10} cfu ml^{-1})		
	0 h	24 h	120 h
10^8 cfu ml^{-1}	3.38 ±0.09	8.12 ±0.40	8.43 ±0.13
10^7 cfu ml^{-1}	3.27 ±0.25	9.01 ±0.33	8.74 ±0.37
10^6 cfu ml^{-1}	3.30 ±0.25	9.49 ±0.21	8.74 ±0.54
10^5 cfu ml^{-1}	3.51 ±0.52	9.48 ±0.17	8.84 ±0.37
10^4 cfu ml^{-1}	3.64 ±0.41	9.51 ±0.23	9.11 ±0.25
No MCCB104	3.83 ±0.23	9.57 ±0.26	9.13 ±0.19

± Standard Error

Table 6. Survival of *Penaeus monodon* postlarvae under probiotic treatment and after challenge with *Vibrio harveyi* MCCB111

Probiotic Treatment	Final survival of Post larvae under Probiotic treatment (%) n=2000	RPS after challenge with *Vibrio harveyi* MCCB111 (Average of three groups of 30 post larvae each)
Control	54.00	
Bacillus MCCB101	70.85*	66.67*
Pseudomonas MCCB102	66.35*	80.00*
Pseudomonas MCCB103	58.30*	86.67*
Micrococcus MCCB104	78.90*	86.67*
Enterotrophotic	69.35*	36.67

*Values significant ($p<0.01$)

Table 7. Larval health assessment by microscopic examination of post larvae (PL30) of probiotic treated and control tanks

Treatment	Larval Health Scores			
	Size in cm (% CV)	Colour	Colour and condition of Hepatopancreas, Gut condition, Fouling, Deformity	Muscle:Gut Ratio (% CV)
Control	2.3 (4.18)	Clear	10	88.33 (0.46)
MCCB101	2.1 (1.92)	Clear	10	88.28 (0.57)
MCCB102	2.1 (2.05)	Clear	10	87.10 (0.59)
MCCB103	2.2 (2.55)	Clear	10	87.34 (0.60)
MCCB104	2.0 (2.16)	Clear	10	84.58 (0.82)
Enterotrophotic	2.0 (3.08)	Clear	10	87.16 (0.48)

CV: Coefficient of Variation

Table 8. Range of physico-chemical parameters observed in the probiotic treated and untreated rearing water of *Peaneus monodon* during the experiment

Treatment	Parameter			
	Temperature (°C)	pH	Total Ammonia (ppm)	Total Nitrite (ppm)
Control	27-29	7.5-8.0	0.06-1.01	0.0063 – 0.0377
Bacillus MCCB101	27-29	7.5-8.0	0.02-1.75	0.0012 – 0.0244
Pseudomonas MCCB102	27-29	7.5-8.0	0.05-1.79	0.0086 – 0.0314
Pseudomonas MCCB103	27-29	7.5-8.0	0.09-1.67	0.0043 – 0.0299
Micrococcus MCCB104	27-29	7.5-8.0	0.03-1.15	0.0028 – 0.0354
Enterotrophotic	27-29	7.5-8.0	0.04-2.28	0.0031 – 0.0458

Table 9. Survival of *Penaeus monodon* post larvae (PL-30) after formalin (100pm) stress test of

Treatment	Survival (n=100)	
	After 1 h in Formalin	After 3 h in normal rearing water
Control	98	98
Bacillus MCCB101	99	99
Pseudomonas MCCB102	99	99
Pseudomonas MCCB103	98	98
Micrococcus MCCB104	100	100
Enterotrophotic	100	100

Table 10. Bacterial population in the rearing water and larvae at different stages during the larval cycle of probiotic supplemented and control (All counts are averages of 3 samples plated in duplicate)

Treatment		Rearing water ($\times 10^3$ cfu/ml)					
		Zoea-1	Zoea-3	Mysis-2	PL-2	PL-8	PL-30
Bacillus MCCB	TPC	270	1200	570	400	150	550
101	TVC	0.042	150	300	11	12	5.2
Pseudomonas	TPC	190	1000	86	250	15	290
MCCB 102	TVC	0.025	0.19	ND	0.18	1.3	5.8
Pseudomonas	TPC	200	1100	49	240	250	290
MCCB 103	TVC	0.008	0.29	ND	0.63	0.95	7.5
Micrococcus	TPC	170	1200	160	390	380	490
MCCB 104	TVC	ND	1.7	ND	8.7	6.0	6.2
Enterotrophotic	TPC	170	910	310	420	390	350
	TVC	0.033	32	8.8	13	9.0	5.8
Control	TPC	260	56	450	360	420	320
	TVC	ND	0.15	4.5	4.3	14	9.7
		Larvae ($\times 10^3$ cfu/larvae)					
Bacillus MCCB	TPC	0.12	9.3	23.0	10.0	12.0	1.8
101	TVC	ND	0.049	0.025	0.022	0.13	0.76
Pseudomonas	TPC	0.14	6.2	3.2	1.8	0.70	1.4
MCCB 102	TVC	ND	ND	ND	ND	0.008	0.088
Pseudomonas	TPC	0.11	11.0	0.12	1.5	2.2	0.75
MCCB 103	TVC	ND	0.017	ND	ND	0.004	0.089
Micrococcus	TPC	0.085	8.6	0.80	1.1	0.57	0.90
MCCB 104	TVC	ND	0.03	ND	0.02	0.03	0.015
Enterotrophotic	TPC	0.86	13.0	10.0	11.0	4.3	0.75
	TVC	ND	0.067	0.005	0.063	2.5	0.08
Control	TPC	0.091	7.4	11.0	11.0	6.6	1.2
	TVC	ND	0.035	0.009	0.028	0.21	0.12

TPC: Total Plate Count TVC: Total Vibrio count ND: Not detected

Table 11. Microbial diversity of probiotic treated and untreated rearing water and larvae of *Penaeus monodon*

Treatment	Rearing Water		Larvae	
	SDI	Genera	SDI	Genera
Bacillus MCCB 101	0.325	*Pseudomonas* sp./ *Moraxella*	1.249	*Pseudomonas* sp./ *Vibrio*/ *Photobacterium*/ *Bacillus*/ *Planococci*
Pseudomonas MCCB 102	0.802	*Pseudomonas* sp./ *Bacillus*/ *Acenitobacter*/ *Moraxella*	1.201	*Pseudomonas* sp./ *Vibrio*/ *Photobacterium*/ *Micrococcus*/ *Planococci*
Pseudomonas MCCB 103	1.094	*Pseudomonas* sp./ *Bacillus*/ Lactobacilli/ Gram positive cocci	1.490	*Pseudomonas* sp./ *Vibrio*/ *Photobacterium*/ *Micrococcus*/ *Planococci*
Micrococcus MCCB 104	0.394	*Pseudomonas* sp./ *Bacillus*/ *Vibrio*	1.595	*Pseudomonas* sp./ *Vibrio*/ *Photobacterium*/ *Micrococcus*/ *Planococci*/ *Acenitobacter*/ Lactobacilli
Enterotrophotic	0.997	*Pseudomonas* sp./ *Bacillus*/ *Vibrio*/ *Photobacterium*	1.333	*Pseudomonas* sp./ *Vibrio*/ *Photobacterium*/ *Bacillus*/ *Micrococci*
Control	1.194	*Pseudomonas* sp./ *Bacillus*/ *Vibrio*/ *Photobacterium*/ *Moraxella*	1.392	*Pseudomonas* sp./ *Vibrio*/ *Photobacterium*/ *Bacillus*/ *Planococci*

SDI: Shannon diversity index

Table 12. Survival of *Penaeus monodon* postlarvae after challenge with *Vibrio harveyi* MCCB111

Probiotic Treatment	RPS after challenge with *Vibrio harveyi* MCCB111 (Average of three groups of 30 post larvae each)
Bacillus MCCB101	66.67*
Pseudomonas MCCB102	80.00*
Pseudomonas MCCB103	86.67*
Micrococcus MCCB104	86.67*
Enterotrophotic	36.67

*Values significant ($p<0.01$)

Table 13. Bacterial population in the rearing water and larvae after challenge with *Vibrio harveyi* MCCB111 (All counts are averages of 3 samples plated in duplicate)

Treatment		Rearing water ($\times 10^3$ cfu/ml)		
		Day1	Day 5	Day 10
Bacillus MCCB 101	TPC	3258.33	2141.67	3858.33
	TVC	341.67	18.17	10.00
	LBC	3258.33	108.33	ND
Pseudomonas MCCB 102	TPC	2800.0	975.0	2816.67
	TVC	366.67	5.58	7.75
	LBC	2800.0	118.33	ND
Pseudomonas MCCB 103	TPC	1916.67	652.5	1683.33
	TVC	254.17	9.33	7.33
	LBC	1916.67	95.83	ND
Micrococcus MCCB 104	TPC	2100.0	1100.0	2608.33
	TVC	516.67	25.83	12.50
	LBC	2100.0	196.67	ND
Enterotrophotic	TPC	1491.67	3475.0	2625.0
	TVC	691.67	17.08	16.75
	LBC	1491.67	150.0	ND
Control	TPC	3191.67	2608.33	3400.0
	TVC	182.50	21.92	64.17
	LBC	3191.67	249.17	ND
		Larvae* ($\times 10^3$ cfu/larvae)		
Bacillus MCCB 101	TPC	1.68	14.42	29.50
	TVC	ND	0.43	0.72
Pseudomonas MCCB 102	TPC	1.40	7.25	6.50
	TVC	0.02	0.03	0.08
Pseudomonas MCCB 103	TPC	3.00	22.55	18.18
	TVC	0.10	0.17	0.52
Micrococcus MCCB 104	TPC	17.33	9.65	13.02
	TVC	0.80	0.23	0.47
Enterotrophotic	TPC	2.85	27.67	30.17
	TVC	ND	0.40	0.30
Control	TPC	1.93	22.83	22.84
	TVC	0.35	0.64	1.43

TPC: Total plate count TVC: Total *Vibrio* count LBC: Luminescent bacterial count
ND: Not detected
*Luminescent bacteria were not detected in any of the larval samples

Table 14. Microbial diversity of probiotic treated and untreated rearing water and larvae of *Penaeus monodon* challenged with *Vibrio harveyi* MCCB111

Treatment	Rearing Water		Larvae	
	SDI	Genera	SDI	Genera
Bacillus MCCB 101	0.731	*Pseudomonas* sp *Photobacterium/ Planococci/ Moraxella*	1.142	*Pseudomonas* sp./ *Bacillus/ Planococci/ Micrococcus*
Pseudomonas MCCB 102	1.151	*Pseudomonas* sp./ *Bacillus/ Enterobacteriaceae/ Planococci*	0.845	*Pseudomonas* sp./ *Vibrio/ Photobacterium/ Micrococcus*
Pseudomonas MCCB 103	1.4667	*Pseudomonas* sp./ *Planococci/ Micrococcus/ Acenitobacter/ Photobacterium*	1.490	*Pseudomonas* sp./ *Micrococcus/Bacillus*
Micrococcus MCCB 104	1.249	*Pseudomonas* sp./ *Bacillus/ Vibrio/*	1.357	*Pseudomonas* sp./ *Vibrio/ Photobacterium/ Micrococcus/ Planococci/* Lactobacilli
Enterotrophotic	0.693	*Pseudomonas* sp./ *Vibrio/ Photobacterium*	0.999	*Pseudomonas* sp./ *Vibrio/ Photobacterium/ Bacillus/ Micrococci*
Control	1.313	*Pseudomonas* sp./ *Bacillus/ Vibrio/ Photobacterium/ Planococci/ Staphylococci*	1.335	*Pseudomonas* sp./ *Enterobacteriaceae/ Bacillus/ Planococci*

SDI: Shannon diversity index

CHAPTER - 4

Isolation and Characterization of Bacteriophages Infective to *Vibrio harveyi*

CHAPTER - 4

Isolation and characterization of bacteriophages infective to *Vibrio harveyi*

4.1 Introduction

Resistance of pathogens to antimicrobials is a serious problem and the search for new chemotherapeutants is time consuming, expensive and at times with little reward especially when bacteria rapidly develop resistance to them (Matsuzaki et al. 2005). This has prompted a search for alternatives and bacteriophages are one of the agents that are actively being pursued. Many studies conducted in the control of pathogens of human and terrestrial animals employing bacteriophages have yielded promising results (Smith & Huggins 1987, Barrow et al. 1998, McNerney & Traoré 2005). They are being explored for application in the control of phytopathogens, food safety and food preservation as well (Schnabel & Jones 2001, Leverentz et al. 2003, Wagenaar et al. 2005). The uniqueness of bacteriophages as therapeutics is that they are self replicating entities (often a single dose being sufficient), specific to their targets and little or no side effects (Matsuzaki et al. 2005). However concern exists with their use because of their ability to be agents of generalized transduction in the environment and thereby mediate transfer of genes among bacteria species.

Attempts of phage therapy in aquaculture systems are a fairly recent development and have been attempted against *Lactococcus garvieae* in yellow tail *Seriola quinqueradiata*, *Pseudomonas plecoglossicida* in *Plecoglossus altivelis* and *V. harveyi* in *P. monodon* larval rearing systems (Nakai et al. 1999, Nakai & Park 2002, Park & Nakai 2003, Vinod et al. 2006). In all these studies improved survival of the animals and protection from the pathogens was obtained. These investigations have shown that phages can act as potent therapeutic agents in the management of pathogens of aquatic animals in culture.

One important issue to be addressed before selecting a phage for therapeutic studies is to characterize the isolates and establish their identity (Summers 2001). This is important because phages selected for therapeutic purposes must not carry virulence genes or properties conferring antimicrobial resistance (Wagner & Waldor 2002). In addition bacteriophage-bacteriophage interactions and their role in the modulation and expression of virulence in bacterial pathogens are coming to light (Boyd et al. 2001, Wagner & Waldor 2002). The following chapter details the isolation of phages against *V. harveyi* and the experiments conducted for partially characterizing them.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Isolation of bacteriophages

Eighty seven *Vibrio harveyi* isolates previously isolated from diseased larvae of *Penaeus monodon* and other hatchery environs from the culture collection at National Centre for Aquatic Animal Health, Cochin University of Science and Technology were chosen for the study. All isolates were cryopreserved in 20-ppt salinity ZoBell's marine broth 2216E (ZB) with 10% glycerol at -80°C. Working cultures were maintained in 20-ppt ZoBell's marine agar (ZA) slants at 28°C. Bacteriophages were isolated from a sediment sample collected from the Vembanad estuary, Kochi, Kerala following an enrichment method (Cerveny et al. 2002). Briefly, 400 ml sediment slurry was prepared by mixing 100g sediment with 200 ml estuarine water (which came along with the sediment) and 200 ml aged seawater of the same salinity. Primary enrichment of bacteriophages in the sample was carried out by mixing 250 ml slurry, 200 ml ZB and 5 ml each overnight cultures of 12 strains of *V. harveyi* MCCB 111, LB6, LB12, LB20, LB28, LB35, LB40, LB45, LB52, LB60, LB65 and LB72. Following an overnight incubation under continuous agitation (120 rpm) at 28°C, the mud and bacteria were removed the next day by centrifugation at 10000 × *g* and supernatant was filter sterilized through 0.2 µm pore-size cellulose-acetate membrane filter (Sartorius) pre-washed in 3 % beef extract. Phage activity in the supernatant was further amplified by mixing an equal volume of static overnight cultures of each of the 12 strains

separately and shaking the cultures (120 rpm) overnight at 28°C. On the next day the cultures were centrifuged and filtered as described above and presence of bacteriophages in the lysates was detected by dropping 10 µl of the filtrates on ZA plates seeded with a lawn of each of the bacterial strains. Plaques appeared on plates seeded with LB6, LB12, LB20, LB28, LB45 and LB65 but not with any other *V. harveyi* cultures.

4.2.2 Determination of phage titres in lysates

Soft agar overlay method was followed to determine the number of phage particles in the lysates. For this the lysates were serially diluted 10-fold upto 10^{10} dilution factor. 200 µl from each dilution was mixed with 100 µl of overnight culture of host strain in 3 ml molten soft agar (50°C, Agar 0.3%, pH 7.5) and dispensed onto ZA plates in duplicate. The plates were incubated overnight and dilutions showing countable plaques (30-300 plaques per plate) were counted for determining the phage titres in the lysates. The assay was standardized using a coliphage (KSW1) isolated from raw sewage on an *Escherichia coli* host strain NCTC 10213.

4.2.3 Purification of phages

The following protocol was followed for the purification of phages. An isolated plaque formed soft agar overlay was picked using a sterile loop and dispensed into 1 h old culture (25 ml vol) of the host *V. harveyi* strain. The culture was incubated at 28°C under continuous agitation (120 rpm) for 8 h. Phages were harvested in the supernatant by centrifugation and filter sterilized as described above. The resulting lysate was serially diluted 10-fold and plated by soft agar overlay to check phage titres and the above process repeated twice. All six phages were purified in this manner and the lysate obtained after the third purification was used for further work. All lysates were stored at 4°C.

4.2.4 Stocking of phages

High titre stocks were prepared following the method described in Rowher et al. (2000) with slight modifications. 500 µl of 10^3 dilution of the purified lysate was mixed with their respective host strain and plated by soft agar overlay. When confluent lysis was observed, 5 ml Salt-Magnesium (SM) buffer (50mM Tris HCl pH 7.5, 100mM NaCl, 10mM $MgSO_4$, 0.1% gelatin) (Sambrook & Russell 2001) was added to plates and allowed to stand for 10 min. The buffer was carefully decanted and saved taking care not to disturb the agar. A further 2 ml was added and the procedure repeated. The decanted and saved material was centrifuged at 10000 × g for 10 min at 4°C and filter sterilized by passing through 0.2 µm pore-size Durapore (Millipore) membrane filter, titre checked by serial dilution and soft agar overlay and stored at 4°C in 1 ml aliquots.

4.2.5 Host-Range of the phages

Host range of the phages was assessed against 162 bacterial isolates spanning 14 species. These were 87 strains of *V. harveyi* isolated in this study, *Bacillus* MCCB101, *Pseudomonas* sp. MCCB 102, 103, 117, 118, 119 *Micrococcus* sp. MCCB104, vibrios and aeromonads isolated from *Macrobrachium reosenbergii* larval rearing systems (Jayaprakash et al. 2006a) viz. *V. alginolyticus* (8 strains MRNL 1-8), *V. cholerae* (11 strains MRCS 11-13, 16, 17, 19, 20, 23, 35, 37, 39), *V. fluvialis* MRCS 26, *V. mediterranei* (7 strains MRCS 15, 18, 22, 32, 34, 36, MRQL 27), *V. nereis* (8 strains MRCS 29-31, MRQL 13, 29, 32, 34, 35), *V. parahaemolyticus* (4 strains MRCS 1, 6, 8, 9), *V. proteolyticus* (2 strains MRCS 4, 10), *V. splendidus* (2 strains MRCS 3, 5), *V. vulnificus* (8 strains, MRQL 5, 9, 10, 19, 20, 23, 33, 36), *Aeromonas* sp. (6 strains MRCS 2, 7 MRNL 9, 10, 16, 17) and the type strains *V. harveyi* LMG4044, *V. alginolyticus* LMG4409, *V. alginolyticus* MTCC4439, *V. cholerae* MTCC, *V. fluvialis* LMG11654, *V. mediterranei* LMG11258, *V. parahaemolyticus* LMG2850, *V. proteolyticus* LMG3372, *V. vulnificus* LMG13545, *P. aeruginosa* MTCC741, *P. aeruginosa* ATCC1934.

An overnight culture (100 µl) of these bacteria was mixed in soft agar and overlayed on ZA plates individually. The plates were allowed to stand for 30 min following which 5 µl of phage stock was spotted adequately separated from each other. The plates were incubated upright at 28°C for 12 hours and appearance of glassy plaque at the spotted area was recorded as positive sign of phage lysis of the plated bacterium.

4.2.6 Lytic efficiency of the phages

4.2.6.1 In microbiological media (ZoBell's broth)

To evaluate the lytic efficiency of the vibriophages against *Vibrio harveyi* the bacterial strains were inoculated into 50 ml ZB at an initial concentration of 0.01 OD_{600} (10^3 cfu ml^{-1}) in triplicate. Following incubation at 28°C under continuous agitation (120 rpm) when the OD600 reached 1.0 (10^8 – 10^9 cfu ml-1), 1 ml phage stock containing 10^9 pfu was inoculated. Bacterial growth was monitored periodically until 24 h by measuring the optical density (OD) at 600 nm wavelength. The efficiency of all phages was checked individually on their hosts. Lytic efficiency of all phages together was also assessed on *V. harveyi* strain LB28. The protocol followed was as given by Yoichi et al. (2004).

4.2.6.2 In plain seawater

The efficiency of all five vibriophages in combination to prevent the growth of *V. harveyi* in plain seawater was evaluated. The *V. harveyi* strain used in this experiment was LB31 since during the host range study it was lysed by all the phages. One ml of an overnight culture of *V. harveyi* LB31 was inoculated in each of six flasks containing 50 ml filter sterilized seawater to obtain a final concentration of 10^6 cfu ml^{-1} of *V. harveyi*. One ml stock of each of the phage containing 10^9 pfu ml^{-1} was added to three of the flasks. The flasks were incubated at 28°C under continuous agitation (120 rpm). *V. harveyi* counts were monitored periodically by drawing 100 µl sample from each of the flask, serially diluting 10-fold and plating 20 µl from each dilution by quadrant

streak method on ZA (Miles & Misra 1938). The plates were incubated at 28°C for 24 h and dilutions having 30-300 colonies counted for the estimation of *V. harveyi* LB31 cell densities.

4.2.7 Characterization of phage

4.2.7.1 Electron microscopy

Phage lysates were negatively and positively stained with 2 % phosphotungstic acid and 2 % uranyl acetate respectively and viewed at magnification ranging from 40000 – 100000 X under a transmission electron microscope (TEM) at All India Institute of Medical Sciences (AIIMS), New Delhi.

4.2.7.2 Phage nucleic acids

Total DNA was extracted by phenol-chloroform method following precipitation of the phages by zinc chloride (Su et al. 1998). To 50 ml bacterium-free phage lysate, DNase was added to final concentration 10 μg ml^{-1} and incubated at 37°C for 1 h to remove contaminating bacterial DNA. One ml 2 M $ZnCl_2$ was added and incubation continued at 37°C for 5 min to allow phage particles to precipitate. The aggregated particles were collected by centrifugation at 6000 × g for 10 min and resuspended in 2 ml SM Buffer. It was centrifuged once again at 8000 × g for 10 min and pellet was resuspended in 700 μl TENS buffer (Tris-HCl 50mM pH 8.0, EDTA 100 mM, NaCl 100 mM, SDS 0.3%). Freshly prepared proteinase K was added to a final concentration of 100 μg ml^{-1} and incubated at 55°C for 4 h. The sample was deproteinated by adding an equal volume of freshly constituted phenol (Tris-equillibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and aqueous layers were separated by centrifugation at 15000 × g for 10 min at 15°C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once more. Following this an equal volume of chloroform:isoamyl alcohol (24:1) mixture was added mixed by gentle inversion and centrifuged at 15000 × g for 10 min at 15°C to separate the aqueous phase which was

transferred to a fresh tube. DNA was precipitated by incubation at -20°C overnight after adding equal volume of ice-cold isopropanol and 60 µl of 3 M sodium acetate. The precipitated DNA was collected by centrifugation at 15000 × g for 10 min at 15°C and the pellet was washed in ice-cold 70% ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes were left open until the pellet dried. The DNA pellet was dissolved in 100 µl MilliQ (MIlipore) grade water. DNA concentration and purity were assessed spectrophotometrically by comparing absorbance at 260 nm and 280 nm as well as on 0.7% agarose gels.

4.2.7.3 Restriction Fragement Length Polymorphism (RFLP) profile

Phage DNA was digested with the restriction enzymes *Eco*R1 and *Xba*1 (New England Biolabs) as per the manufacturers recommendations. A double digest with the same enzymes was also done. Phage λ DNA provided by the manufacturer was incorporated as reaction control.

4.2.7.4 Polymerase chain reaction (PCR)

A primer sequence PH102, which identifies the *V. harveyi* phage VHS1 was used (Pasharawipas et al. 2005). The primers (Forward 5'AAACGACTTCGCGCATGTT3', Reverse 5'GACTCGCTTTTAACTGCTA3') amplify a 2.5 kb fragment of the VHS1 genome encoding putative pyruvate decarboxylase transporter, DNA polymerase and capsid portal protein. PCRs were performed in a final volume of 25 µl containing 1X PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X100, 20mM Tris HCL pH 8.8), 1.5 mM $MgCl_2$, 250 µM each of dATP, dGTP, dTTP, dCTP, 10 pmol of each primer, 100 ng DNA template and 2.5 units of Taq polymerase (New England Biolabs). The amplification protocol consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 2 min, and extension at 72°C for 2 min, except for a final extension at 72°C for 10 min. MilliQ water was used as negative control.

PCR products and restriction digests were separated electrophoresing on 1% agarose gels in 1X Tris-Actetate EDTA (TAE) Buffer, at 100V for 75 min, stained with ethidium bromide (10 μg ml^{-1}) and visualized on a UV transilluminator.

4.2.7.5 SDS PAGE

Phages were precipitated from 50 ml lysates by adding 1 ml of 2 M $ZnCl_2$ and were incubated at 37°C for 5 min. The precipitated phages were collected by centrifugation at 6000 × g for 10 min at 4°C. The pellet was resuspended in 1 ml SM buffer and centrifugation repeated. The final pellet was resuspended in SM Buffer and maintained at 4°C until use. The precipitate (20mg) was treated with 100 μl of treatment buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.2 M dithiotreitol, 0.02% bromophenol blue, pH 6.8) and incubated at 95°C for 2 min. The structural proteins of the phages were compared on 8.5% and 12% SDS-PAGE gels by running the samples in Tris-Glycine buffer system at 10 mA for 2.5 h on a mini vertical gel electrophoresis unit following Laemmli (1970). Following electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 overnight. The gels were destained and the bands formed detected.

4.3 Results and Discussion

From the sediment sample taken from Vembanad estuary we obtained six phages against *V. harveyi* isolates LB6, 12, 20, 28, 45, and 65. So accordingly the phages were labeled Viha6, Viha12, Viha20, Viha28, Viha45 and Viha65 respectively indicative of the strain against they were originally isolated. Isolation protocols usually suffer from phage:host incompatibility problems i.e. if the bacterial strain used is not susceptible to phages present in the sample, or if the phage to bacteria ratio is too low. In both cases, the sample could erroneously be classified as to not have phages (DePaola et al. 1997). Therefore enrichment techniques using multiple strains tend to yield better results. Using this method however, enumeration of phages in the sample is not possible;

therefore we cannot ascertain the phage population in the sample. But when the aim is to obtain phages then this methodology is extremely useful. Hence we used the enrichment protocol of (Cerveny et al. 2002) and were able to obtain phages against *V. harveyi* from the sediment sample. In ecological studies, total virus/ phage or counts are made either by direct observations using TEM, fluorescent labeling, flow cytometry and pulse-field gel electrophoresis wherein results are conditional of the advantages and disadvantages of each technique (Fuhrman 1999).

The phage titres of lysates obtained after secondary enrichment are given in Table 15. All phages formed plaques of uniform diameter at 2 mm (Fig. 4). All phages except Viha6 stored well at 4°C without suffering any decline in their titres. Unfortunately after 6 months of storage however Viha6 became no longer detectable on soft agar overlays as well as in spot inoculations and enrichments attempted with its host strain. Therefore this phage could not be studied further.

The lytic efficiency of the five phages and in combination was similar (Fig. 5a-f). In all cases the phages were able to arrest the growth of *V. harveyi* for about 12 h after which phage resistant forms emerge and begin to dominate. When phage lytic activity on *V. harveyi* LB31 was tested in plain seawater also an initial suppression of growth was noticed but after 12 h resistant forms growth remerged ostensibly due to the development of resistant forms. It was expected that a cocktail of all phages together might be able to prevent the emergence of resistance bacterial forms or at least delay their onset as was observed by Yoichi et al. (2004) in their studies on biocontrol of *E. coli* O157:H7 using bacteriophages. Yoichi et al. (2004) had also observed that phage resistant cells of *E. coli* were existed in the culture prior to phage infection. This however was not observed with this set of vibriophages in ZoBell's broth or plain seawater. Previous experiments with phage therapy have shown that in vitro development of phage-resistant bacterial forms could not be observed in vivo (Park et al. 2000). In vivo, the bacterium is under other pressures like host defense, nutrient limitation, environmental factors other than phages. It is also hypothesized that resistance to a phage might be costly for bacteria physiologically since it involves

loss/modification of a receptor protein which could put it at a competitive disadvantage particularly under low nutrient conditions normally seen in the environment (Fuhrman 1999). Studies of O'Flynn et al. (2004) have observed reversion of phage-resistant mutants of *E. coli* O157:H7 after just 50 generations. This implies phage-resistance of bacteria could be a transient feature and native state is advantageous. Since in the rich media used in this experiment the bacteria were not under nutrient limitation, resistant forms could dominate even if they are competitively disadvantaged. Phages in this experiment were introduced when the OD_{600} of the cultures neared 1.0 which corresponds to a cell density of 10^8 cfu ml^{-1} and the bacteria were in the mid-log phase of growth. The phages were able to suppress bacterial growth for 10-12 h even at this high numbers seldom found in nature. Therefore it may still be worthwhile to proceed with therapeutic application of these phages and evaluate their performance and development of bacterial resistance forms in vivo.

The phages showed broad spectrum lytic activity when screened on 162 bacterial isolates including 87 *V. harveyi* isolates (Table 16 & 17). Phages Viha45 and 65 lysed 62.5 and 63.6 % of *V. harveyi* strains tested respectively, while Viha12, 20 and 20 each lysed only 18.2% of the strains tested. Only Viha65 had activity against *V. harveyi* type strain LMG4044. A few strains of *V. cholerae, V. mediterranei* and *V. vulnificus* tested were inhibited by phages Viha20, 28, and 65. Interestingly all phages were lytic to the strains of two other members of the *V. harveyi* phylogenetic core-group namely, *V. alginolyticus*, *V. parahaemolyticus*. Both these organisms are pathogens of shrimp and other animals in culture systems (Gomez-Leon et al. 2005, Cai et al. 2006, Jayaprakash et al. 2006b) therefore it could be possible to evaluate the potential of controlling their populations in shrimp culture systems. *V. harveyi* infecting siphovirus like phages have been reported earlier (Pasharawipas et al. 2005, Vinod et al. 2006, Shivu et al. In Press), but their lytic activity was limited to *V. harveyi* strains alone. The vibriophage KVP40 which belonged to the family *Myoviridae* is a well characterized broad host range phage reported to infect eight *Vibrio* species (Miller et al. 2003). The broad host range of the vibriophages isolated in this study further accentuates their case as potential candidates as therapeutic agents against *V. harveyi* in shrimp culture systems.

Transmission electron microscopic studies revealed the phages to posses an isometric head and a non-contractile tail with a terminal bulb (Fig. 7a-e). The capsid and tail dimensions (Table 18) are similar to those ascribed to phages belonging to the family *Siphoviridae* (Ackermann 2001). Hence the phages can be placed in the family *Siphoviridae* of the order *Caudovirales*. None of the phages seem to have a collar therefore cannot be classified under the family *Myoviridae*. All were double-stranded DNA phages (Fig 8).

The restriction enzyme *Eco*R1 digested the DNA of all the phages (Fig. 9a). With this enzyme, Viha12, Viha20, and Viha28 had similar bands while band pattern of Viha45 and Viha65 were different from each other as well as others. Xba1 digested the DNA of all the phages and here Viha20 showed only 1 band which means only 1 cutting site for the enzyme (Fig. 9b). The band pattern of Viha12 and 28 was similar while that of Viha45 and Viha65 was different from each other as well as others. The λ DNA gave the expected 5 bands with *Eco*R1 and 1 band with *Xba*1 indicating reaction conditions followed were acceptable. Since the DNA of all the phages could be digested with the restriction enzymes proves the double stranded nature of their genome. From these profiles it would be erroneous to make estimations of the total genome size. The restriction profile obtained shows these phages to be very dissimilar to the *Siphovirus* reported by Pasharawipas et al. (2005) and Shivu et al. (In Press).

Pasharawipas et al. (2005) and Khemayan et al. (2006) have reported that the *Vibrio harveyi* infecting Siphovirus-like phage (VHS1) isolated from *P. monodon* culture systems was found to increase the virulence of *V. harveyi*. Since the isolates obtained in this study are also belong to the same group, it was important to assess the similarity between the two isolates despite the geographical isolation. Hence an attempt to check the presence of the similar amplicon to VHS1 in the phages isolated in this study was made. The expected product size with the primer set PH102 was 2.5 kb but we obtained a 1.5 kb fragment with only Viha45 and Viha65 while there was no amplification with others (Fig. 10). The annealing temperature of the PCR reaction had to be optimized

and the best amplification was obtained at the temperature 49°C much below the 60°C used by Pasharawipas et al. (2005) and Khemayan et al. (2006). Since the PCR results deviate from that obtained by Khemayan et al. (2006) similarities of all the phages isolated here to the virulence modulator VHS1 phage reported by Pasharawipas et al. (2005) can ruled out. Sequencing of the PCR product will allow further identification and similarity of the phages.

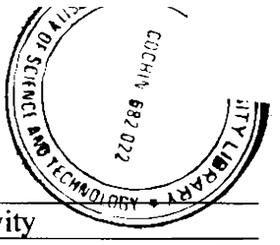
The structural protein comparison profile comparison could be done only with phages Viha12, Viha45 and Viha65 (Fig. 11 a&b). The profiles of Viha20 and Viha28 were not clear, therefore could not be compared. A slight difference in the band pattern of the three phages Viha12, Viha45 and Viha65 between 43-66 kDa range in the 12% PAGE gel was observed which indicated their dissimilarity. Further studies however are required for confirmation.

Tailed phages belonging to *Myoviridae*, *Siphoviridae* and *Podoviridae* seem to be the most abundant in marine systems. This is evident from the fact that out of 5100viruses reported so far, only 186 are not tailed phages. Of the tailed phages 61% belong to *Siphoviridae*. Out of 183 vibriophages reported so far, 65 belong to *Myoviridae*, 59 to *Siphoviridae* and 59 to *Podoviridae*. Only 10 filamentous phages infective to vibrios have been reported so far. Therefore with the current available information tailed phages appear to be the dominant phage type infective to vibrios (Ackermann 2001). All the 5 phages isolated in this study were tailed and had double stranded DNA as their genetic material. Based on morphological features they could be grouped under *Siphoviridae*. The broad host range of the phage isolates in this study makes them ideal candidates for therapeutic application once it is proven that they are not agents of virulence in *V. harveyi*.

Table 15. Phage titres obtained after secondary enrichment and purification

Phage Lysate	Host Strain	Titre ($\times 10^8$ pfu ml^{-1})
Secondary Enrichment		
Viha6	LB6	2.76
Viha12	LB12	473
Viha20	LB20	268
Viha28	LB28	760
Viha45	LB45	12
Viha65	LB65	295
Purified Lysates (after 3rd purification)		
Viha6	LB6	235
Viha12	LB12	5.75
Viha20	LB20	8.5
Viha28	LB28	167
Viha45	LB45	110
Viha65	LB65	12

Table 16. Host range of vibriophages



Culture No	Organism	Phage lytic acivity				
		Viha12	Viha20	Viha28	Viha45	Viha65
LB1	*Vibrio harveyi*	-	-	-	-	+
LB2	*V. harveyi*	+	+	+	-	+
MCCB111	*V. harveyi*	-	-	-	-	+
LB4	*V. harveyi*	-	-	-	-	+
LB5	*V. harveyi*	-	-	-	-	+
LB6	*V. harveyi*	-	-	-	-	+
LB7	*V. harveyi*	+	+	+	-	+
LB8	*V. harveyi*	+	+	+	-	+
LB9	*V. harveyi*	-	-	-	+	-
LB11	*V. harveyi*	-	-	-	-	+
LB12	*V. harveyi*	+	+	+	-	+
LB13	*V. harveyi*	-	-	-	+	+
LB14	*V. harveyi*	-	-	-	-	-
LB15	*V. harveyi*	+	+	+	-	+
LB16	*V. harveyi*	+	+	+	-	+
LB17	*V. harveyi*	-	-	-	+	-
LB18	*V. harveyi*	-	-	-	-	-
LB19	*V. harveyi*	-	-	-	-	+
LB20	*V. harveyi*	+	+	+	-	+
LB21	*V. harveyi*	+	+	+	-	+
LB22	*V. harveyi*	-	-	-	-	+
LB23	*V. harveyi*	+	+	+	-	+
LB24	*V. harveyi*	+	+	+	-	+
LB25	*V. harveyi*	-	-	-	+	+
LB26	*V. harveyi*	-	-	-	+	-
LB28	*V. harveyi*	+	+	+	-	+
LB29	*V. harveyi*	+	+	+	-	+
LB30	*V. harveyi*	-	-	-	+	+
LB31	*V. harveyi*	+	+	+	+	+
LB32	*V. harveyi*	+	+	+	+	+
LB33	*V. harveyi*	+	+	+	-	+
LB34	*V. harveyi*	+	+	+	+	+
LB35	*V. harveyi*	-	-	-	-	+
LB36	*V. harveyi*	-	-	-	+	+
LB37	*V. harveyi*	-	-	-	-	-
LB38	*V. harveyi*	-	-	-	-	+
LB39	*V. harveyi*	-	-	-	+	+
LB40	*V. harveyi*	-	-	-	+	+
LB41	*V. harveyi*	-	-	-	+	+
LB42	*V. harveyi*	-	-	-	+	+
LB43	*V. harveyi*	-	-	-	+	-

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Table 16 (continued)

Culture No	Organism	Phage lytic acivity				
		Viha12	Viha20	Viha28	Viha45	Viha65
LB44	*V. harveyi*	-	-	-	+	+
LB45	*V. harveyi*	-	-	-	+	-
LB46	*V. harveyi*	-	-	-	+	+
LB47	*V. harveyi*	-	-	-	+	+
LB48	*V. harveyi*	-	-	-	+	+
LB49	*V. harveyi*	-	-	-	+	+
LB50	*V. harveyi*	-	-	-	+	+
LB51	*V. harveyi*	-	-	-	+	+
LB52	*V. harveyi*	-	-	-	+	+
LB53	*V. harveyi*	-	-	-	+	+
LB54	*V. harveyi*	-	-	-	+	+
LB55	*V. harveyi*	-	-	-	+	+
LB56	*V. harveyi*	-	-	-	+	+
LB57	*V. harveyi*	-	-	-	-	+
LB58	*V. harveyi*	-	-	-	-	+
LB59	*V. harveyi*	-	-	-	+	+
LB60	*V. harveyi*	-	-	-	+	+
LB61	*V. harveyi*	-	-	-	+	+
LB62	*V. harveyi*	-	-	-	+	+
LB63	*V. harveyi*	-	-	-	+	+
LB64	*V. harveyi*	-	-	-	+	-
LB65	*V. harveyi*	-	-	-	-	+
LB66	*V. harveyi*	-	-	-	-	-
LB67	*V. harveyi*	-	-	-	-	+
LB68	*V. harveyi*	-	-	-	+	-
LB69	*V. harveyi*	-	-	-	+	-
LB70	*V. harveyi*	-	-	-	+	-
LB71	*V. harveyi*	-	-	-	+	-
LB72	*V. harveyi*	-	-	-	+	-
LB73	*V. harveyi*	-	-	-	+	-
LB74	*V. harveyi*	-	-	-	+	-
BL2	*V. harveyi*	-	-	-	+	-
BL4	*V. harveyi*	-	-	-	+	-
BL5	*V. harveyi*	-	-	-	+	-
BL6	*V. harveyi*	-	-	-	+	-
BL7	*V. harveyi*	-	-	-	+	-
BL8	*V. harveyi*	-	-	-	+	-
BL9	*V. harveyi*	-	-	-	+	-
BL11	*V. harveyi*	-	-	-	+	-
BL12	*V. harveyi*	-	-	-	+	-
BL13	*V. harveyi*	-	-	-	+	-

Table 16 (continued)

Culture No	Organism	Phage lytic acivity				
		Viha12	Viha20	Viha28	Viha45	Viha65
BL14	*V. harveyi*	-	-	-	+	-
BL15	*V. harveyi*	-	-	-	+	-
BL16	*V. harveyi*	-	-	-	+	-
BL17	*V. harveyi*	-	-	-	+	-
BL18	*V. harveyi*	-	-	-	-	-
MCCB101	*Bacillus* sp.	-	-	-	-	-
MCCB102	*Pseudomonas* sp.	-	-	-	-	-
MCCB103	*Pseudomonas* sp.	-	-	-	-	-
MCCB104	*Micrococcus* sp.	-	-	-	-	-
MCCB117	*P. aeruginosa*	-	-	-	-	-
MCCB118	*P. aeruginosa*	-	-	-	-	-
MCCB119	*P. aeruginosa*	-	-	-	-	-
MTCC741	*P. aeruginosa*	-	-	-	-	-
ATCC1934	*P. aeruginosa*	-	-	-	-	-
LMG4409	*V. alginolyticus*	-	-	-	-	+
MTCC4439	*V. alginolyticus*	-	-	-	-	+
MTCC	*V. cholerae*	-	-	-	-	-
LMG4044	*V. harveyi*	-	-	-	-	+
LMG11654	*V. fluvialis*	-	-	-	-	-
LMG11258	*V. mediterranei*	-	-	-	-	-
LMG2850	*V. parahaemolyticus*	-	-	-	-	-
LMG3372	*V. proteolyticus*	-	-	-	-	-
LMG13545	*V. vulnificus*	-	-	-	-	-
MRCS 2	*Aeromonas* sp.	-	-	-	-	-
MRCS 7	*Aeromonas* sp.	-	+	+	-	-
MRNL 9	*Aeromonas* sp.	-	-	-	-	-
MRNL 10	*Aeromonas* sp.	-	-	-	-	-
MRNL 16	*Aeromonas* sp.	-	-	-	-	-
MRNL 17	*Aeromonas* sp.	-	-	-	-	-
MRNL 1	*V. alginolyticus*	+	+	+	+	+
MRNL 2	*V. alginolyticus*	+	+	+	+	+
MRNL 3	*V. alginolyticus*	+	+	+	+	+
MRNL 4	*V. alginolyticus*	-	-	-	-	-
MRNL 5	*V. alginolyticus*	+	+	+	+	+
MRNL 6	*V. alginolyticus*	+	+	+	+	+
MRNL 7	*V. alginolyticus*	+	+	+	+	+
MRNL 8	*V. alginolyticus*	+	+	+	+	+
MRCS 11	*V. cholerae*	-	+	+	-	+
MRCS 12	*V. cholerae*	-	-	-	-	-
MRCS 13	*V. cholerae*	-	-	-	-	-
MRCS 16	*V. cholerae*	-	-	-	-	-

Table 16 (continued)

Culture No	Organism	Phage lytic acivity				
		Viha12	Viha20	Viha28	Viha45	Viha65
MRCS 17	*V. cholerae*	-	-	-	-	-
MRCS 19	*V. cholerae*	-	-	-	-	-
MRCS 20	*V. cholerae*	-	+	+	-	+
MRCS 23	*V. cholerae*	-	-	-	-	-
MRCS 35	*V. cholerae*	-	-	-	-	+
MRCS 37	*V. cholerae*	-	+	+	-	+
MRCS 39	*V. cholerae*	-	+	+	-	+
MRCS 26	*V. fluvialis*	-	-	-	-	-
MRCS 15	*V. mediterranei*	-	-	-	-	-
MRCS 18	*V. mediterranei*	-	-	-	-	-
MRCS 22	*V. mediterranei*	-	+	+	-	+
MRCS 32	*V. mediterranei*	-	+	+	-	+
MRCS 34	*V. mediterranei*	-	-	-	-	-
MRCS 36	*V. mediterranei*	-	+	+	-	+
MRQL 27	*V. mediterranei*	-	-	-	-	-
MRCS 28	*V. nereis*	-	-	-	-	-
MRCS 29	*V. nereis*	-	-	-	-	-
MRCS 30	*V. nereis*	-	-	-	-	-
MRCS 31	*V. nereis*	-	-	-	-	-
MRQL 13	*V. nereis*	-	-	-	-	-
MRQL 29	*V. nereis*	-	-	-	-	-
MRQL 32	*V. nereis*	-	-	-	-	-
MRQL 34	*V. nereis*	-	-	-	-	-
MRQL 35	*V. nereis*	-	-	-	-	-
MRCS 6	*V. parahaemolyticus*	+	+	+	-	-
MRCS 8	*V. parahaemolyticus*	+	+	+	+	+
MRCS 9	*V. parahaemolyticus*	+	+	+	+	+
MRCS 1	*V. parahaemolyticus*	+	+	+	+	+
MRCS 4	*V. proteolyticus*	-	-	-	-	-
MRCS 10	*V. proteolyticus*	-	-	-	-	-
MRCS 3	*V. splendidus*	-	-	-	-	-
MRCS 5	*V. splendidus*	-	-	-	-	-
MRQL 5	*V. vulnificus*	-	+	+	-	+
MRQL 9	*V. vulnificus*	-	-	-	-	-
MRQL 10	*V. vulnificus*	-	+	+	-	+
MRQL 19	*V. vulnificus*	-	-	-	-	-
MRQL 20	*V. vulnificus*	-	-	-	-	-
MRQL 23	*V. vulnificus*	-	-	-	-	-
MRQL 33	*V. vulnificus*	-	-	-	-	-
MRQL 36	*V. vulnificus*	-	-	-	-	-

Table 17. Summary of the spectrum of lytic activity of the vibriophages

Organism	Number of strains lysed/Number of strains screened				
	Viha12	Viha20	Viha28	Viha45	Viha65
V. harveyi	16/88	16/88	16/88	55/88	56/88
V. alginolyticus	7/10	7/10	7/10	7/10	9/10
V. parahaemolyticus	4/4	4/4	4/4	3/4	3/4
V. cholerae	0/12	3/12	3/12	0/12	4/12
V. mediterranei	0/8	3/8	3/8	0/8	3/8
Aeromonas sp.	0/6	1/6	1/6	0/6	0/6
V. vulnificus	0/9	2/9	2/9	0/9	2/9
V. proteolyticus	0/3	0/3	0/3	0/3	0/3
V. fluvialis	0/2	0/2	0/2	0/2	0/2
V. neries	0/9	0/9	0/9	0/9	0/9
V. splendidus	0/2	0/2	0/2	0/2	0/2
P. aeruginosa	0/7	0/7	0/7	0/7	0/7
Bacillus sp.	0/1	0/1	0/1	0/1	0/1
Micrococcus sp.	0/1	0/1	0/1	0/1	0/1

Table 18. Morphological characteristics of the isolated vibriophages

Phage	Characteristics	Capsid dimensions (nm)	Tail dimensions (Length x Diameter nm)
ViHa12	Isometric capsid, Non-contractile Tail	80	108 x 27
ViHa20	Isometric capsid, Non-contractile Tail	80	Not Clear
ViHa28	Isometric capsid, Non-contractile Tail	83	Not Clear
ViHa45	Isometric capsid, Non-contractile Tail	80	90 x 20
ViHa65	Isometric capsid, Non-contractile Tail	120	170 x 25

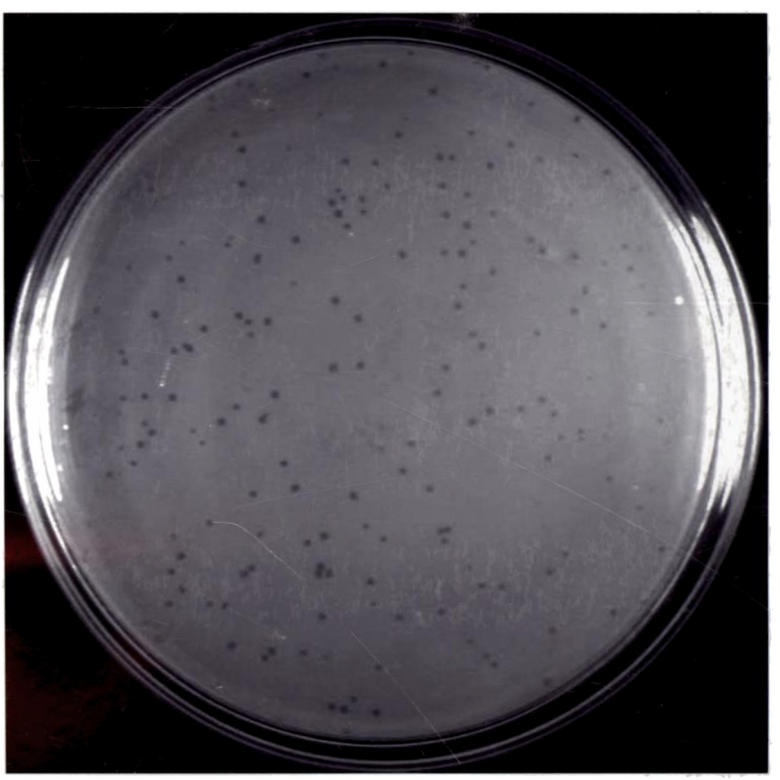

Fig.4.Plaques formed by vibriophage Viha12 on its host *Vibrio harveyi* LB 12

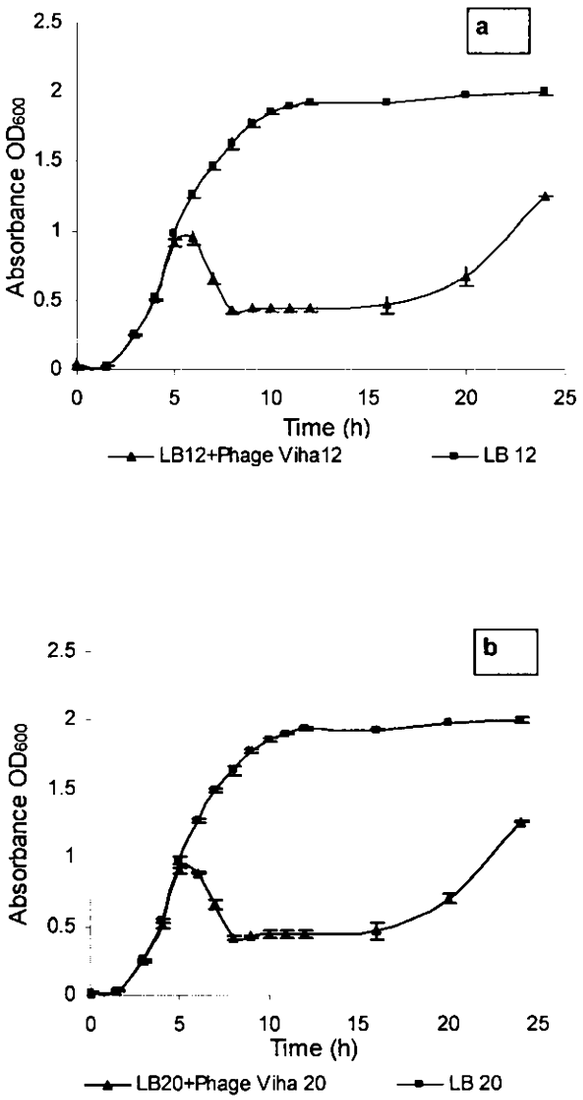

Fig. 5 a-f. *Vibrio harveyi* cell lysis by infection of vibriophages individually on their host and in combination in ZoBell's broth.

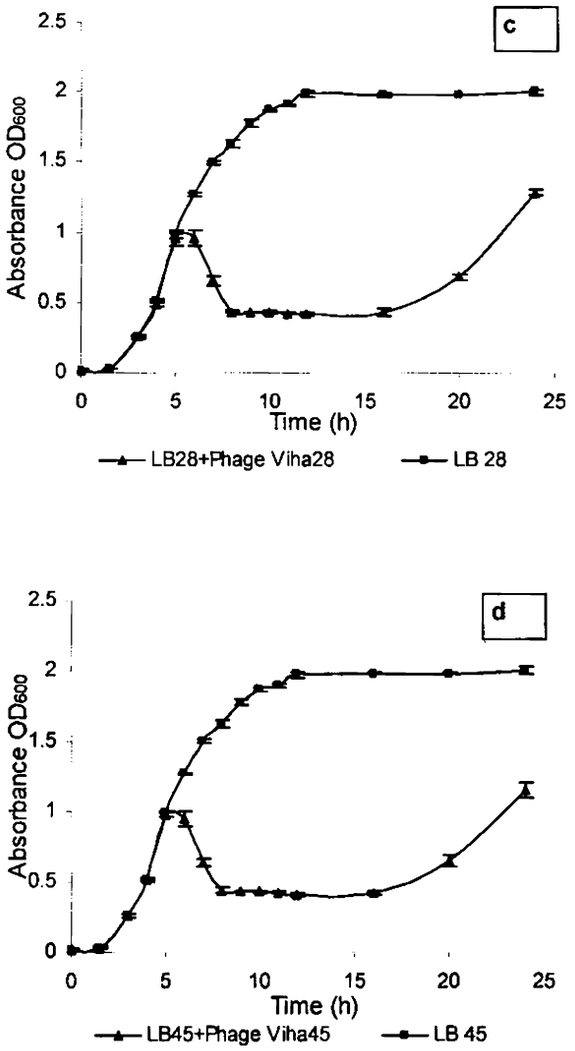

Fig. 5 a-f. (Contd) *Vibrio harveyi* cell lysis by infection of vibriophages individually on their host and in combination in ZoBell's broth.

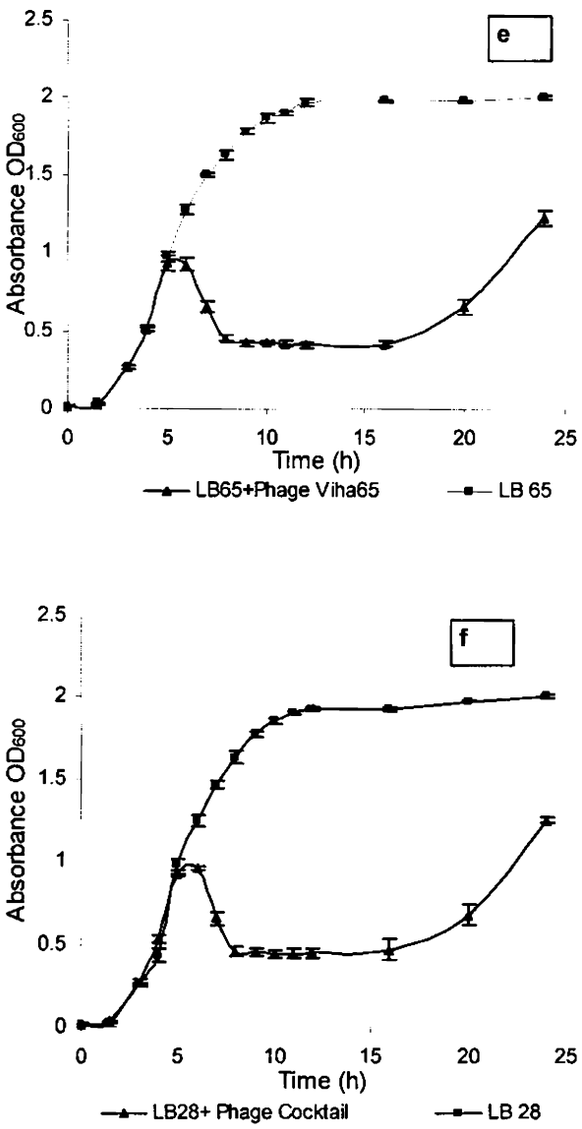

Fig. 5 a-f. (Contd) *Vibrio harveyi* cell lysis by infection of vibriophages individually on their host and in combination in ZoBell's broth.

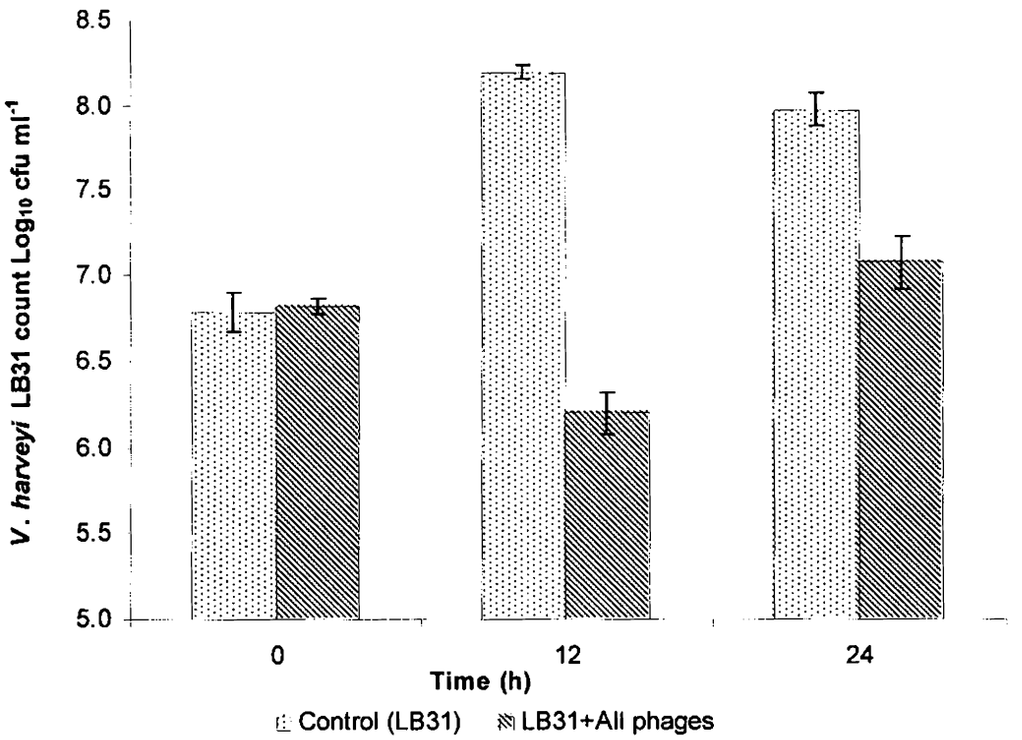

Fig. 6. *Vibrio harveyi* LB31 cell lysis by infection of vibriophages in combination in filtered seawater.

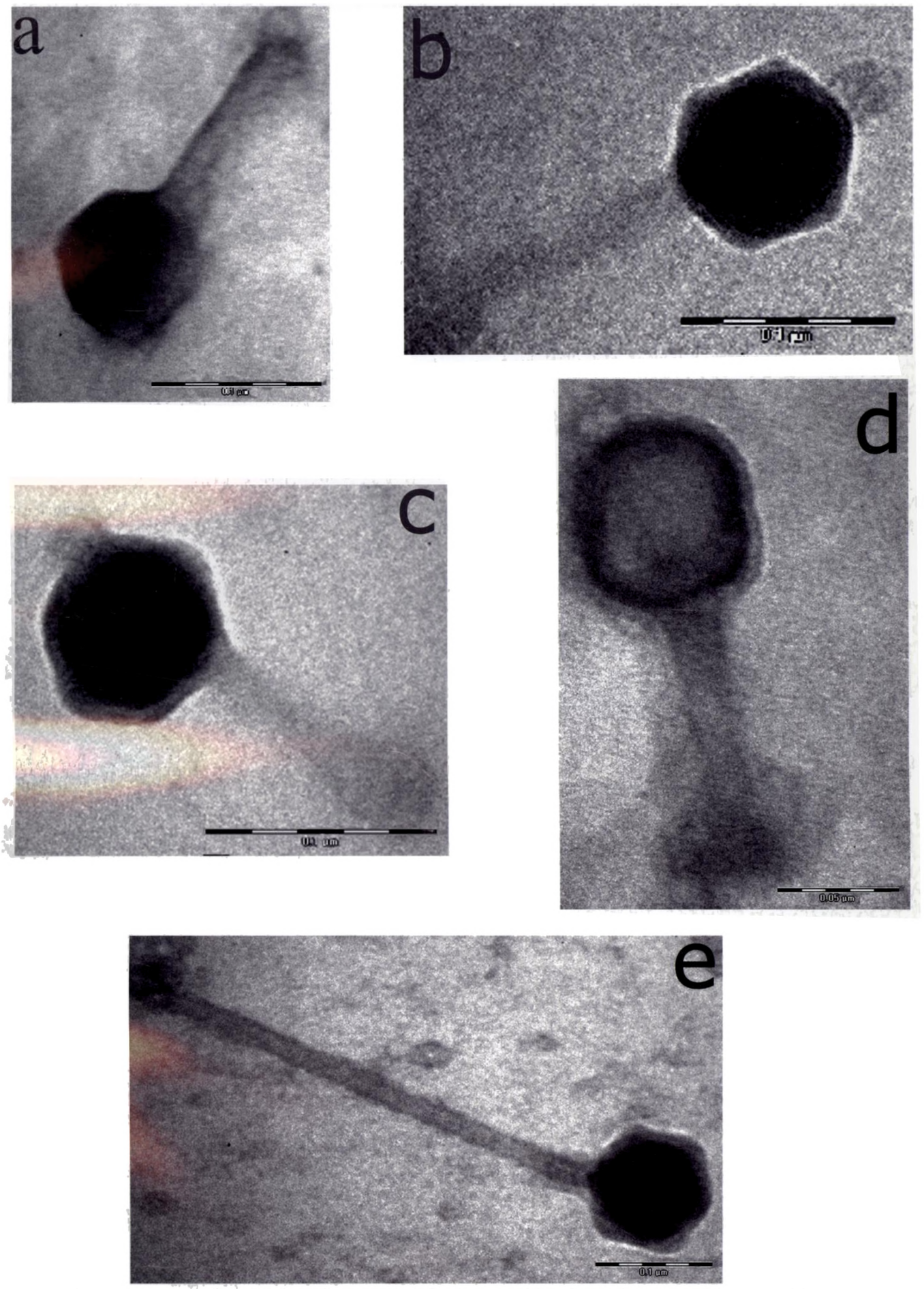

Fig.7a-e Morphology of the vibriophages. a:Viha 12, b:Viha20,c:Viha28, d:Viha45, e:Viha65

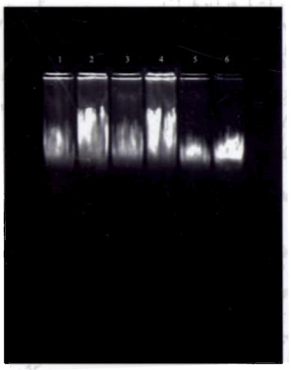

Fig 8. Genomic DNA of the vibriophages. Lane 1-6: Viha12, Viha20, Viha28,Viha45,Viha65, *E. coli* phage KSW1 respectively.

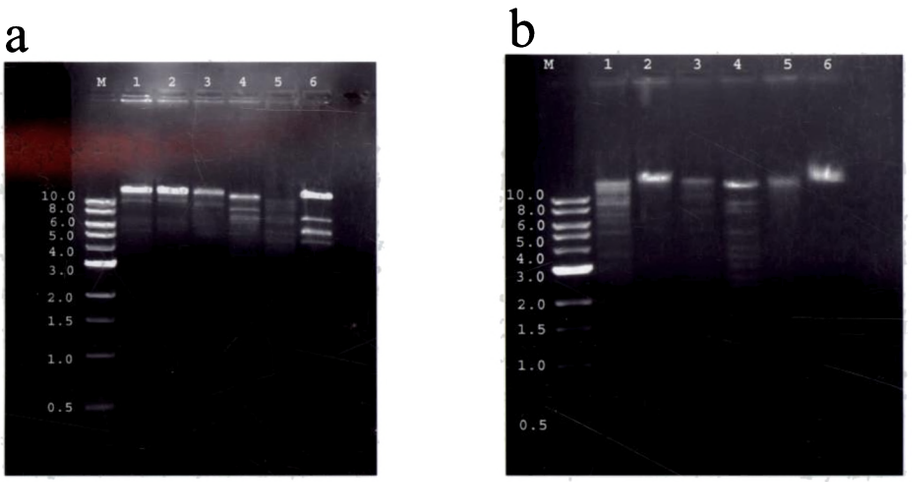

Fig. 9 a&b. Restriction profile of the vibriophages with the restriction enzyme *Eco*R1 (a) and *Xba*1 (b). Lane M: Marker 1kb ladder, Lanes 1-6: Viha12, Viha20, Viha28, Viha45, Viha65, λ DNA (Control) respectively.

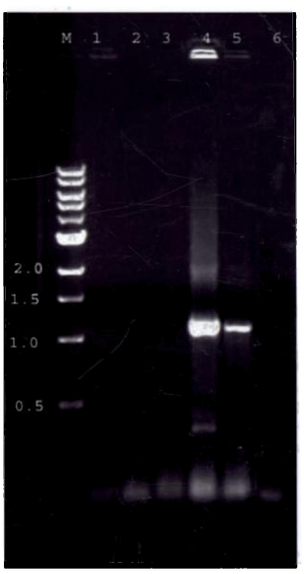

Fig. 10. Amplification of the DNA of vibriophages with the primers PH102 specific for VHS1 siphophage. Lane M: Marker 1 kb ladder, Lanes 1-6: Viha12, Viha20, Viha28, Viha45, Viha65 and Negative control respectively.

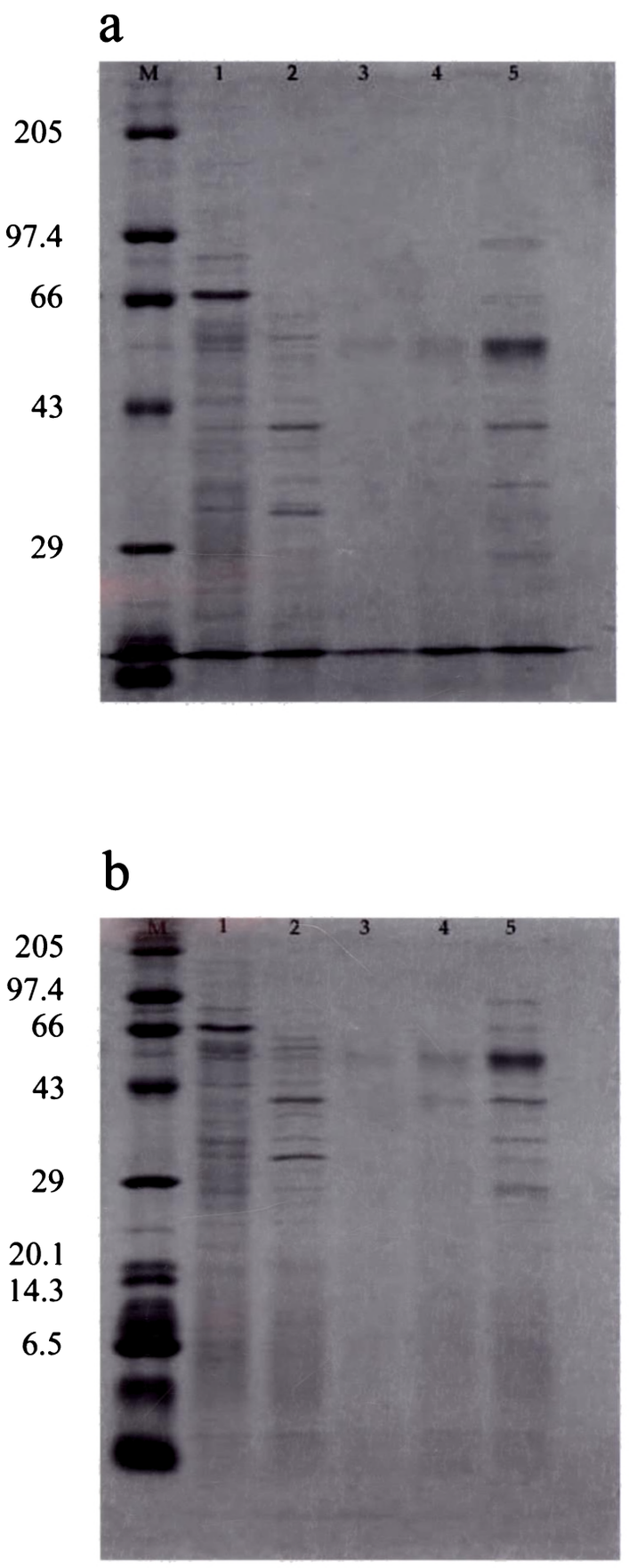

Fig. 11a&b. SDS polyacrylamide gel electrophoresis profiles of structural proteins a.8% b.12%. Lane M: Protein molecular weight marker (kDa). Lane 1-6: Viha65 Viha45, Viha28, Viha20, Viha12 respectively.

CHAPTER - 5

Conclusions and Future Research

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The aim of this work was to develop biological control alternatives to chemotherapeutants such as antibiotics for use in peaneid larval rearing systems against vibrios with special reference to *V. harveyi*. Eighty seven luminescent bacterial isolates were obtained from shrimp hatchery systems of both East and West Coast of India. They were identified as *Vibrio harveyi* based on phenotypic characters. Source of *V. harveyi* was found to be the incoming nauplii, crabs used for feeding broodstock and sand around the raw sea water intake points. Luminescent bacteria could also be isolated from diseased larvae and larval rearing water and the hatchery drain off wastewater. The latter suggested the ineffectiveness of treatment of wastewater. Even though LB could not be detected in treated water, its presence in VBNC state could not be ruled out as the organisms were stressed during the treatment process of chlorination, dechlorination etc.

The antibiotic resistance among the isolates was extremely high. The similarity level ranged from 40 to 90 %. This implies a high heterogenous nature in the resistance profile and that the process of resistance acquisition is independent of the use and consequence of presence of the antibiotics in the system. However, the isolates had a very high MAR index at an average of 0.63 with little variation between those from different samples. This situation poses an additional threat of antimicrobial resistance to be acquired by human pathogenic bacteria in the environment. In the present study as antibiotic residues were not analyzed, the MAR index could not be linked to the widespread application of antibiotics in shrimp hatcheries in India. However, the study strongly suggests the need for developing biological control measures as alternatives for improving sustainability and productivity of shrimps in culture because antibiotic administration by and large is a futile exercise as demonstrated by the heterogenous nature of the resistance profile of *V. harveyi*.

This justifies the evaluation of putative probionts and isolation of vibriophage for the control of vibrios as viable alternatives of antibiotics.

Four putative probiotics such as *Pseudomonas* MCCB102 and MCCB103, *Micrococcus* MCCB104, and *Bacillus* MCCB101 were evaluated for their probiotic potential against 87 isolates of *V. harveyi*. The two isolates of *Pseudomonas* and the isolate of *Micrococcus* were found to inhibit all isolates of *V. harveyi* tested by disc diffusion assay, but *Bacillus* MCCB101 was not. Additionally, the pseudomonads were inhibiting the *Bacillus*, but not *Micrococcus*. This led to the development of a combination of *Bacillus* MCCB101 and *Micrococcus* MCCB104 to evaluate their combination to inhibit *V. harveyi* in vivo.

In cocultures, *Pseudomonas* MCCB102 and MCCB103 could eliminate *V. harveyi* at high initial cell counts (10^6 cfu ml^{-1}). But neither the *Bacillus* MCCB101 nor *Micrococcus* MCC104 could do so. This trend was seen in vivo also. However, the larval survival in these tanks was not related to the vibriocidal property of the pseudomonads as the survival in those tanks supplemented with them was 66.35 and 58.3 % respectively, while being more than 70% in tanks treated with *Micrococcus* MCCB104, *Bacillus* MCCB101 and their combination (Enterotrophotic), suggesting a complex relationship between *Vibrio* and probiotic population and larval survival. The observation could be confirmed by the challenge experiment where the highest RPS was in tanks supplemented with *Micrococcus* MCCB104, *Bacillus* MCCB101, *Pseudomonas* MCCB102 and MCCB103 and Enterotrophotic in the diminishing order of RPS at comparatively higher *Vibrio* count.

The observations pointed to the existence of a 'probiotic effect' exerted on the larvae of *P. monodon* by the four probionts when supplemented to the rearing water where their presence beneficially impacted larval survival. This implied that probiotics could favour the host animal in ways other than antagonism of pathogens as evidenced by *Bacillus* MCCB101 which improved larval survival even though it wasn't exhibiting in vitro antagonism to *V. harveyi*. Probiotic effect is a subject worth unraveling.

The study restablished the importance of higher generic diversity in the rearing water and in larvae for better survival. Even in the control tanks (not treated with probiotics), the survival was reasonably higher and *V. harveyi* could not be recovered from the larvae after challenge.

Based on the study *Micrococcus* MCCB104 can be reasonably recommended as the best probiotic in shrimp larval rearing system amongst the four studied when applied to the rearing water at a final density greater than 10^6 cfu ml^{-1}. Trials in larger commercial systems have to be accomplished before commercialization.

During the in vivo studies recovery of the probiotics added could not be accomplished and whenever recovered, it was too close to the time of application and was found to taper off as the time from application lapsed. Therefore it is suggested that investigations on the fate of probiotics in larval rearing systems and in aquaculture as a whole have to be made an immediate requirement.

The second alternative to antibiotic treatment investigated was the vibriophages isolated from sediment of backwaters of Kochi. Based on their lytic efficiency five phages could be brought under study. They showed broad spectrum lytic activity when screened on 162 bacterial isolates including 87 *V. harveyi*.

Based on their morphology, the phages were placed in the family *Siphoviridae* of the order *Caudovirales*. All of them were double stranded DNA phages. Based on the banding pattern subsequent to digestion with restriction enzymes such as *Eco*R1 and *Xba*1 the phages were grouped into four distinct types.

The lytic efficiency of the five phages and in combination was similar. In all cases phages were able to arrest the growth of *V. harveyi* for about 12 hours after which phage resistant forms emerged and began to dominate. When phage lytic activity on

Vibrio harveyi LB31 (a strain found susceptible to all the phages during host range study) in plain seawater was tested an initial suppression of growth was noticed.

As there were reports of *V. harveyi* infective siphovirus-like phage (VHS1) enhancing virulence of *V. harveyi* strains to *P. monodon*, the similarity of the phages isolated here was compared with the former by checking for the presence of similar amplicons. The expected product size with the primer set PH102 was 2.5 kb but the amplified product size was only 1.5 kb and that too with only two of the phages. Even though this has apparently ruled out the similarity of these phages to VHS1, much work needs to be done in this direction.

Based on this study it can be categorically stated that sustainable shrimp larval culture can be maintained by the application of probiotics without resorting to any antibiotic treatment. The probionts such as *Pseudomonas* MCCB102 and MCCB103, *Micrococcus* MCCB104 and *Bacillus* MCCB101 are all potent candidates, but *Micrococcus* MCCB104 seems to be the best option. Vibriophage system remains an attractive alternative to antibiotics but it is too early to bring it into the field as two issues such as virulence mediation in *V. harveyi* and development of resistance forms need to be addressed. In fact the complex phage-bacterium relationship also needs to be elucidated.

To sum up probiotics and bacteriophages will take the shrimp larval rearing technology a long way to sustainability.

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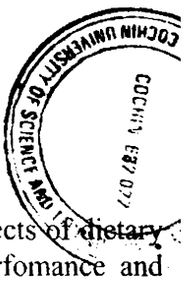

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Appendix – 1

Appendix - 1

Publications from this work

1. High level of multiple antibiotic resistance and heterogeneity amongst *Vibrio harveyi* isolated from *Penaeus monodon* larval rearing systems (Communicated to Letters in Applied Microbiology)

2. Improved survival of *Penaeus monodon* larvae bypreemptive treatment of rearing water with four probiotic bacteria (Communicated to Diseases of Aquatic Organisms)

3. Isolation and characterization of bacteriophages infective to *Vibrio harveyi* from estuarine sediments (Communicated to Archives of Virology)