Bioprocess Technology for Antagonistic *Pseudomonas* MCCB 102, 103, *Micrococcus* MCCB 104, and Evaluation of *Synechocystis* MCCB 114, 115 as Probiotics for the Management of *Vibrio* in Shrimp Culture Systems

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In
ENVIRONMENTAL BIOTECHNOLOGY

Under
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By
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Certificate

This is to certify that the research work presented in this thesis entitled "**Bioprocess Technology for Antagonistic *Pseudomonas* MCCB 102, 103, *Micrococcus* MCCB 104, and Evaluation of *Synechocystis* MCCB 114, 115 as Probiotics for the Management of *Vibrio* in Shrimp Culture Systems**" is based on the original work done by Ms. R. Preetha under my guidance, in the School of Environmental Studies, at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, 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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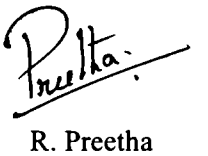
August 2006

Declaration

I hereby do declare that the work presented in this thesis entitled "**Bioprocess Technology for Antagonistic *Pseudomonas* MCCB 102, 103, *Micrococcus* MCCB 104, and Evaluation of *Synechocystis* MCCB 114, 115 as Probiotics for the Management of *Vibrio* in Shrimp Culture Systems**" is based on the original work done by me under the guidance of Dr. I.S. Bright Singh, Professor in Microbiology, School of Environmental Studies, Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, 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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General Introduction

Chapter-1

General Introduction

Expansion of commercial aquaculture resulted in concomitant appearance of infectious and non-infectious diseases in culture systems. Among them vibriosis has been one of the most common ailments in finfish and shellfish culture in both marine and estuarine environments, spread across large number of species world over (Lightner 1996). In shrimp culture vibriosis is still a major disease problem, which results in high mortality and economic loss to all shrimp producing countries (Lightner et al. 1992; Mohney et al. 1994; Lavilla-Pitago 1995; Lightner 1988). Vibriosis caused by Gram-negative bacteria in the family Vibrionaceae (Lightner 1996, Thompson et al. 2004). Other reported names of the disease are penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis, red leg disease etc. The disease affects all stages in its life cycle such as larvae, juvenile, adults and brood stock. Signs of the disease are tissue lesion, necrosis, malformation, low growth etc., besides septicemia and death. The severity relates to the species, culture environment, feed quality, aquaculture practices adopted etc., (Lightner 1996; Aguirre-Guzman et al. 2001).

With the intensification of aquaculture practices the favorable environment required for pathogenic bacterial growth gets generated in the culture systems (Aguirre-Guzman and Felipe 2000). Pathogenic bacteria take advantage of such ecological niches introduced in the aquaculture systems, which in due course lead to the emergence of diseases (Skjermo and Vadstein 1999). Most of the bacterial species, involve in the outbreak of diseases, are part of the autochthonous flora of the culture species (Hameed 1993; Singh et al. 1995; Singh et al. 1998 and Arias et al. 1999) and therefore form a

constant source of possible infections. Besides, the aquaculture practices by and large, stocking density, high organic matter, temperature fluctuations, low oxygen level, high suspended solids perturb the bacterial community and stimulate the growth of opportunistic pathogens.

Gram-negative bacteria, prominent in marine environment (Thompson et al. 2004) are the normal flora in both cultivated and wild penaeid marine shrimp (Lightner et al. 1992; Singh et al. 1998). However, negative interactions between shrimp larvae and the normal bacterial flora often causes diseases leading to mass mortality (Hisbi et al. 2000). While a number of bacterial species have been implicated as aetiologic agents in penaeids, reports of infections by *Vibrio* are far more than any.

More than thirty species of *Vibrio* have been identified (Vadenberghea et al. 2003; Thompson et al. 2004). They are considered as a part of normal flora of sea water which can invade marine animals (Olafsen. 2001), act as opportunistic pathogens causing mortality under stress (Lightner 1992; Hisbi, et al. 2000). Stress, which either lowers the resistance of the host or enhances the effect of pathogens on the host, is an important factor in the disease process (Lightner et al. 1992), with *Vibrio* as the aetiology. *V.harveyi, V. parahaemolyticus, V. alginolyticus, V. vulnificus, V. anguillarum, V. damsela, V. penaeicida, V. neris, V. fluvialis and V. tubiasahi* have been described as the principal pathogenic species of *Vibrio* to penaeid shrimp (Lightner 1996). Larval mortalities associated with the presence of *V. harveyi* have been reported in *P. monodon* and *P. vannami* in Indonesia (Sunaryanto and Mariam 1986), Thailand (Jirvanchpaisal et al. 1994), India (Karunasagar et al. 1994), Philippines (Baticados et al. 1990; Lavilla- Pitogo et al. 1990) and Australia (Pizzutto and Hirst 1995). Disease outbreaks attributed to the other *Vibrio* sp. such as *V. alginolyticus, V. damselei, V. parahaemolyticus, V. vulnificus, V. penaeicida* have been observed in nursery and grow out ponds

of *P. vannamei*, *P. monodon*, *P. japonicus*, *P. stylirostris* and *P. orientalis* in Ecudor (Lightner 1992), Philippines (Alapide-Tendencia and Dureza 1997), New Caledonia (Costa et al. 1998, Mermound et al. 1998) and in People's Republic of China (Sudheesh and Xu 2001).

Vibrio harveyi, a luminous species of *Vibrio*, has been recognized as a tropical pathogen of importance especially in shrimp culture (Owens et al. 1992; Karunasagar et al. 1994; Jirvanichpaisal et al. 1994; Abraham and Manly 1995). The species is known to cause losses in hatcheries and required to be the major cause of death in grow out systems (Nithimathachoke et al. 1995). It can elicit disease and significant mortality in shrimp larvae at 10^2-10^3 CFU/ml (Lavilla Pitago et al. 1990; Karunasagar et al. 1994). From shrimp with red disease *V. harveyi* and *V. parahamolyticus* have been isolated as the most dominant species (Eleonor et al. 1997). *V.harveyi* has been reported frequently associated with mass mortality of cultured giant tiger prawns in Taiwan (Chen et al. 1992), Australia (Pizzatto and Hirst 1995), India (Karunasagar et al. 1994), Indinesia (Sunaryanto and Mariam 1986), Philippanes (Baticados et al. 1990, Lavilla-pitogo 1990) and Tailand (Jiravanichpaisal et al. 1994). The organism has been isolated from the haemolymph and tissues of cultured *P.vannamei* in Ecudor and Texas (Lightner et al. 1992; Mohney et al. 1994).

Antibiotics have been in use for a long time for the management of *Vibrio* as prophylaxis and therapy (Baticados and Paclibare 1992; Karunasagar et al. 1994; Aguirre-Guzman et al. 2000; Hameed et al. 2003). May be because of this practice, multiple antibiotic resistant strains of *V. harveyi* have been found common in hatchery environment (Tendencia & de la Pena 2001). However, in natural ecosystem occurrence of antibiotic-resistant forms has been less frequent (Toranzo et al. 1992). During the course of antibiotic application those strains which carry resistant genes alone survive and their

competitors are removed. Virulent strains may re-enter the hatchery systems perhaps from within biofilm or from water pipes, air line or guts of the animal where they have been protected from the antibiotics. Subsequently, exchange of genetic information with the resistant bacteria could take place and as a result they might survive further doses of antibiotics leading to evolution of antibiotic resistant strains of the pathogen quickly (Moriarty 1997). Another limitation of the use of antibiotics in shrimp disease control is its inhibition of useful micro-algae in the rearing water (Hameed 1994). Several reports suggest that the use of antibiotics has become ineffective in controlling the disease due to various reasons such as limited efficiency of existing and readily available drugs, the possible development of resistant bacterial strains and the limited tolerance of shrimp larvae to the drugs. The concern on drug resistant microorganisms led to the development of alternative disease prevention strategies, including the use of non-pathogenic bacteria as probiotic biocontrol agents (Austin et al.1995; Moriarty 1998).

1.1 Probiotics

The concept of biological disease control, particularly the use of nonpathogenic bacterial strains for disease prevention, has received widespread attention during the last decade. Fuller (1989) defined probiotics as "a live microbial feed supplement, which beneficially affect the host animal by improving its intestinal microbial balance". However, in the case of aquatic organisms microbial flora of the aquatic environment, skin and gill microflora of the organism must also be contributing to disease prevention. There have been very few studies in aquaculture that focus on bacteria that prevent the growth of pathogenic organisms (Westerdahl et al. 1991; Nogami and Maeda, 1992; Olsson et al. 1992; Austin et al. 1995; Riquelme et al.1997, Jayaprakash et al. 2005).

Many marine heterotrophic bacteria are known to produce antibacterial compounds of significance. Large number of marine bacterial isolates exhibited antagonistic properties against other pelagic bacteria and studies of antibiosis at the phylogenetic level are usefull for formulating strategies for pathogen control in aquatic environment (Long and Azam 2001). According to them α-proteobacteria are potential sources of probiotic that are active against members of the Bacteroides. Moreover members of Vibrionales and Alteromonadales may also be good probiotics that are active against pathogenic *Vibrio* spp. and this has been recognized by Verschuere et al. (2000).

1.1.1 Different types of probiotics

1.1.1.1 Lactic acid bacteria

Lactic acid bacteria are Gram-positive usually non-motile, non-sporulating bacteria which produce lactic acid as major product by fermentation. Members of this group consist both rods (*Lactobacillus* and *Carnobacterium*) and cocci (*Streptococcus*). *Streptococcus, Leuconostoc, Lactobacillus* and *Carnobacterium* belong to the normal microbiota of the gastrointestinal tract in healthy fish (Ringϕ and Gatesoupe 1998).

Lactic acid bacteria have been tested as probiotics in warm-blooded animals, and attempts have also been made to use them as antagonists of fish pathogens (Gastesoupe 1994; Joborn et al. 1997). Joborn et al. (1997) isolated a strain of *Carnobacterium inhibens* KI from gastrointestinal tract of Atlantic salmon which produced inhibitory substances against fish pathogens *in vitro*. Robertson et al. (2000) reported a strain of *Carnobacterium* sp. isolated from the intestine of Atlantic salmon which showed antagonism against *A. hydrophilla, A. salmonicida, Flavobacterium psychrophilum, Photobacterium damselae, Streptococcus milleri* and *V. anguillarum* and could reduce mortality on subsequent challenge with *A. salmonicida,*

V. ordalii and *Yersinia rukeri.* Enhancement of immune response was also reported by a probiotic strain of *Lactobacillus rhamnosus* in rainbow trout (Nikoskelainen et al. 2003). *V. alginolyticus* load in Artemia culture could be reduced by six isolates of *Lactobacillus* and *in vitro* studies showed that extracellular products from *Lactobacillus* inhibited the growth of *V. alginolyticus* especially those from *Lactobacillus brevis* (Villamil et al.2003).

1.1.1.2 *Bacillus*

Bacillus, aerobic Gram positive, endospore forming bacteria have been extensively used as probiotics in aquaculture (Moriarty 1998; Rengipat et al.1998; Rengipat et al. 2000; Vaseeharan and Ramasamy 2003). *Bacillus* spores have been tested as probiotics against opportunistic pathogenic strains of *Vibrio* sp. in turbot larvae, *Scophtalmus maximus* by Gastesoupe (1991). Rengipat et al. (1998) isolated *Bacillus* S II which provided 100 % survival after challenging with a shrimp pathogen, *V. harveyi* and a significant difference was obtained in the overall survival between probiotic treated and the control group. Moriarty (1998) observed that population size of *Vibrio,* especially luminous *Vibrio,* was small in ponds where a large abundance of *Bacillus* sp. was maintained in the water column. Vaseeharan and Ramasamy (2003) reported three isolates of *Bacillus*, including *B. subtilis*, having inhibitory activity against *V. harveyi, V. anguillarum, V. vulnificus* and *V. damsela* in an antagonism assay. *B. subtilis* inhibited *V. harveyi*, both *in vivo* and *in vitro.*

1.1.1.3 *Pseudomonas*

Pseudomonads are common inhabitants of the aquatic environment including shrimp ponds (Otta et al. 1999) and have been commonly associated with gills and intestinal tract of live fish (Cahill 1990). Lemos et al. (1985) studied 200 epiphytic isolates from intertidal seaweeds and 38 had the ability

to inhibit growth of other bacteria. All these isolates belonged to the *Pseudomonas/Alteromonas* group, and the isolates inhibited growth of many fish pathogens including *V. anguillarum* and *Aeromonas salmonicida*. A strain of *Pseudomonas flurescence* was successfully used to reduce the frequency of stress-induced infections by *A. salmonicida* in fish (Smith and Davey 1993). Torrento and Torrens (1996), reported five strains of *Pseudomonas,* which showed inhibitory activity against *V. harveyi*. *P. flurescence* has also been reported to inhibit *Saprolegnia* sp. in finfish culture (Bly et al. 1997). Gram et al. (1999) observed that bathing rainbow trout for 6 days in *P. flurescence* AH2, isolated from *Lates niloticus*, reduced mortality from 47 to 32 % following challenge with *V. anguillarium*. Spanggaard et al. (2001) recorded 1018 bacterial and yeast isolates from skin, gill and intestine of rainbow trout, and 45 isolates of which inhibited *V. anguillarum* in disc diffusion assay. The dominant antagonist was *Pseudomonas*, which improved the survival of rain bow trout against vibrios following a challenge by inoculating in the rearing water. Chythanya et al. (2002) reported a strain of *Pseudomonas* I-2, antagonistic to shrimp pathogenic *V. harveyi, V. fluvialis, V. parahaemolyticus, V. vulnificus* and *Photobacterium damselae* by means of secreting low molecular weight inhibitor.

1.1.1.4 *Vibrio*

Austin et al. (1995) found that a strain of *V. alginolyticus* could reduce mortality of Atlantic salmon, considerably subsequent to a challenge with *A. salmonicida* and to a lesser extent with *V. anguillarum* and *V. ordalii*. De Schrijver and Olivier (2000) reported improved protein digestion in juvenile turbort after oral administration of *V. proteolyticus*. In addition, Irianta and Austin (2002) reported that the cultures of *A. hydrophila* and *V. fluvialis* were effective in controlling infections by *A. salmonicida* in rainbow trout.

1.1.1.5 Other bacteria

Austin and Billaud (1990) reported a marine isolate of *Planococcus* which inhibited the fish pathogen *Serratia liquefaciens* by producing a water soluble bacteriocidal antibiotic. Another Gram positive cocci, *Micrococcus luteus* was found to have potential in combating *A. salmonicida* infection in rainbow trout, *Oncorhynchus mykiss* (Irianto and Austin 2002). *Enterococcus faecium* was used as gut probiotic in European eels (*Anguilla anguilla* L.) and significantly reduced mortality among eels on experimental challenge with *Edwardsiella tarda* (Chang and Liu 2002). Brunt and Austin (2005) reported *Aeromonas sobria* as probiotic against diseases caused by *Lactococcus garvieae* and *Streptococcus iniae* in rainbow trout.

1.1.1.6 Microalgae

A heterotrophically grown, spray dried unicellular algae, *Tetraselmis suecica*, have been used as feed for penaeids and as a feed-additive for salmonids. The data generated revealed a reduction in the severity of bacterial diseases, might be due to the presence of unspecified antimicrobial compounds in the algal cells (Austin and Day 1990; Austin et al. 1992). Spray dried powder of *Tetraselmis suecica* inhibited prawn pathogenic strains of *V. alginolyticus*, *V. anguillarm*, *V. parahaemolyticus* and *V. vulnificus* and reduced mortality in Atlantic salmon caused by *A. samonicida, V. anguillarum, V. salmonicida, Y. rukeri* etc. (Austin and Day 1990; Austin et al. 1992). Microalgae employed in shrimp hatcheries usually have a natural heterotrophic bacterial load between 10^{4} and 10^{7} CFU m l^{-1} but rarely of vibrios (Lizarraga-Partida et al. 1997). There is a positive correlation between growth of microalgae and the associated heterotrophic bacteria. Accordingly enhanced growth of the microalgae *Tetraselmis chuii* was obtained when cultured with the commonly associated bacteria with (Cañizares-Villanueva and Onti-veros-Arredondo, 1993). The same effect was observed for

C.gracilis when grown with *Flavobacterium* sp. where significant improvement in the specific growth rate of the microalgae was obtained and the stationary growth phase lasted longer (Suminto and Hirayama, 1997). Tendencia and de la Pena (2003) reported that *Chlorella* sp., a unicellular green alga with high chlorophyll content inhibited the growth of *V. harveyi* in co-culture experiments. They (Tendencia et al. 2005) also reported that the presence of micro algae such as *Chetoceros, Thalassiosira, Navicula, Nitszchia, Melosira* and *Fragilaria* in the experimental tanks reduced the *Vibrio* count and improved the survival of shrimps.

1.1.2 Mode of action of probiotics

Bulk of literature is available on different types of probiotics and their application in aquaculture, but the mode of action is not well understand. However, for their appropriate application a clear understanding of the way in which they act upon the pathogen and improve health of the cultured animals and upgrade quality of their environment is essential. Different possible ways by which probiotics act upon are either through production of inhibitory compounds, competition for nutrients, competition for adhesion sites, enhancement of immune response, improvement of water quality on interaction with phytoplankton or through combination effect of more than one of the mechanisms.

1.1.2.1 Production of inhibitory compounds

Inhibition of antagonistic bacteria, which produce inhibitory substances, can effectively eliminate pathogens in the aquatic environment or intestine of the host. In most of the cases the antagonistic property is due to the production of antibiotics, bacteriocins, siderophores, lysozyme, proteases, hydrogen peroxide, alteration of pH by organic acid production etc. either singly or in combination (Verschuere et al. 2000).

Lactic acid bacteria are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms (Hong et al. 2005). Nair et al. (1985) reported a large number of marine bacteria producing bacteriolytic enzymes against *V. parahaemolyticus*. Imada et al. (1985) characterized an *Alteromonas* sp., which had inhibited protease production in *Aeromonas hydrophila* and *V. anguillarum*. A marine isolate of *Pseudomonas* AI-J 25a inhibited the growth of many isolates of *Vibrio* including *V. parahaemolyticus* by means of a proteolytic enzyme (Than et al. 2004). Undissociated weak acids such as lactic acid produced by Lactic acid bacteria possess the ability to cross link membranes of micro-organisms, dissociated inside and acidify the interior causing the expulsion of H^+ ions. This weakens the cells and makes them more susceptible to bacteriocins and other bacteriocidal compounds (Nykanen et al. 1998). Extracellular products (ECPs) of *Lactobacillus* also showed antibacterial property, for example *Lactobacillus brevis* inhibited the growth of *V. alginolyticus* (Villamil et al. 2003). Chythanya et al. (2002) reported that a marine isolate of *Pseudomonas* I-2 inhibited *V. harveyi, V. fluvialis, V. parahaenolyticus, V. damsela* and *V. vulnificus* by a low molecular weight substances.

1.1.2.2 Competition for nutrients or energy

Competition for nutrients / available energy is very common among microbial population in aquatic environment and it plays an important role in the structure of microbiota of the aquatic systems (Verschuere et al. 2000). Rico-Mora et al. (1998) introduced a bacterial isolate (SK-05), with active growth in organically-poor substrates into a *Skeletonema costatum* culture and then added *V. alginolyticus*. Even though SK-05 exerted no *in vitro* inhibitory action against *V. alginolyticus*, it could eliminate *V. alginolyticus* due to its ability to utilize the exudates of the diatom.

Siderophores are the low molecular weight ferric ion chelating compounds; which bind with ferric irons and make it available for siderophore producing organisms and there happens by a competition between siderophore producing organisms and the others (Prescott et al. 2005). Many probiotics are capable of producing siderophores and there by they can selectively eliminate pathogens. Smith and Davey (1993) reported that a fluorescent *Pseudomonas* is capable of inhibiting the growth of *Aeromonas salmonicida* in culture media due to competition for free iron. Pybus et al. (1994) observed that a strain of *V. anguillarum* inhibited pathogenic *V. ordalii,* only in iron deficient media. Gram et al. (1999) reported that under iron-limited conditions alone *P.fluorescens* AH2 was inhibitory to *V. anguillarum in vitro* and addition of iron to sterile-filtered supernatant of the strain AH2 eliminated the inhibitory activity. Even though there is no evidence for siderophore induced antagonistic activity, the cell free supernatant of a siderophore producing *Pseudomonas* PS-102 was found to be antagonistic to *V. harveyi, V. alginolyticus, V. fluvialis, V. vulnificus, V. parahaemolyticus* and *Aeromonas* spp. (Vijayan et al. 2006).

1.1.2.3 Competition for adhesion sites

Attachment of probiotics to intestinal mucus and wall surface is important for their establishment within the gut of the host. Olsson et al. (1992) reported different isolates which inhibited *V. anguillarum in vitro.* Studies showed greater capability of these organisms for adhesion and growth in fish intestinal mucosa than the pathogen and skin mucosa isolates. *Carnobacterium* strain K1 (Joborn et al. 1997) has been reported as probiotic with adhesion capacity and growth on the intestinal wall. The application of another probiotic (AP5) prevented the attachment of *V. alginolyticus* in the mucus membrane and thus the growth of the pathogen in the digestive tract (Vine et al. 2004). Chabrillon et al. (2005) reported three isolates, which reduced adhesion of *Photobacterium damselae* Sole (*Solea senegalensis*

Kaup) in the intestinal mucosa when assayed under exclusion, displacement and competition conditions. In 2006 they (Chabrillon et al. 2006) reported probiotic strains with higher ability to adhere to the mucus membrane than the pathogens in gilthead seabream (*Sparus aurata*). Oral administration these probiotics protected *Sparus aurata* on subsequent challenge with *V. anguillarum.*

1.1.2.4 Improvement of water quality

Gram-positive bacteria, especially *Bacillus* spp. are more efficient in converting organic matter to CO_2. More over they are capable of converting a greater percentage of organic carbon to bacterial biomass or slime (Verschuere et al. 2000). During the production cycle higher population size of gram-positive bacteria can minimize dissolved and particulate organic carbon (Balcazar et al. 2006). There are several other reports stating that *Bacillus* sp. can improve water quality, survival and growth rate of animals, and reduced pathogenic vibrios in culture system (Dalmin et al. 2001).

In intensive/semi intensive shrimp culture systems high concentrations of ammoniacal nitrogen adversely affect productivity. Shan and Obbard (2001) used indigenous nitrifying bacteria immobilized on porous clay pellets for removing excess ammoniacal nitrogen. Bender and Philips (2004) reported application of microbial mats, which include cyanobacteria, anoxygenic photoautotrophs (purple bacteria), sulfur-reducing bacteria etc. for water quality improvement in aquaculture ponds.

1.1.2.5 Enhancement of immune response

Shrimps and other invertebrates have a prominent nonspecific immune system. There are many reports on bacterial compounds or nonliving cells as immunostimulants which can activate the non specific immune system. However, the immuno stimulatory property of probiotics are not well studied (Verschuere et al. 2000). Enhanced resistance of rainbow trout,

Oncorhynchus mykiss (Walbaum) to *Yersinia ruckeri* challenge following oral administration of probiotics such as *B. subtilis* and *B. licheniformis* (Raida et al. 2003) has been reported. Macey and Coyne (2005) reported putative probiotics, which improve disease resistance and phagocytic activity in *Haliotis midae*. They observed increased survival after challenge with *V. anguillarum* and higher growth rate in the probiotic fed animals.

1.2. Bioprocess technology

1.2.1. Media development

Over the years, substantial progress has been made in designing media to facilitate commercial production of several microorganisms. In most of the industrial fermentation, the product used to be something other than the cell mass demanding two distinct biological requirements in medium design. Primarily the medium has to supply enough nutrients for the growth of the organisms and secondarily it should provide nutritional requirements to maximize the product formation (Dahod 1999). Other than the biological requirements, for the selection of nutrients, one has to consider cost-effectiveness, availability, consistency in quality etc. In addition to that the media components should not interfere down stream processes also.

Complex non-synthetic media are more productive than chemically defined ones and are economical also. But there are cases were the complex media interfere with down stream processes. During the course of fermentation a completely defined medium is necessary for studying nutritional control and nutritional requirements of the product formation (Dahod 1999).

The choice of carbon source, generally influence secondary metabolism and therefore the product formation, say for example antibiotic production (Doull and Vining 1990; Spizec and Tichy 1995; Marwick et al. 1999). In rich

media, faster growth of microbes leads to suppression of secondary metabolism and there by reduce product yield (Stanbury 1997). Many marine bacteria have been reported to exhibit the above property (Riqueleme et al. 1996). As a general principle it has been reported that easily metabolizeable substances such as glucose may provide maximum cell density but reduce production of many secondary metabolites (Marwick et al. 1999). Kaiser et al. 1994 found that the total yield of manamycin from *Streptomyces* was increased when glycerol was fed during the production phase.

Alike carbon sources high concentrations of nitrogen sources have been reported to reduce antibiotic production (Doull and Vining 1990; Spizec and Tichy, 1995; Marwick et al. 1999). For an example isopenicillin production by *Actinomycetes* was reported to be sensitive to ammonium level (Demain 1986). However, in *Streptomyces griseofuocus*, ammonium stimulated the antibiotic production (Zhang et al. 1996). There are repots of certain amino acids improving the product yield, for example, addition of cystine increased phenazine-1-carboxylic acid yield in *Pseudomonas fluorescens* (Slininger and Jackson 1992). At the same time, use of unsuitable amino acids as nitrogen source inhibited the synthesis of secondary metabolites (Martain and Demain, 1980). These observations suggest that response of an organism to a nitrogen source varies with the type of compound used.

Phosphate is essential for the growth of microorganisms but at certain level it will inhibit secondary metabolism (Spizek and Tichy 1995). Turner and Messenger (1986) reported that phosphate limitation stimulated phenazine-1-carboxylic acid synthesis by *Pseudomonas aurofaciens*. However, phenazine production by *Pseudomonas phenazinium* was not regulated by phosphate concentration (Messenger and Turner 1983). These observations suggest that involvement of phosphate in regulating secondary metabolism is species specific.

There is varied response to elemental requirements. In some marine bacteria bromide concentration has significant role in antibiotic production as bromide is incorporated in certain marine antibiotics (van-Pee 1996). Yamaski et al. (1998) reported that there was no secondary metabolism in *Burkholderia glumae*, where $MgCl_2$ was replaced by $MgSO_4$. Zinc sulphate was found to increase phenazine production by *P. fluorescens* (Slininger and Jackson, 1992). KNO_3 and $FeCl_3$ increased phytotoxin produced by *P. syringaei* (Palmer and Bender 1993).

In addition to nutritional factors physical factors such as temperature, pH, oxygen, salinity, pressure etc. play an important role in growth and secondary metabolite production (Marwick et al. 1999). A marine *Alteromonas* sp. grew best at 28°C but yielded more antiviral compound at 25°C (Myogga et al. 1995). Temperature was found to be an important factor in the secondary metabolism of marine isolates of *Streptomyces*, *Aspergillus* etc. (James et al. 1991; Tepsic et al.1997). Phenazine production of *P. fluorescens* declined rapidly at pH 8 and found to be optimum at pH 7 (McCarthy et al.1985). Hays et al. (1997) reported the influence of pH on methylomycin synthesis of *Streptomyces* sp. Oxygen was found to be an important factor which influenced secondary metabolite synthesis by *Streptomyces parvulus*, *Saccharopolyspora erytherea* etc. (Kaiser et al.1994; Clark et al, 1995). The effect of salt concentration on secondary metabolism has not been investigated in detail but some marine bacteria required salt for growth (Kogure 1998). Okami et al. (1980) reported the effect of salinity on the production of aplasmomycin from a marine *Streptomyces*. Alteration in the pressure in bioreactor reported to improve cell dry weight (Yang and Wang 1992) and pressure also acted as stressor, and improved secondary metabolite production in some cases (Wright et al.1999).

The above discussion points to the need of optimization of culture conditions and composition of medium for enhanced production of biomass and the products of interest on one to one basis.

1.2.2. Statistical methods in bioprocess technology

The classical method of optimization of fermentation media involves varying one parameter at a time and keeping the others constant and is still employed in bioprocess technology (Strobel and Sullivan 1999). However, this conventional optimization technique is extremely laborious and time-consuming (Wang and Lu 2004), and often does not bring about the effect of interaction of various parameters (Weruster-Botz 2000). But the statistical experimental design techniques in fermentation process involve study of the main and interacting effect of factors and their by it can improve product yield and reduce process variability, time, cost etc. (Elibol 2004).

The procedure applied in statistical experimental design for optimization of fermentation media involves identification of the most important media components (screening), identification of the optimum variable ranges (narrowing), identification of the optimum (optimum search) and experimental verification of the identified optimum (Weruster-Botz 2000). For identification of most important media components Plankett-Burman experimental designs are used, where it is assumed that no interaction between the different media components in the range of variables under consideration. More over in this design the significant levels are determined and non-significant media components are screened out. For the identification of the optimum variable ranges (partial) factorial experimental designs are often used and to identify an optimum within the optimal variable range identified, the response surface method (RSM) has been used.

Response surface methodology (RSM) is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. This technique was used for designing media, optimization of culture conditions and for the production of bacteriocins (Leal-Sanchez et al. 2002), enzymes (Beg et al. 2003), antibiotics (Adinarayana et al. 2003), polysaccharides (Wang and Lu 2004) organic acids (Xiong et al. 2005) etc. However, it has not yet been reported for probiotics, especially in aquaculture.

1.3 Bioprocess technology for probotics

Bulk of literature is available on different types of probiotics and its application in aquaculture. However, bioprocess technology and commercialization of probiotics are not well exploited. In the present study we made an attempt to develop bioprocess technology for the mass production of already identified probiotics such as *Pseudomonas* MCCB 102 (Vijayan et al. 2006), *Pseudomonas* 103 (Jayaprakash 2005) and *Micrococcus* MCCB 104 (Jayaprakash et al. 2005). In addition, a study on the nature of the inhibitory compounds and their role in eliminating pathogens is needed for efficient application and commercialization of probiotics. Hence we have also made an attempt to characterize antagonistic compounds produced by *Pseudomonas* MCCB 102 and 103.

Since green water culture systems and application of microalgae for controlling pathogens have got wide spread attention, the third objective was to evaluate unicellular marine cyanobacterial systems as putative probiotics in shrimps.

1.4 Objectives

The objectives focused at in this research are the following

I. Optimization of culture conditions and media designing for the mass production of antagonistic probiotics *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103.

II. Isolation, purification and characterization of antivibrio compounds from *Pseudomonas* MCCB102 and *Pseudomonas* MCCB 103.

III. Optimization of culture conditions and media designing for the mass production of antagonistic probiotic *Micrococcus* MCCB 104.

IV. Evaluation of *Synechocystis* MCCB 114 and *Synechocystis* MCCB 115 as probiotic for the management of *Vibrio* in shrimp culture systems.

Chapter-2

Optimization of Culture Conditions and Media Designing for the Mass Production of Antagonistic Probiotics Pseudomonas MCCB 102 and Pseudomonas MCCB 103

Chapter 2

Optimization of Culture Conditions and Media Designing for the Mass Production of *Pseudomonas* MCCB 102 and *Pseudomonas* 103

2.1 Introduction

In shrimp larval rearing systems vibriosis has been designated as systemic bacterial infection caused by several species of *Vibrio*, such as *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus* etc. (Singh et al.1989; Lavilla-Pitogo et al.1990; Karunasagar et. al.1994; Abraham and Manely, 1995) and has been controlled to some extent by means of antibiotics. Prophylactic and therapeutic application of antibiotics for the control of the aquatic pathogens has led to multiple antibiotic resistance among vibrios and under such situations these strains have been found to be more virulent (Karunasagar et al. 1994; Abraham et al. 1997, Tendencia and de la Pena 2001) and are liable to be transferred to human pathogens too. Since antibiotics do not support sustainable aquaculture, biological control agents have been in vogue for some time in place of antibiotics. Among them the use of antagonistic probiotics (Gomez-Gil et al. 2000) has become popular in aquaculture in the recent years. Antagonistic probiotics modify the microbial flora in its niche and there by control growth of pathogenic forms in the system and improve the conditions in the culture environment.

Torrento and Torrens (1996), reported five strains of *Pseudomonas*, which showed inhibitory activity against *V. harveyi*, the causative agent of luminescent vibriosis in *Penaeus monodon*. Subsequently *P. flurescence* was reported to inhibit *Saprolegnia* sp. in finfish culture (Bly et al. 1997). Gram et al. (1999) demonstrated the protection of rainbow trout administered with

P. fluorescens AH 2 when challenged with *V. anguillarum*. Later, Chythanya et al. (2002) reported *Pseudomonas* 1-2, which antagonized shrimp pathogenic *V. harveyi, V. fluvialis, V. parahaemolyticus, V. vulnificus* and *Photobacterium damselae.*

Recently Vijayan et al. (2006) and Jayaprakash (2005) isolated and identified native probiotic strains, such as *Psudomonas* PS-102 and *Psudomonas* MCCB 103 antagonistic to a range of vibrios in penaeid and non-penaeid rearing systems. However, to bring forth the probiotic to an application mode at commercial level an appropriate bioprocess technology is imperative. For developing such a technology the prime requirement is optimization of growth conditions to maximize production of both biomass and antagonistic activity. The first objective of the present study was to optimize culture conditions such as sodium chloride concentration, pH and temperature for the production of the probiotic *Pseudomonas* PS-102, renumbered as *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 based on *in vitro* cell growth and antagonistic activity against *V. harveyi* MCCB 111. Subsequently, for facilitating commercial production of the probiotics, optimizations of carbon and nitrogen sources and other growth factors were to be accomplished for bioprocessing. These objectives were accomplished by employing the statistical modeling technique, Response Surface Methodology (RSM).

The classical optimization method, required for the bioprocess, involves the process of varying one parameter at a time and keeping the others constant. However, such an approach does not bring about the effect of interaction of various parameters. In this context Response Surface Methodology (RSM) can be used to evaluate the effect of several factors influencing the responses by varying them simultaneously in limited number of experiments, and their by, it can improve product yield and reduce process

variability, time, cost etc. Further, in this study RSM based on five-level central composite design (CCD) was employed for the optimization of above culture conditions.

2.2 Materials and methods

2.2.1. Probiotic organisms

Two isolates of probiotics, such as *Pseudomonas* PS 102 and *Pseudomonas* MCCB103 were used for the present study. The first isolate described previously by Vijayan et al. (2006) and was obtained from Central Institute of Brackish Water Aquaculture, Indian council of Agricultural Research, Chennai, India and maintained in National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology, Cochin, Kerala, India under the code *Pseudomonas* MCCB102. The second organism used was *Pseudomonas* MCCB103, previously described by Jayaprakash (2005), which also formed part of the microbial culture collection of NCAAH.

2.2.2 Experimental design for optimization of salinity pH, and temperature for the mass production of *Pseudomonas* MCCB 102 and 103

2.2.2.1 Culture medium and inoculum preparation

The organisms were grown in ZoBell's marine broth 2216 E for optimization of culture conditions such as sodium chloride concentration, pH and temperature. A single colony of each isolate (*Pseudomonas* MCCB 102 and *Pseudomonas* MCCB103) grown on ZoBell's agar 2216 E (Oppenheimer and ZoBell 1952) was inoculated in 100 ml ZoBell's broth and incubated at 28°C for twenty-four hours and used as the inoculum.

2.2.2.2 Shake flask experiments

Optimization was carried out in Erlenmeyer flasks (250 ml capacity) with 100 ml ZoBell's marine 2216 E broth. For optimization of sodium

chloride concentration, the medium was prepared in different concentrations of sodium chloride such as 0, 5, 10, 15, 20, 25, 30 and 35 g l^{-1}. The influence of pH on growth and antagonistic activity was assessed at pH of 6, 6.5, 7, 7.5 and 8. pH was adjusted by employing a pH meter (Scientific Tech., India) initially. During incubation pH was controlled daily by the aseptic addition of sterile 1mol l^{-1} NaOH and 1mol l^{-1} HCl, using narrow range pH paper. Temperature was optimized in ZoBell's marine broth at 25, 30, 35 and 40 ± 0.1^{0}C. All flasks through out the study, in 100 ml aliquots, were inoculated with the culture to a final concentration equivalent to 0.01 optical density (OD) at A_{600} (10^3 CFU m l^{-1}). Incubations were in a temperature controlled rotary shaker (Orbitek, Scigenics Biotech. (Pvt.) Ltd., India) at 100 rev. min^{-1}.

2.2.2.3 Analysis of the samples

2.2.2.3.1 Biomass determination and antagonism assay

Samples were aseptically withdrawn from the flasks at 24-hour interval. The cells were removed by centrifugation at 10,000 × g for 15 minute and pH of the supernatant adjusted to 7 using 1mol l^{-1} HCl, filter sterilized using cellulose acetate membrane of 0.22 µm porosity. The cell free supernatant was used for antagonism assay towards *Vibrio harveyi* MCCB 111, the target bacterial culture. *V. harveyi* grown on ZoBell's marine Agar slants were harvested in saline (0.85% sodium chloride, w/v) and optical density adjusted to 1.5 at A_{600} and 500 µl plated on ZoBell's marine 2216 E plates. Optical density of cell suspension used for seeding the plates was kept constant through out the experiment. Aliquots of supernatant from the culture (20 µl) were spotted and activity measured after 18 hours of incubation (at 28°C) by measuring the inhibition zone using a Hi Antibiotic Zone Scale (Himedia, India) as described earlier (Jayaprakash et al., 2005). The pellets were repeatedly washed in sterile saline (0.85% NaCl), resuspended and OD at $A_{600\ nm}$ determined spectrophotometrically (UV-1601, Shimadzu Corporation,

Japan) and converted to cell dry mass (CDM) using a standard curve (Guerra and Pastrana 2002).

2.2.2.4 First step optimization of salinity, pH, and temperature for biomass and antagonistic activity for *Pseudomonas* MCCB 102 and 103

One-dimensional screening was done initially to find out the range that has to be used for further optimization experiment. Different concentrations of sodium chloride such as 0, 5, 10, 15, 20, 25, 30 and 35 g l^{-1}, pH of 6, 6.5, 7, 7.5 and 8, and temperature of 25, 30, 35 and 40 ± 0.1^{0}C were screened one at a time. Biomass and antagonistic activity were determined daily for one week. Considering both biomass and activity, regression analysis was done to find out the optimum range of each parameter.

2.2.2.5 Second step optimization of salinity pH, and temperature using statistically designed experiments for *Pseudomonas* MCCB 102 and 103

Response surface methodology (RSM) is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously (Dirk 2000). The most popular RSM design is the central composite design (CCD) and it was used to find out the optimum biomass production and antagonistic activity at different combinations of sodium chloride concentration, pH and temperature. CCD has three groups of design points: two-level factorial or fractional factorial design points, axial points (sometimes called "star" points) and centre points. CCD's are designed to estimate the coefficients of a quadratic model. The experiments were done using a soft ware Design Expert (version 6.0.9, State Ease, Minneapolis, M N).

2.2.2.6 Experimental verification of the identified optima from the model for *Pseudomonas* MCCB 102 and 103

Experiments were repeated at the optimum concentration of sodium chloride, pH and temperature obtained from the model equations for biomass and antagonistic activity for *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103.

2.2.3 Experimental design for optimization of media for the mass culture of *Pseudomonas* MCCB 102 and 103 using RSM

2.2.3.1 Screening of C-sources, N-sources, amino acids and vitamins

Twenty-four C-sources (glucose, sucrose, galactose, maltose, fructose, lactose, arabinose, cellobiose, mannose, ribose, trehalose, xylose, rhamnose, mannitol, sorbitol, starch, succinic acid, dextrin, glycerol, potassium sodium tartarate, pyruvic acid, sodium acetate, sodium citrate, and sodium gluconate, Hi-Media, India) were screened in a basal medium (Oliver 1982) as sole source of carbon for growth of *Pseudomonas* MCCB 102 and 103. All sugars were added to a final concentration of 0.02% (w/v) and other carbon sources were added in a final concentration of 0.1% (w/v). Tubes without carbon sources were also kept as control. All tubes were inoculated uniformly and incubated for 24-48 hours at 28°C. Growth was measured based on turbidity against control. Subsequently seven of them (glucose, glycerol, mannitol, pyruvic acid, sodium acetate, sodium citrate and sodium gluconate) were selected for probiotics based on their role as sole source of carbon for further screening at 10 g l^{-1} level in a mineral medium (Chang and Blackwood 1969) supplemented with 10 g l^{-1} sodium chloride, the concentration of which was obtained from the previous experiment. Five different mineral media (Anne 1967; Chang and Blackwood 1969; Kanner 1978; Stephen et al.1981; Oliver et al.1982) were screened for both biomass and antagonistic compound for *Pseudomonas* MCCB 102 and 103, and selected the one described by

Chang and Blackwood (1969). Composition of the mineral medium was $MgCl_2.6H_2O$: 4.1 g l^{-1}, Na_2SO_4: 7.1 g l^{-1}, K_2HPO_4: 0.1 g l^{-1} and Urea: 2 g l^{-1}. Ammonium chloride, ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate, sodium nitrate and urea were screened as sole N-source at 2 g l^{-1} level in the same mineral based media with 10 g l^{-1} glucose and without N-source. Twenty-four amino acids (DL- alanine, DL-2-amino-n-butyric acid, L-arginine monohydrochloride, DL-aspartic acid, L-cystine hydrochloride, L-cystine, 3-(3,4-dihydroxyphenyl) DL-alanine, L-glutamic acid, glycine, L-histidine monohydochloride, L-hydroxyproline, L-lucine, L-iso leucine, DL-Nor-lucine, L-lysine monohydrochloride, L-methionine, DL-ornithinie monohydrochloride, L-proline, DL-β-phenylalanine, D-serine, DL-threonine, L-tryptophan, L-tyrosine and DL-valine- Hi-Media, India) and Casamino acid (BD Biosciences) were screened as growth factors at 0.2 g l^{-1} level and vitamins such as ascorbic acid, biotin, cyanocobalamine, folic acid, inositol, pantothenic acid, riboflavin and thiamine (Hi-Media, India) at 0.02 g l^{-1} level in a pattern of one at a time for biomass and antagonistic activity. All incubations were done for ninety-six hours.

2.2.3.2. Shake flask experiment

Primary screening of nutrients (one-at-a-time) and the final optimization of selected ingredients were carried out in Erlenmeyer flasks (250 ml capacity) with 100 ml mineral medium without ferric sulphate (Chang and Blackwood 1969). Sugars, amino acids and vitamins were filter sterilized using cellulose acetate membrane (Millipore, India) having 0.22 µm porosity and were then added to sterile mineral medium. After adding the nutrients pH was adjusted by the addition of sterile 1mol l^{-1} NaOH and 1mol l^{-1} HCl, employing narrow range pH paper. All flasks through out the study were inoculated with the culture to a final concentration equivalent to 0.01 at A_{600} (10^3 CFU m l^{-1}) in 100 ml aliquots. Incubations were done in a temperature

controlled rotary shaker (Orbitek, Scigenics Biotech. (Pvt.) Ltd,India) at 120 rev min^{-1}

Samples were aseptically withdrawn from the flasks at 24 hours interval, biomass and antagonistic compound production was determined as described below.

2.2.3.3 Analysis of the sample

The cells were removed by centrifugation at 10,000 × g for 15 minute and the supernatant was filter sterilized using cellulose acetate membrane (Millipore, India) having 0.22 μm porosity and used for the determination of antagonistic compound. Cell free supernatant (5 ml) was extracted into 3 ml chloroform and then reextracted into 1ml of 0.2 mol l^{-1} HCl to give a pink to deep red solution and measured spectrophotometrically (UV-1601, Shimadzu Corporation, Japan) at 520 nm and the concentration of antagonistic compound was determined as described by Essar et al.(1990). The pellets were repeatedly washed in sterile saline (8.5 g l^{-1} NaCl) and biomass was calculated as described in the previous experiment.

2.2.3.4. Experimental design and optimization of media using RSM

The medium for the production of probiotic has been first optimized by a 'one-variable-at-a-time' approach. The minimum and maximum ranges of selected variables were investigated. The medium composition that resulted in the maximum biomass production and antagonistic activity was further optimized by response surface methodology (RSM) using central composite design (CCD) and a set of fifty experiments was carried out. The experiments were done using a soft ware Design Expert as described above (version 6.0.9, Stat Ease, Minneapolis, MN).

2.2.3.5. Experimental verification of biomass and antagonistic compound in newly designed media for *Pseudomonas* MCCB 102 and 103

Experiments were conducted at the optimum concentrations of ingredients obtained from the model equations for both biomass and antagonistic compound production for *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103. The experimental values were subsequently compared with predicted values obtained from the model equations.

2.3 Results

2.3.1 Optimization of salinity pH, and temperature for the mass production of *Pseudomonas* MCCB 102

2.3.1.1 First step optimization of salinity, pH, temperature and for biomass and antagonistic activity for *Pseudomonas* MCCB 102

One-dimensional screening results were used to find out the optimum level of each parameter. Regression analysis was done using biomass as X and antagonistic activity as Y for pH, temperature and sodium chloride concentration (Table 1) and wherever regression was found to be highly significant, the corresponding factor levels were taken for further analysis, such as 5-20 g l^{-1} for sodium chloride concentration, 6-8 for pH and 25 to 35 ^{0}C for temperature. The antagonistic activity was negligible at 30 g l^{-1} sodium chloride and no activity at 35 g l^{-1}.

2.3.1.2 Second step optimization of salinity, pH, temperature and using statistically designed experiments for *Pseudomonas* MCCB 102

The coded values of the independent variables such as sodium chloride concentration (X_1), pH (X_2) and temperature (X_3), are given in Table 2 along with the experimental and predicted values of biomass and antagonistic activity. The CCD matrix were analysed by standard analysis of variance (ANOVA) as approximate to the experimental design used. The ANOVA of

the quadratic regression model demonstrates that the model is highly significant (P< 0.0001) for both biomass and activity (Table 3 and 4). The Model F-value is 123.14 for biomass and 80.24 for activity and implies that the model is significant. In the case of biomass A, B, C, A^2, B^2, C^2, AC, BC are significant model terms. Where 'A' is salinity, 'B' is pH and 'C' is temperature. When we consider activity A, B, C, A^2, B^2, C^2, AB, AC and BC are significant model terms.

The goodness of fit of the model was checked by coefficient of determination (R^2). R^2 = 0.9911 in the case of biomass and 0.9863 in the case of activity. It can be expressed in percentage also and then it is interpreted as the percent variability in the response in the given model. As per the model, sample variation of 99.11 % for biomass and 98.63% for antagonistic activity was attributed to the independent variables and the model did not explain only 0.89% and 1.37% respectively of the total variation. The lack-of-fit test was insignificant for both biomass (0.1777) and activity (0.0673). For biomass, correlation coefficient (R) is equal to 0.9955 and for activity it is 0.9931. An adequate precision was also observed, 29.639 for biomass and 22.770 for activity respectively. In the case of biomass "Pred R-Squared" 0.9459 is in reasonable agreement with the "Adj R-Squared" of 0.9830 and in the case of antagonistic activity the "Pred R-Squared" of 0.9117 is in reasonable agreement with the "Adj R-Squared" of 0.9740.

The RSM gave the following regression equations for the biomass (Y_1) and antagonistic activity (Y_2) as a function of salinity (X_1), pH (X_2) and temperature (X_3).

Final Equations in Terms of Coded Factors are:

$$Y_1 = +1.16 + 6.261E\text{-}003\ X_1 + 7.356E\text{-}003\ X_2 + 7.123E - 003\ X_3 - 0.059\ X_1^2 - 0.019\ X_2^2 - 0.014\ X_3^2 + 1.25E - 003\ X_1 X_2 + 8.750E\text{-}003\ X_1 X_3 + 0.051\ X_2 X_3 \quad \text{.....Eq.1}$$

$$Y_2 = +16.33 - 2.31\ X_1 + 0.92\ X_2 - 5.12\ X_3 - 4.02\ X_1^2 - 0.98\ X_2^2 - 3.07\ X_3^2 - 2.42\ X_1 X_2 - 0.92 X_1 X_3 + 1.58\ X2\ X_3 \quad \text{.....Eq.2}$$

The two-dimensional contour plots and its respective response surface plots on biomass showed the interactive effect of sodium chloride concentration and temperature (Fig. 1 A) at the optimum pH (6), and that of pH and temperature (Fig.1 B) at the optimum concentration of sodium chloride (12.24 g l^{-1}). Fig. 2 showed similar plots on antagonistic activity, having the interactive effect of sodium chloride concentration and temperature (Fig. 2 A) at the optimum pH (6.5), that of pH and temperature (Fig.2 B) at the optimum concentration of sodium chloride (12.9 g l^{-1}) and, the interactive effect of sodium chloride concentration and pH (Fig.2 C) at optimum temperature (25°C).

The predicted optimum conditions for biomass are, sodium chloride 12.2 g l^{-1}, pH 6 and temperature 25 °C and those for antagonistic activity are 12.9 g l^{-1}sodium chloride, pH 6.5 and temperature 25 ° C.

2.3.1.3 Experimental verification of biomass and antagonistic compound in newly designed medium for *Pseudomonas* MCCB 102

The optimum culture conditions such as sodium chloride concentration, pH, and temperature required for *Pseudomonas* MCCB 102 were found out for

maximum biomass and antagonistic activity from the regression equations (Eq.1 and Eq. 2), and were experimentally verified as shown in the Table 5. After optimization biomass was found significantly increased by 8.4% and activity by 25.6%.

2.3.2 Optimization of salinity, pH, and temperature for the mass production of *Pseudomonas* MCCB 103

2.3.2.1 First step optimization of salinity, pH, and temperature for biomass and antagonistic activity for *Pseudomonas* MCCB 103

Regression analysis was done (Table 6) and the optimum ranges were fixed for each parameter as described above, for *Pseudomonas* MCCB 103 (5-20 g l^{-1} for sodium chloride, 6-7 of pH and temperature of incubation 25 to 35 ^{0}C).

2.3.2.2 Second step optimization of salinity, pH, and temperature using statistically designed experiments for *Pseudomonas* MCCB 103

A central composite design (CCD) of response surface methodology was used to maximize the biomass production and antagonistic activity, and to determine the interaction between these parameters. The coded values of the independent variables [sodium chloride concentration (X_1), pH (X_2) and temperature (X_3)] are given in Table 7 along with the experimental and predicted values of biomass and antagonistic activity. The CCD matrix was analyzed by standard analysis of variance (ANOVA) as approximate to the experimental design used. The ANOVA of the quadratic regression model demonstrates that the model is highly significant ($P< 0.0001$) for both biomass and activity (Table 8 and 9). The Model F-value is 22.34 for biomass and 33.78 for activity and implies that the model is significant. In the case of biomass, A, B, C, A^2, B^2, C^2, AC and BC are significant model terms. Where 'A' is sodium chloride concentration, 'B' is pH and 'C' is temperature. When we consider activity A, B, C, A^2, C^2, AB and AC are significant model terms.

The goodness of fit of the model was checked by coefficient of determination (R^2). R^2 = 0.9524 in the case of biomass and 0.9682 in the case of activity. Coefficient of determination gives a measure of how much variability in the observed response value can be explained by the experimental factors and their interactions. It can be expressed in percentage also and then it is interpreted as the percent variability in the response in the given model. As per the model, sample variation of 95.24 % for biomass and 96.82% for antagonistic activity were attributed to the independent variables and the model did not explain only 4.76% and 3.18%, respectively of the total variation. A higher value of correlation coefficient (R) indicates an excellent correlation between independent variables but a relatively low value of coefficient of variation (CV) shows the reliability and improved precision of the experiments. For, biomass R is equal to 0.9759 and for activity it is 0.984. The coefficient of variation is 2.59% and 9.91% for biomass and activity respectively. The purpose of statistical analysis is to determine the experimental factors, which generate signals, large in comparison to the noise. Adequate precision measures signal to noise ratio and a ratio grater than 4 is desirable. Accordingly, an adequate precision was obtained, 15.196 and 20.549 for biomass and activity respectively. In the case of biomass "Pred R-Squared" 0.7799 is in reasonable agreement with the "Adj R-Squared" of 0.9096 and the "Pred R-Squared" of 0.8003 is in reasonable agreement with the "Adj R-Squared" of 0.9395, in the case of activity.

The RSM gave the following regression equations for the biomass (Y_1) and antagonistic activity (Y_2) as a function of sodium chloride concentration (X_1), pH (X_2) and temperature (X_3).

Final Equations in Terms of Coded Factors are:

$$\text{Biomass} = +0.86 - 0.022 X_1 + 0.016 X_2 - 0.031 X_3 + 0.065 X_1^2 + 0.023 X_2^2 + 0.041 X_3^2 + 0.000 X_1 X_2 + 0.025 X_1 X_3 - 0.042 X_2 X_3 \quad \text{.....Eq.3}$$

$$\text{Activity} = +16.08 - 1.30 X_1 - 1.28 X_2 - 2.84 X_3 - 1.34 X_1^2 - 0.28 X_2^2 - 4.05 X_3^2 - 1.75 X_1 X_2 - 1.16 X_1 X_3 - 0.80 X_2 X_3 \quad \text{.....Eq.4}$$

Response surface plot (Fig.3.A) of biomass shows the interaction between sodium chloride concentration and temperature on biomass production and was found to be maximum at 25°C and 5 g l^{-1} sodium chloride. Fig.3.B shows the effect of pH and temperature at the optimum concentration of sodium chloride (5g l^{-1}). The biomass was found to be increasing with pH and was maximum at 7. It was also clear from the plots that the maximum biomass production was at 25°C, and it decreased with increasing temperature.

The Fig. 4 shows that both at lower and higher concentrations of sodium chloride (5 g l^{-1} and 20 g l^{-1}) antagonistic activity increases with decreasing temperature and was found to be maximum at 25.11 ^{0}C at the optimum pH 7. Both biomass and activity show more or less similar results due to the possible influence of biomass on antagonistic activity.

2.3.2.3 Experimental verification of biomass and antagonistic compound at the optimum pH, temperature and sodium chloride concentration of *Pseudomonas* MCCB 103

The optimum pH, temperature and sodium chloride concentration were found out for maximum biomass and antagonistic activity from the regression equations (Eq.3 and Eq. 4) and were experimentally verified as shown in the Table 10. After optimization, biomass was found increased by 13.26% (1.145

± 0.17 to 1.32 ± 0.06, dry weight in g l^{-1}) and activity by 31.75% (9.33±0.58 to 13.67±0.58, diameter of the halo zone in mm).

2.3.3 Optimization of media for the mass culture of *Pseudomonas* MCCB102 using RSM

2.3.3.1 Screening of C-source, N-source, amino acids and vitamins for biomass and antagonistic activity of *Pseudomonas* MCCB 102

Variables such as glucose and glycerol were selected as carbon sources (Table 11 and Fig. 5), urea as the nitrogen source (Fig. 6), sodium chloride (from the previous experiment) and mineral salt solution described by Chang and Blackwood, 1969 (Fig. 7) were chosen for the study by a 'one – variable – at – a - time method'. The minimum and maximum limits of the variables were, glucose: 2 - 20 g l^{-1}, glycerol: 2-20 g l^{-1}, sodium chloride: 5 - 15 g l^{-1}, urea: 0.1 - 4 g l^{-1} and mineral salt solution: 5- 20 ml l^{-1}. Growth factors such as aminoacids and vitamins did not have much effect on enhanced production of biomass and activity (Fig.8-10).

2.3.3.2 Second step optimization of ingredients of media for *Pseudomonas* MCCB 102

The coded values of the independent variables (glucose: X_1, glycerol: X_2, sodium chloride: X_3, Urea: X_4 and mineral salt solution: X_5) along with the experimental and predicted values of biomass and antagonistic activity are given in Table 12. The CCD matrix was analyzed by standard analysis of variance (ANOVA). The ANOVA of the quadratic regression model demonstrates that the model is highly significant ($P< 0.0001$) for both biomass and activity (Table 13 and 14). The Model F-value, 65.0 for biomass and 43.85 for the antagonistic compound production, implies that the model is significant.

Coefficient of determination (R^2) is equal to 0.9782 in the case of biomass and 0.9680 in the case of antagonistic compound production. It can be

expressed in percentage also and then it is interpreted as the percent variability in the response in the given model. As per the model, sample variation of 97.82 % for biomass and 96.8% for the antagonistic compound production were attributed to the independent variables and the model did not explain only 2.18% and 3.2%, respectively of the total variation. For biomass, correlation coefficient (R) is equal to 0.9890 and for the antagonistic compound production it is 0.9838. An adequate precision was obtained, 31.41 and 31.95 for biomass and the antagonistic compound production respectively, indicating an adequate signal as it measures the signal-to-noise ratio. A ratio greater than 4 is desirable (Wang and Lu 2004). The model can be used to navigate the design space. In the case of biomass "Pred R-Squared" 0.9245 is in reasonable agreement with the "Adj R-Squared" of 0.9632 and in the case of antagonistic compound production the "Pred R-Squared" of 0.8846 is in reasonable agreement with the "Adj R-Squared" of 0.9459 also.

The RSM gave the following regression equations for the biomass and antagonistic compound production as functions of glucose (A), glycerol (B), sodium chloride (C), urea (D) and mineral salt solution (E).

Final Equations in Terms of Coded Factors are:

Biomass= + 1.53+0.26A + 0.036 B + 0.004324C + 0.14D + 0.27E-0.038 A^2 + 0.046 B^2 + 0.041 C^2-0.18 D^2-0.14 E^2-0.11 AB + 0.088 AC - 0.022 AD-0.040 AE - 0.025 BC + 0.068BD - 0.00844 BE - 0.053 CD-0.013 CE - 0.015 DE

.....Eq.5

Antagonistic compound = + 65.90 - 15.40 A + 2.05 B-5.47 C - 2.60 D + 4.82 E + 5.22A^2 - 9.32 B^2 -6.15 C^2-11.34 D^2-10.01E^2-1.67 AB+6.47 AC+10.03 AD-2.79 AE-3.28 BC-1.79 BD+3.42 BE-0.050 CD-3.63 CE+1.34 DE

.....Eq.6

The P value is used as a tool to test significance of each coefficient and the pattern of interaction between the coefficient on both biomass and activity.

The smaller the P value, the more significant is the corresponding coefficient (Rao et al. 2000). Linear coefficients such as A, B, D & E, quadratic coefficients A^2, B^2, C^2, D^2 & E^2 and interaction coefficients such as AB, AC, AE, BD and CD are highly significant for biomass. In the case of the antagonistic compound production linear coefficients A, B, C, D & E, quadratic coefficients A^2, B^2, C^2 & D^2 and interaction coefficients such as AC, AD, AE, BC, BE and CE are the significant model terms.

Effect of interaction of varying concentrations of glucose and glycerol, glucose and sodium chloride, glucose and mineral salts solution, glycerol and urea, sodium chloride and urea on biomass production when all other parametres at optimum are presented in Fig. 11 A, B, C, D, and E. Based on the data generated the optimum concentration of the ingredients were glucose:19.8 g l^{-1}, glycerol : 4.1 g l^{-1}, sodium chloride : 14.5 g l^{-1}, urea :2.4 g $l^{-1}$and mineral salts solution 15.5 ml l^{-1}.

The interaction between nutrients and their effect on antagonistic compound production were also studied (Fig.12). Effect of interaction of varying concentrations of ingredients as listed above on antagonistic compound production when all the other parametres kept optimum are presented in Fig. 12 A, B, C, D, E and F. The optimum concentrations of the ingredients for maximum antagonistic compound production were glucose: 2 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5 g l^{-1}, urea :2.1 g $l^{-1}$and mineral salts solution 17.4 ml l^{-1}.

2.3.3.3 Experimental verification of biomass and antagonistic compound in newly designed media for *Pseudomonas* MCCB 102

The concentration of ingredients of the media were found out from the regression equations (Eq 5 and Eq. 6) for both biomass and antagonistic compound production. Experiments were conducted at the above-mentioned compositions and

found that quantity of the biomass (1.99±0.08 g l^{-1}) and concentration of the antagonistic compound (100.33 ± 4.5 mg l^{-1}) were closer to the predicted values (2 g l^{-1} biomass and 96 mg l^{-1} antagonistic compound), validating the model.

2.3.4. Optimization of media for the mass culture of *Pseudomonas* MCCB 103 using RSM

2.3.4.1 Screening of C-sources, N-sources, amino acids and vitamins on biomass and antagonistic activity for *Pseudomonas* MCCB 103

Variables such as mannitol and glycerol were selected as carbon sources (Table 15 and Fig. 13), ammonium chloride as nitrogen source (Fig.14), sodium chloride (from the previous experiment) and mineral salt solution described by Chang and Blackwood (1969), (Fig. 15) were chosen for the study by a 'one variable-at-a-time method', and the range of sodium chloride concentration was taken from the previous experiment. The minimum and maximum limits of the variables were, mannitol: 2-20 g l^{-1}, glycerol: 2-20 g l^{-1}, sodium chloride: 5-15 g l^{-1}, Urea: 0.1-4 g $l^{-1}$and mineral salt solution: 5 - 20 ml l^{-1}. Growth factors such as amino acids and vitamins did not have any effect on biomass and activity (Fig. 16-18).

2.3.4.2. Second step optimization of ingredients of media for *Pseudomonas* MCCB 103

The most popularly used central composite design (CCD) of RSM was employed to maximize the biomass production and antagonistic activity. The interactive effect of nutritional factors on both biomass and activity were also investigated. The coded values of the independent variables (mannitol: A, glycerol: B, sodium chloride: C, urea: D and mineral salt solution: E) along with the experimental and predicted values of biomass and antagonistic activity are given in Table-16.The CCD matrix was analyzed by standard analysis of variance (ANOVA). The ANOVA of the quadratic regression model demonstrates that the model is highly significant ($P< 0.0001$) for both biomass and activity (Table-17 and 18). The Model F-value is 390.48 for

biomass and 27.99 for antagonistic compound production, which implies that the model is significant.

The 'lack of fit 'value is insignificant for both biomass and activity and the goodness of fit of the model was checked by coefficient of determination (R^2). R^2 is equal to 0.9963 in the case of biomass and 0.9507 in the case of antagonistic compound. It can be expressed in percentage also and then it is interpreted as the percent variability in the response in the given model. As per the model, sample variation of 99.63 % for biomass and 95.07% for antagonistic compound was attributed to the independent variables and the model did not explain only 0.37% and 4.93%, respectively of the total variation. A higher value of correlation coefficient (R) indicates an excellent correlation between independent variables. For biomass R is equal to 0.9981 and for antagonistic compound it is 0.9750. The purpose of statistical analysis is to determine which experimental factors generate signals, large in comparison to the noise. Adequate precision measures signal to noise ratio and, a ratio grater than 4 is desirable (Wang and Lu 2004). Accordingly an adequate precision was obtained such as 90.378 and 21.306 for biomass and antagonistic compound respectively. In the case of biomass "Pred R-Squared", 0.9845, is in reasonable agreement with the "Adj R-Squared", 0.9937, and in the case of antagonistic compound production the "Pred R-Squared", 0.7944, is in reasonable agreement with the "Adj R-Squared" 0.9168.

The RSM gave the following regression equations for the biomass and antagonistic activity as a function of mannitol (A), glycerol (B), and sodium chloride(C), Urea (D) and mineral salt solution (E).

Final Equations in Terms of Coded Factors are:

Biomass = + 0.75 + 0.034 A - 0.006. B - 0.041 C + 0.073 D + 0.40 E + 0.061 A^2 + 0.013 B^2 - 0.0062 C^2 - 0.086 D^2 + 0.030 E^2 - 0.0068 AB - 0.029 A C + 0.0068 A D + 0.016 A E - 0.014 B C - 0.005 B D + 0.015 B E - 0.006 C D - 0.033 C E - 0.043 .....Eq.7

Antagonistic compound = + 65.53 - 1.69 A + 2.69 B - 9.77 C + 9.15 D + 22.93 E-0.66 A^2 - 2.81 B^2 - 4.01 C^2 - 3.74 D^2 - 2.64 E^2 - 0.82 A B - 2.86 A C + 3.00 A D-3.81 A E - 1.99 B C + 0.57 B D + 1.49 B E - 0.11 C D-2.71 C E - 4.47 D E Eq.8

The P values are used as a tool to test significance of each coefficient and the pattern of interaction between the coefficient on both biomass and activity. The smaller the P value, the more significant is the corresponding coefficient (Rao *et al.* 2000). Linear coefficients such as A, C, D & E, quadratic coefficients A^2, B^2, D^2 & E^2 and interaction coefficients such as AC, AE, BC, BE, CE and DE are highly significant for biomass. In the case of antagonistic compound production linear coefficients B, C, D & E, quadratic coefficients B^2, C^2, D^2& E^2 and interaction coefficients such as AC, AD, AE and DE are the significant model terms. Since it is an hierarchiel model the insignificant coefficients were not omitted from the Eq. 7 and Eq.8, (Wang and Lu 2004).

The response surface plots of the significant interactions of nutrients are given in Fig. 19 and Fig. 20 Effect of interaction of varying concentrations of manitol and sodium chloride, manitol and mineral solution, glycerol and sodium chloride, glycerol and mineral solution, sodium chloride and mineral solution, urea and mineral solution on biomass production when all other

parametres at optimum are presented in Fig. 19 A, B, C, D, E and F. Based on the regression equation (Eq. 7), the optimum concentration of the ingredients were mannitol: 20 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5 g l^{-1}, urea :3.3 g $l^{-1}$and mineral salts solution 20 ml l^{-1}.

The interaction between nutrients and their effect on antagonistic compound production were also studied (Fig.20). Effect of interaction of varying concentrations of ingredients as listed above on antagonistic compound production when all other parametres kept optimum are presented in Fig. 20 A, B, C and D. The optimum concentrations of the ingredients for maximum antagonestic compound production from the regression equation (Eq. 8) were mannitol: 2 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5.1 g l^{-1}, urea :3.6 g $l^{-1}$and mineral salts solution 20 ml l^{-1}.

2.3.4.3 Experimental verification of biomass and antagonistic compound in newly designed media for *Pseudomonas* MCCB 103

The validation was carried out in shake flasks under optimum conditions predicted by the model. The experimental values for biomass (1.44 ± 0.05 g l^{-1}) and antagonistic compound (101.37 ± 2.76 mg l^{-1}) were closer to the predicted values (biomass: 1.4 g l^{-1} and antagonistic compound: 100.32 mg l^{-1}), validating the model. More over, at the above concentrations; biomass could be increased by 19% (1.21 ± 0.04 to 1.44 ± 0.05 g l^{-1}) with five-fold increase of antagonistic compound (20 ± 2.23 to 101.37 ± 2.76 mg l^{-1}).

2.4. Discussion

The results showed that culture conditions such as sodium chloride content, pH and temperature had an important role in the microbial biomass production and antagonistic activity of both *Pseudomonas* MCCB 102 and 103. A full factorial central composite design (CCD) of response surface methodology was used in the next step to maximize the biomass production

and antagonistic activity of the aquaculture probiotics. Subsequently the quadratic regression model was found to be highly significant for both biomass and antagonistic activity. The goodness of fit of the model was checked by coefficient of determination (R^2) that which gave a measure of how much variability in the observed response value could be explained by the experimental factors and their interactions, which was very high for both biomass and antagonistic activity in the case of *Pseudomonas* MCCB 102 and 103. Closer the value of R to one, the stronger the model and better will be the response predicted by the model (Wang and Lu 2004). For *Pseudomonas* MCCB 102 and 103 it was closer to one for both biomass and antagonistic activity. More over higher values of correlation coefficient (R) for both probiotic organisms indicated an excellent correlation between independent variables. Adequate precision was obtained for both biomass and antagonistic activity, which measured signal to noise ratio and the ratio grater than four was desirable (Wang and Lu 2004).

The 3D response surface and the 2D contour plots are the graphical representations of the regression equations (Elibol et al. 2004). Response surface and contour plots of the above mentioned models provided a method to visualize the relation between the response and experimental levels of each variable, and the type of interactions between the test variables (Sen and Swaminathan 1997). This methodology has been reported for optimizing the production of bacteriocins (Leal-Sanchez *et al.* 2002), enzymes (Beg *et al.* 2003), antibiotics (Adinarayana et al. 2003), polysaccharides (Wang and Lu 2004) organic acid (Xiong et al. 2005) etc. Shape of the contour plot, circular or elliptical, indicates whether the mutual interactions between the corresponding variables are significant or not. If it is circular the interaction between the variables are negligible and if it is elliptical the interaction between the variables are significant (Wang and Lu 2004). In the case of *Pseudomonas* MCCB 102, linear and quadratic effects of sodium chloride and

pH were significant but interaction effect between sodium chloride and pH was not significant. However, in the case of temperature linear, quadratic and all interaction effects were significant. Therefore temperature played an important role in biomass production. Meanwhile the model for antagonistic activity of *Pseudomonas* MCCB 102 implied that all the three factors ie, sodium chloride, pH and temperature had significant effect on antagonistic activity. Biomass was found to be increasing with sodium chloride concentration up to 12.5 (g l^{-1}) and activity up to 12.9 g l^{-1}after wards it decreased. Both biomass production and antagonistic activity were high at low temperature (25°C), which decreased with increasing temperature. A slightly acidic pH was preferred for both biomass production and antagonistic activity. However, the antagonistic compound was slightly alkaline in nature (Vijayan et al., 2006). Optimum requirements for both biomass production and antagonistic activity showed more or less similar values and, that might be due to the influence of biomass on antagonistic activity. Optimum values of sodium chloride concentration, pH and temperature were found to be with in the range and the experimental verification at the optimum increased biomass production by 8.4% and activity by 25.6%.

In the case of *Pseudomonas* MCCB 103 also, temperature played an important role in biomass production, linear, quadratic and all interaction effects of temperature were found to be significant. Since linear, quadratic and all interaction effects of sodium chloride were found to be significant for antagonistic activity of *Pseudomonas* MCCB 103, sodium chloride had important role on antagonistic activity. However, quadratic effect of pH and interaction between pH and temperature were not significant. It was observed that the optimum of each variable was with in the range ie. 5.01 g l^{-1} for sodium chloride, pH 7 and temperature 25 °C for biomass and sodium chloride 5 g l^{-1}(5 % w/v), pH 7 and temperature 25.32 °C for antagonistic activity of *Pseudomonas* MCCB 103. Incidentally, optimum requirements for both

biomass production and antagonistic activity showed more or less similar values and, that might be due to the possible influence of biomass on antagonistic activity. The response of growth (biomass) showed similar pattern with the response surface of the antagonistic activity for *Pseudomonas* 103 indicating a strong correlation between the biomass production and antagonistic activity (Liong and Shah 2005).

Subsequently the model was successfully validated by conducting experiments at the optimum conditions obtained from the model it self for maximizing the biomass and antagonistic activity. Biomass was increased by 13.26 % and antagonistic activity by 31.75 % for *Pseudomonas* MCCB 103 at the optimum conditions predicted from the model proving response surface methodology as power full tool for optimization of growth conditions.

Different nutritional factors such as carbon sources, nitrogen sources and growth factors including aminoacids and vitamins were screened initially in 'one-variable-at-a-time' approach for both *Pseudomonas* MCCB 102 and 103. Selected ingredients were further optimized by the most popularly used design; central composite design (CCD) of response surface methodology for maximizing the biomass and antagonistic compound production. The ANOVA of the quadratic regression models of *Pseudomonas* MCCB 102 and 103 demonstrates that the models were highly significant and the 'lack of fit 'values were insignificant for both biomass and the antagonistic compound production, and the goodness of fit of the models for both probiotics were checked by coefficient of determination (R^2). A higher value of correlation coefficient (R) for both *Pseudomonas* MCCB 102 and 103 indicates a good agreement between experimental and predicted values of both biomass and antagonistic compound production and thus suggesting a high significance of the model (Huang et al. 2006). More over closer the R value to one, the

stronger the model and better will be the response predicted by the model (Wang and Lu 2004).

In the case of *Pseudomonas* MCCB 102, a linear effect was observed on biomass, which increased with increasing concentration of glucose up to 19.8 g l^{-1} sodium chloride up to 14.5 g l^{-1}, urea up to 2.4 g l^{-1}, and mineral solution up to 15.5 ml $l^{-1}$when all other parametres were at optimum. Same pattern was observed on antagonistic compound production, which increased with increasing concentration of urea up to 2.1 g l^{-1} and mineral salt solution up to 17.4 ml l^{-1}.

It was clear from the regression equation (equation-5) that glucose was more essential for biomass production of *Pseudomonas* MCCB 102 than glycerol. However, antagonistic compound production was maximum at low concentration of glucose (2 g l^{-1}) and decreased with increasing concentration of glucose irrespective of the presence of sodium chloride, urea and mineral salt solution. Glycerol concentration had an important role in antagonistic compound production, which increased with increasing concentration of glycerol up to 20 g l^{-1}. Easley metabolizable substances provided maximum cell density but reduced the production of many secondary metabolites (Marwick et al. 1999) that might be true in the case of glucose, which had not much influence on antagonistic compound production. However, glycerol played an important role in antagonistic compound production and the same observation was reported in the case of antibiotics produced by *Streptomyces* (Kaiser et al. 1994).

Sodium chloride played an important role in antagonistic compound production than biomass production of *Pseudomonas* MCCB 102. Linear, quadratic and interaction effects (sodium chloride vs glucose and sodium chloride vs urea) were found to be significant for antagonistic compound

production. Even though there was no significant linear effect on biomass production quadratic and interaction effects (sodium chloride vs glucose, sodium chloride vs glycerol and sodium chloride vs mineral salts solution) were significant. Optimum concentration of sodium chloride for maximum growth of *Pseudomonas* MCCB 102 (biomass production) was 14.5 g l^{-1}, a quite possible observation as it was isolated from brackish water (Vijayan et al. 2006) but for the antagonistic compound the organism preferred low salinity (5 g l^{-1}) and decreased with increasing concentration of sodium chloride at optimum concentration of other nutrients.

In the case of *Pseudomonas* MCCB 103, composition of the medium obtained from the model for biomass production contained mannitol: 20 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5 g l^{-1}, urea :3.3 g $l^{-1}$and mineral salts solution 20 ml l^{-1} and the one for antagonistsic compound production contained mannitol: 2 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5.1 g l^{-1}, urea :3.6 g $l^{-1}$and mineral salts solution 20 ml l^{-1}. In the medium for biomass production the linear and quadratic effects of mannitol, urea and mineral salt solution were more significant than the other factors. This suggested that mannitol, urea and mineral salt solution had direct relationship with biomass production. Liong and Shah (2005) reported better growth of a probiotic strain of *Lactobacillus* in the presence of mannitol and subsequent increase for cholestrol removal. Sodium chloride showed a linear effect on biomass production and this observation was in accordance with the previous study (Vijayan et al. 2006). Even though the linear coefficient of glycerol in the medium for biomass production was not significant it showed significant quadratic effect and interaction effect on sodium chloride concentration and mineral salt solution. This justifies inclusion of glycerol in the medium for biomass production.

In the medium designed for the antagonistic phenazine compound (pyocyanin) production of *Pseudomonas* MCCB 103; ingredients such as sodium chloride, urea and mineral salts solution were significant at their linear and quadratic level. This implies that they can act as limiting factors and minor variations in their concentration may alter the rate of product formation. In the case of carbon sources, glycerol showed significant effect (both linear and quadratic) on the antagonistic compound production. Even though mannitol failed to show any direct effect on the antagonistic compound production, its interaction effect with urea and mineral salts solution were significant. Moreover, it played an important role in biomass production and there by enhanced antagonistic secondary metabolite production as the latter was growth dependent (Marwick et al. 1999). These observations justified the inclusion of mannitol in the medium for the antagonistic compound production. Both biomass and antagonistic compound production of *Pseudomonas* MCCB 103 was maximum at low concentration of sodium chloride (5g l^{-1}) exhibiting a negative correlation. This observation was strengthened by the argument of Vijayan et al. (2006), who reported a brackish water isolate of *Pseudomonas* PS-102, showing maximum antagonistic activity in ZoBell's marine broth with 5 g l^{-1} sodium chloride.

Response surface plots are the graphical representations of regression equations (Wang and Lu 2004). Accordingly response surface plots of the above models provided a method to visualize the interaction of nutrients and the optimum concentration of each nutrient required for maximum biomass and antagonistic compound production. In the present study, response surface of biomass of *Pseudomonas* MCCB 103 showed similar pattern with the response surface of antagonistic compound indicating a strong correlation between the biomass and antagonistic compound production (Liong and Shah 2005).

To our knowledge there are no reports of accomplishment of optimization of the production of aquaculture probiotics using RSM. The methodology used was efficient and could improve product yield and reduce time, cost etc. More over validation of the experiments showed the experimental values very close to the predicted ones for maximum biomass and antagonistic compound production, there by validating the models for both *Pseudomonas* MCCB 102 and 103.

Table 1 Factor levels used for regression analysis and their significance in the screening experiment of *Pseudomonas* MCCB 102

Sodium Chloride concentration ($g\ l^{-1}$)	Significance F	pH	Significance F	Temperature (^0C)	Significance F
0	0.933	6	0.029	25	0.0058
5	0.007	6.5	0.025	30	0.033
10	0.017	7	0.011	35	0.036
15	0.012	7.5	0.0094	40	0.935
20	0.025	8	0.0058		
25	0.0681				

Table 2 Central composite design matrix of the three variables along with the experimental and predicted values of biomass and antagonistic activity *Pseudomonas* MCCB 102

	Sodium Chloride ($g\ l^{-1}$)	pH	Temper-ature (°C)	Biomass ($g\ l^{-1}$)		Activity (Diameter of inhibition zone in mm)	
				Experim-ental	Predicted	Experim-ental	Predicted
1	5	6	25	1.12	1.11	12.66	13.03
2	20	6	25	1.1	1.10	15.33	15.07
3	5	8	25	1.02	1.02	16	16.55
4	20	8	25	1.02	1.02	10	8.92
5	5	6	35	1	1.00	0	1.46
6	20	6	35	1.03	1.03	0	0.17
7	5	8	35	1.12	1.12	10.66	11.3
8	20	8	35	1.14	1.15	0	0.0025
9	0	7	30	0.98	0.99	10.66	9.03
10	25.11	7	30	1.01	1.0	0	1.08
11	12.5	5.32	30	1.09	1.09	12.66	12.01
12	12.5	8.68	30	1.12	1.12	15	15.12
13	12.5	7	21.59	1.1	1.11	15.83	16.27
14	12.5	7	38.41	1.14	1.13	0	0.96
15	12.5	7	30	1.16	1.16	15.33	16.33
16	12.5	7	30	1.16	1.16	17.22	16.33
17	12.5	7	30	1.16	1.16	16	16.33
18	12.5	7	30	1.17	1.16	16.66	16.33
19	12.5	7	30	1.16	1.16	16.66	16.33
20	12.5	7	30	1.15	1.16	16	16.33

Table 3 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass of *Pseudomonas* MCCB 102

Source	SS	DF	MS	F-value	Probability P>F
Model	0.076	9	0.0084	123.14	< 0.0001
Residual (error)	0.00068	10	0.000068		
Lack of fit	0.00048	5	0.000097		
Pure error	0.0002	5	0.00004		
Cor total	0.076	19			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=0.75 %, R^2=0.9911,R=0.9955

Table 4 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic activity of *Pseudomonas* MCCB 102

Source	SS	DF	MS	F-value	Probability P>F
Model	853.95	9	94.88	80.24	< 0.0001
Residual (error)	11.82	10	1.18		
Lack of fit	9.6	5	1.92	4.31	0.0673
Pure error	2.23	5	0.45		
Cor total	865.77	19			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=10.04 %, R^2=0.9863, R=0.9931

Table 5 Experimental verification of identified optimum from the model of *Pseudomonas* MCCB 102

Variable	Levels before Optimiz-ation	Levels after optimization		Biomass (g/L)		Activity (Diameter of halo zone in mm)	
		For biomass	For activity	Initial	After Optimization	Initial	After Optimization
Sodium chloride (gl^{-1})	30	12.24	12.9	1.07 ±0.04	1.16 ±0.01	13.25 ±1	16.64 ±0.5
pH	7.2±0.5	6	6.5				
Temperat-ure (°C)	28	25	25				

Table 7 Central composite design matrixes of the three variables along with the experimental and predicted values of biomass and antagonistic activity of *Pseudomonas* MCCB 103

	Sodium Chloride ($g\ l^{-1}$)	pH	Temper-ature (°C)	Biomass ($g\ l^{-1}$)		Activity (diameter of inhibition zone in mm)	
				Experim-ental	Predicted	Experim-ental	Predicted
1	5	6	25	1.03	1.01	12.67	12.13
2	20	6	25	0.94	0.92	14	15.35
3	5	7	25	1.14	1.13	14.2	14.67
4	20	7	25	1.05	1.04	10	10.88
5	5	6	35	0.98	0.99	10.5	10.37
6	20	6	35	0.99	0.99	8.67	8.95
7	5	7	35	0.92	0.93	10.33	9.72
8	20	7	35	0.93	0.94	0	1.29
9	0	6.5	30	1.08	1.08	13.67	14.53
10	25.11	6.5	30	1	1.01	12	10.10
11	12.5	5.66	30	0.89	0.90	17.67	17.46
12	12.5	7.34	30	0.96	0.96	14	13.15
13	12.5	6.5	21.59	1	1.03	10.33	9.41
14	12.5	6.5	38.41	0.95	0.93	0	-0.134
15	12.5	6.5	30	0.84	0.86	16	16.08
16	12.5	6.5	30	0.85	0.86	17.67	16.08
17	12.5	6.5	30	0.89	0.86	16	16.08
18	12.5	6.5	30	0.9	0.86	15.67	16.08
19	12.5	6.5	30	0.86	0.86	15.67	16.08
20	12.5	6.5	30	0.85	0.86	15.33	16.08

Table 8 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass of *Pseudomonas* MCCB 103

Source	SS	DF	MS	F-value	Probability P>F
Model	0.12	9	0.014	22.24	< 0.0001
Residual (error)	0.0061	10	0.00061		
Lack of fit	0.00315	5	0.00063	1.07	0.1472
Pure error	0.0029	5	0.00059		
Cor total	0.13	19			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=2.59 %, R^2=0.9524,R=0.9759

Table 9 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic activity of *Pseudomonas* MCCB 103

Source	SS	DF	MS	F-value	Probability P>F
Model	445.7	9	49.52	33.78	< 0.0001
Residual (error)	14.66	10	1.47		
Lack of fit	11.23	5	2.25	3.27	0.1099
Pure error	3.44	5	0.69		
Cor total	460.36	19			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=9.91 %, R^2=0.9682, R=0.984

Table 10 Experimental verification of identified optimum from the model of *Pseudomonas* MCCB 103

Variable	Levels before optimizat-ion	Levels after optimization		Biomass ($g\ l^{-1}$) (Cell Dry Mass, CDM)		Activity (Diameter of halo zone in mm)	
		For biomass	For activity	Initial	After Optimization	Initial	After Optimization
Sodium chloride (gl^{-1})	30	5.01	5	1.15 ±0.17	1.32 ±0.06	9.33 ±0.58	13.67 ±0.58
pH	7.2±0.5	7	7				
Temper-ature (°C)	28	25	25.32				

Table 12 CCD matrix of the variables (g l^{-1}) along with the experimental and predicted values of biomass and antagonistic activity of *Pseudomonas* MCCB 102

Expt. No.	Glucose	Glyce -rol	Sodium chloride	Urea	Mineral salts solution	Biomass (mg l^{-1})		Antagonistic compound (mg l^{-1})	
						Experim -ental	Predi -cted	Experimental	Predicted
1	0.2	0.2	0.5	0.1	0.5	0.45	0.41	60.3	58.95
2	2	0.2	0.5	0.1	0.5	1.18	1.09	7.65	4.06
3	0.2	2	0.5	0.1	0.5	0.63	0.63	60.63	69.68
4	2	2	0.5	0.1	0.5	0.85	0.89	10.24	8.12
5	0.2	0.2	1.5	0.1	0.5	0.5	0.42	52.04	48.99
6	2	0.2	1.5	0.1	0.5	1.49	1.46	23.35	19.99
7	0.2	2	1.5	0.1	0.5	0.6	0.54	49.17	46.6
8	2	2	1.5	0.1	0.5	1.25	1.16	9.96	10.94
9	0.2	0.2	0.5	0.4	0.5	0.64	0.74	30.61	34.7
10	2	0.2	0.5	0.4	0.5	1.34	1.34	20.27	19.91
11	0.2	2	0.5	0.4	0.5	1.31	1.22	34.82	38.27
12	2	2	0.5	0.4	0.5	1.5	1.4	18.96	16.81
13	0.2	0.2	1.5	0.4	0.5	0.63	0.54	21.95	24.53
14	2	0.2	1.5	0.4	0.5	1.42	1.49	32.51	35.65
15	0.2	2	1.5	0.4	0.5	0.87	0.93	14.27	14.99
16	2	2	1.5	0.4	0.5	1.51	1.46	13.95	19.43
17	0.2	0.2	0.5	0.1	2	1.22	1.1	69.74	71.91
18	2	0.2	0.5	0.1	2	1.59	1.63	3.48	5.87
19	0.2	2	0.5	0.1	2	1.29	1.29	108.08	96.33
20	2	2	0.5	0.1	2	1.46	1.39	23.41	23.61
21	0.2	0.2	1.5	0.1	2	0.93	1.07	44.6	47.41
22	2	0.2	1.5	0.1	2	1.99	1.95	17.21	7.27
23	0.2	2	1.5	0.1	2	1.24	1.15	53.11	58.71
24	2	2	1.5	0.1	2	1.68	1.61	10.45	11.89
25	0.2	0.2	0.5	0.4	2	1.3	1.37	56.96	53.03
26	2	0.2	0.5	0.4	2	1.82	1.81	27.41	27.09
27	0.2	2	0.5	0.4	2	1.84	1.83	58.09	70.29
28	2	2	0.5	0.4	2	1.8	1.84	32.71	37.68
29	0.2	0.2	1.5	0.4	2	1.17	1.12	26.63	28.33
30	2	0.2	1.5	0.4	2	1.98	1.92	26.15	28.29
31	0.2	2	1.5	0.4	2	1.49	1.48	29.43	32.48
32	2	2	1.5	0.4	2	1.8	1.85	29.71	25.77
33	0	1.1	1	0.25	1.25	0.64	0.68	140.06	132.05
34	3.24	1.1	1	0.25	1.25	1.87	1.94	54.27	58.79
35	1.1	0	1	0.25	1.25	1.71	1.69	3.86	8.31
36	1.1	3.24	1	0.25	1.25	1.75	1.87	26.02	18.07
37	1.1	1.1	0	0.25	1.25	1.73	1.75	47.19	44.14
38	1.1	1.1	2.19	0.25	1.25	1.67	1.77	18.57	18.12
39	1.1	1.1	1	0	1.25	0	0.15	0	7.91
40	1.1	1.1	1	0.61	1.25	0.86	0.82	6.97	-4.44
41	1.1	1.1	1	0.25	0	0	0.11	0	-2.2
42	1.1	1.1	1	0.25	3.03	1.4	1.4	22.03	20.74
43	1.1	1.1	1	0.25	1.25	1.35	1.53	74.98	65.9
44	1.1	1.1	1	0.25	1.25	1.5	1.53	65.13	65.9
45	1.1	1.1	1	0.25	1.25	1.53	1.53	61.87	65.9
46	1.1	1.1	1	0.25	1.25	1.56	1.53	66.47	65.9
47	1.1	1.1	1	0.25	1.25	1.51	1.53	70.8	65.9
48	1.1	1.1	1	0.25	1.25	1.59	1.53	63.35	65.9
49	1.1	1.1	1	0.25	1.25	1.59	1.53	66.72	65.9
50	1.1	1.1	1	0.25	1.25	1.5	1.53	60.18	65.9

Table 13 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass of *Pseudomonas* MCCB 102

Source	SS	DF	MS	F-value	Probability P>F
Model	11.5	20	0.56	63.59	< 0.0001
Residual (error)	0.25	29	0.00877		
Lack of fit	0.21	22	0.00969	1.65	0.2593
Pure error	0.041	7	0.00588		
Cor total	11.41	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=7.24 %, R^2=0.9782,R=0.9890

Table 14 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic compound production of *Pseudomonas* MCCB 102

Source	SS	DF	MS	F-value	Probability P>F
Model	36967.23	20	1848.36	41.33	< 0.0001
Residual (error)	1297.01	29	44.72		
Lack of fit	1134.16	22	51.55	2.22	0.1518
Pure error	162.85	7	23.26		
Cor total	38264.24	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=17.11%, R^2=0.968, R=0.9838

Table 16 CCD matrix of the variables (g l^{-1}) along with the experimental and predicted values of biomass and antagonistic compound of *Pseudomonas* MCCB 103

Expt. No.	Mann -itol	Glyc -erol	Sodium chloride	Urea	Mineral salts solution	Biomass (cell dry mass, mg l^{-1})		Antagonistic compound (mg l^{-1})	
						Experimental	Predicted	Experimental	Predicted
1	2	2	5	1	5	0.24	0.21	20.16	16.66
2	20	2	5	1	5	0.3	0.3	22.16	22.26
3	2	2	5	1	5	0.24	0.22	27.03	23.53
4	20	2	5	1	5	0.29	0.29	28.93	25.84
5	2	2	15	1	5	0.28	0.29	14.87	12.47
6	20	2	15	1	5	0.29	0.27	10.01	6.61
7	2	20	15	1	5	0.26	0.24	14.83	11.4
8	20	20	15	1	5	0.2	0.19	5.07	2.26
9	2	2	5	4	5	0.44	0.45	33.6	36.97
10	20	2	5	4	5	0.56	0.57	51.32	54.58
11	2	20	5	4	5	0.43	0.44	40.06	46.12
12	20	20	5	4	5	0.5	0.53	56.3	60.45
13	2	2	15	4	5	0.5	0.5	28.18	32.33
14	20	2	15	4	5	0.5	0.51	34.36	38.49
15	2	20	15	4	5	0.42	0.44	31.4	33.54
16	20	20	15	4	5	0.42	0.42	30.83	36.41
17	2	2	5	1	20	1.09	1.09	86.45	81.5
18	20	2	5	1	20	1.28	1.25	76.23	71.86
19	2	20	5	1	20	1.18	1.16	95.59	94.34
20	20	20	5	1	20	1.3	1.29	85.6	81.42
21	2	2	15	1	20	1.08	1.04	68.86	66.48
22	20	2	15	1	20	1.1	1.08	50.36	45.38
23	2	20	15	1	20	1.08	1.05	78.65	71.37
24	20	20	15	1	20	1.08	1.07	47.94	47
25	2	2	5	4	20	1.16	1.16	80.65	83.94
26	20	2	5	4	20	1.34	1.34	80.52	86.32
27	2	20	5	4	20	1.2	1.21	98.4	99.06
28	20	20	5	4	20	1.38	1.36	94.6	98.15
29	2	2	15	4	20	1.1	1.08	67	68.47
30	20	2	15	4	20	1.13	1.15	55.86	59.39
31	2	20	15	4	20	1.08	1.08	70.61	75.65
32	20	20	15	4	20	1.11	1.12	62.07	63.28
33	0	11	10	2.5	12.5	0.98	1.01	64.19	65.85
34	32.4	11	10	2.5	12.5	1.18	1.18	60.35	57.8
35	11	0	10	2.5	12.5	0.82	0.84	43.91	43.21
36	11	32.4	10	2.5	12.5	0.8	0.81	56.2	56.01
37	11	11	0	2.5	12.5	0.8	0.81	67.74	66.08
38	11	11	21.9	2.5	12.5	0.6	0.62	18.85	19.62
39	11	11	10	0	12.5	0	0.091	0	22.62
40	11	11	10	6.1	12.5	0.5	0.44	89.66	66.14
41	11	11	10	2.5	0	0	-0.02	0	-3.94
42	11	11	10	2.5	30.3	1.81	1.86	102.08	105.13
43	11	11	10	2.5	12.5	0.74	0.75	61.25	65.53
44	11	11	10	2.5	12.5	0.75	0.75	63.58	65.53
45	11	11	10	2.5	12.5	0.73	0.75	62.21	65.53
46	11	11	10	2.5	12.5	0.78	0.75	74.09	65.53
47	11	11	10	2.5	12.5	0.74	0.75	75.87	65.53
48	11	11	10	2.5	12.5	0.76	0.75	63.37	65.53
49	11	11	10	2.5	12.5	0.72	0.75	63.64	65.53
50	11	11	10	2.5	12.5	0.76	0.75	60.84	65.53

Table 17 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass of *Pseudomonas* MCCB 103

Source	SS	DF	MS	F-value	Probability P>F
Model	8.05	20	0.4	390.48	< 0.0001
Residual (error)	0.03	29	0.00103		
Lack of fit	0.027	22	0.00124	3.41	0.0505
Pure error	0.00255	7	0.00036		
Cor total	8.08	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=4.22 %, R^2=0.9963,R=0.9981

Table 18 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic compound production of *Pseudomonas* MCCB 103

Source	SS	DF	MS	F-value	Probability P>F
Model	34924.36	20	1746.22	27.99	< 0.0001
Residual (error)	1809.13	29	62.38		
Lack of fit	1565.61	22	71.16	2.05	0.1676
Pure error	162.85	7	23.26		
Cor total	36733.5	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=14.76%, R^2=0.9507, R=0.9750

Chapter-3

Isolation, Purification and Characterization of Anti-vibrio Compounds from Pseudomonas MCCB 102 and Pseudomonas 103

Chapter 3

Isolation, Purification and Characterization of Anti-vibrio Compounds from *Pseudomonas* MCCB 102 and *Pseudomonas* 103

3.1 Introduction

Members of the genus *Pseudomonas* are common inhabitants of soil, fresh water and marine environments and are known to produce a wide range of secondary metabolites inhibiting a wide range of pathogenic bacteria (Raaijmakers et al. 1997). *P. fluorescens* and *P. aeruginosa* have been used widely in agriculture as microbial control agents, alternative to synthetic chemicals for combating plant diseases (Raaijmakers et al. 1997; Anjaiah et al. 1998; Kumar et al. 2005). Production of an antibiotic phenazine –1-carboxylic acid by fluorescent *Pseudomonas* sp. in the rhizosphere of wheat protected the plant from an important root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow et al. 1990). O'Sullivan and O'Gara (1992), in their review described different mechanisms by which fluorescent *Pseudomonas* spp. suppressed plant root pathogens including the production of different antibiotics. Bacteriocin produced by *Pseudomonas* sp. inhibited different human pathogens including *V. cholerae* (Hubert et al. 1998). An antifugal compound isolated from *Pseudomonas aeruginosa* K-187 inhibited thirty-six isolates of fungi including the plant pathogen *Fusarium oxysporum*. *P. fluorescens* Pf-5, a soil bacterium suppressed plant pathogens by the production of an antibiotic, pyoluteorin (Whistler et al. 2000). Novel α-Pyrone antibiotics isolated from *P. fluorescens* (Chu et al. 2002), and a marine *Pseudomonas* sp. F92S91 (Singh et al. 2003), were found to be effective against human pathogens. A detailed study of the genes involved in the production of plant lectin-like bacteriocin, from a rizosphere colonizing *Pseudomonas* was conducted by Parret et al. (2003). Different isolates of

Pseudomonas produce an array of secondary metabolites, including pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and hydrogen cyanide that inhibit plant pathogens (Brodhagen et al. 2004). *P. aeruginosa*, an n-alkane degrader frequently isolated from petroleum-contaminated sites inhibited other microbial flora by means of a secondary metabolite, pyocyanin (Norman et al. 2004). Kumar et al (2005) isolated an antifungal metabolite, phenazine –1-carboxamide (PCA) producing *P. fluorescens,* which showed antagonistic activity against many plant pathogens.

Antagonism is not uncommon in aquatic environment but the ecological importance and nature of the antagonistic compounds are not well investigated. A bulk of literature is available on the occurrence of bacterial strains showing *in vitro* antagonism towards known pathogens in aquaculture (Olsson 1998; Rengipat et al.1998; Robertson et al. 2000; Villamil et al. 2003; Jayaprakash et al. 2005). These antagonistic bacteria bring forth alternations in the composition of microbial flora by releasing chemical substances that have bactericidal or bacteriostatic effect (Chythanya et al. 2002; Jayaprakash et al. 2005) or by competition for nutrients or available energy (Gram et al.1999). Gram et al. (1999) observed inhibition of *V. anguillarum*, a fish pathogen by a siderophore producing probiotic, *P. fluorescens* AH2. Later, Chythanya et al. (2002), reported a heat stable, chloroform soluble, low molecular weight compound produced by *Pseudomonas* I-2, and it was found to be effective against shrimp pathogenic vibrios including *V. harveyi, V. fluvialis, V. parahaemolyticus, V. vulnificus* and *Photobacterium damselae.*

Jayaprakash (2005), and Vijayan et al. (2006), isolated *Pseudomonas* MCCB 103 and 102 respectively, both antagonistic to a range of vibrios such as *V. harveyi, V. alginolyticus, V. angulliarum, V. proteolyticus, V. fluviallis, V. parahaemolyticus, V. neris, V. vulnificus, V. mediterranei, V. cholerae* and *Aeromonas spp.* in shrimp and prawn culture systems. Subsequently the

phenomenon of antagonism became a subject of research of this group, as investigations on the nature of the inhibitory compounds and their role in eliminating pathogens were found inevitable for their application. This chapter deals with isolation of the antagonistic compound produced *in vitro* by *Pseudomonas* MCCB 102 and 103, their purification and structural elucidation.

3.2 Materials and Methods

3.2.1 Organisms and their culture

Pseudomonas MCCB 102 and *Pseudomonas* MCCB 103, previously described by Vjayan et al. (2006) and Jayaprakash (2005) were used for the production of the antivibrio molecule. These isolates form part of the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, India. Organisms were grown in ZoBell's marine broth 2216 E (sodium chloride: 12.9 g l^{-1}, pH: 6.5 and incubation temperature: 25 °C for *Pseudomonas* MCCB 102 and sodium chloride: 5 g l^{-1}, pH 7 and incubation temperature: 25 °C for *Pseudomonas* MCCB 103). Cultures of antagonistic bacteria (3-5 days old) were centrifuged at 10,000 g at 4 °C for twenty minutes. The supernatant was filtered through a series of filters including glass microfiber filter (GF/C, Whatman), cellulose acetate membrane (0.45 µm) and PVDF membrane (0.22 µm), and used for further analysis.

3.2.2 Heat stability and pH stability of cell-free supernatants

Heat sensitivity of the inhibitory substance was tested by heating the cell-free supernatant of *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 in a water bath for 30 min at 60 °C, 80 °C, 100 °C and autoclaving for 15 minutes at 121 °C. Each of the treated and untreated supernatant was tested for antagonistic activity against *V. harveyi* by disc diffusion method as mentioned earlier in the Chapter 2. pH stability of the inhibitory substance of

Pseudomonas MCCB 102 and *Pseudomonas* MCCB 103 was tested by adjusting pH of the filter sterilized supernatant to 2, 4, 6, 8 and 10 using 1 mol l^{-1} HCl and 1 mol l^{-1} NaOH, with the help of a pH meter and incubated for two hours. The pH was subsequently readjusted to 7 and tested the activity against *V. harveyi* as described above. Cell-free supernatant was alkaline and its pH was adjusted to 7 and used as control.

3.2.3 Bacteriostatic/cidal action of cell-free supernatants

V. harveyi (18-24 hours old) was harvested into 20 ppt sterile seawater and optical density adjusted spectrophotometrically to 0.1 at 600 nm. The cell suspension was serially diluted in sterile sea water (20 ppt) and determined the viable count on 20 ppt ZoBell's marine agar plates. Accordingly suspension of *V. harveyi* having 10^6 cells ml^{-1} (0.1 OD at 600 nm) were centrifuged (10 ml each) at 10,000 g for 20 minutes at 4 °C, supernatants discarded and cells resuspended in cell-free culture supernatants of *Pseudomonas* MCCB 102 and 103. Viability of *V. harveyi* was tested by inoculating an aliquot of 100 µl in fresh ZoBell's broth at 6 hour intervals and incubated for 96 hours.

3.2.4 Shelf life of cell-free supernatants

Cell-free supernatants were stored at room temperature (28 ± 0.5°C), 4 °C and –20 °C for 12-14 months and tested for antagonistic activity against *V. harveyi* at regular intervals by the disc diffusion assay as mentioned earlier in the Chapter 2.

3.2.5 Extraction of anti-vibrio molecule

Solvent extraction was done using water-immiscible solvents such as 1-butanol, ethyl acetate, dichloromethane, chloroform and hexane. Cell-free supernatant of *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 were mixed with the solvents in 1:1 ratio using cyclomixer for 10 minutes. After mixing properly, the solvent layer was pipetted out and 20 µl of the solvent fraction was impregnated on sterile filter paper disc and allowed to dry for 30

minutes. The process was repeated to get 40 µl extract impregnated per disc and dried in vacuum desiccator for 24 hours. Control discs were kept with solvents alone. The discs were placed on ZoBell's marine agar plates seeded with *V. harveyi*. Diameters of the halo zones were measured after 18-24 hours incubation as described earlier in the Chapter 2. The culture supernatant left over after solvent extraction was also impregnated in discs and checked for the antagonistic activity. During the process of solvent extraction certain fractions turned blue and they alone exhibited anti-vibrio property. Based on previous reports on the pigments isolated from *Pseudomonas* sp. and the color of the supernatants, we assumed that the blue pigments of the culture under study might be phenazine derivatives (Fernandez and Pizarro 1997). Hence, the solvent extraction protocol for the specific separation of phenazines given by Chang and Blackwood (1969) was implemented. Accordingly the supernatant was acidified to pH 2 using 4 mol l^{-1} HCl and mixed with chloroform in a 1:1 ratio using cyclomixer for 10 minutes. Subsequently the chloroform layer was pipetted out and readjusted the pH of the supernatant to neutral using 1 mol l^{-1} $NaHCO_3$ and re-extracted with chloroform. Antivibrio property of choroform fractions extracted at acidic and neutral pH of the supernatant and, that of cell free supernatant after chloroform extractions were determined as described above.

3.2.6 Thin layer chromatographic separation of the anti-vibrio compound

Filter sterilized supernatant was concentrated by vacuum rotary evaporation and was spotted on TLC plates (silica gel G) having 0.2 mm thickness. Different solvent systems used for separation of fractions were: (a) 4 1-butanol : 1 acetic acid: 1 water (Wright 1998), (b) 1 chloroform: 1 methanol (Knight et al. 1979) and (c) 3 ethyl acetate: 2 acetic acid: 1 water (Knight et al. 1979).

For testing the anti-vibrio property, different spots from the TLC plates were scraped off and dissolved in double-distilled water and centrifuged at 10,000 g for 15 minutes at 4 °C. Antibacterial activity of each fraction was tested by disc diffusion method as mentioned earlier in the Chapter 2. Corresponding Rf value of the active spots were measured for each solvent system. Parallel TLC plates were run and the regions corresponding to the active spot were observed under UV transilluminator, sprayed with reagents such as ninhydrin for bacteriocin (Knight 1979), phenolite and iron reagent for siderophores (Cox and Graham 1979) and kept in iodine vapour for the detection of non-specific bioactive spots. (Austin and Billaud 1990).

3.2.7 Purification of anti-vibrio compound by thin layer chromatography and solubility of the active compound in different solvents

Broth cultures of the antagonistic bacteria (3-5 days old) were centrifuged at 10,000 g at 4° C for twenty minutes. Supernatant was passed through a series of filters mentioned above and pH of the supernatant adjusted to 7 and mixed with chloroform in a separating funnel. Chloroform layer was collected and concentrated by vacuum rotary evaporation at 40°C. Concentrated solution was dried over silica in a vacuum desiccator overnight at room temperature, re-dissolved in chloroform and applied on TLC plates. Preparative TLC plates (0.5 mm thickness), with silica gel G as sorbent were used for primary purification. Solubility of the bioactive compound was tested in different solvents (water, methanol, ethanol, 1-propanol and acetone) and antagonistic activity tested subsequently following the disc diffusion method mentioned earlier in the Chapter 2.

3.2.8 HPLC analysis of anti-vibrio compound

The anti-vibrio compounds obtained from *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 purified on silica gel G were analyzed by HPLC according to a method previously described by Fernandez and Pizarro

(1997). Analysis was carried out on a Merck-Hitachi L6200 instrument fitted with 250 × 4.6 mm ODS column and a UV detector tuned at 280 nm with the help of Multi SHM manager software. A gradient method was used for eluting samples employing solvent system A and B. Solvent A was water - trifluroacetic acid (100:0.04, v/v) and solvent B acetonitrile - water - trifluoroacetic acid (90:10:0.04, v/v/v). Authentic pyocyanin (supplied by Colour Your Enzymes, Ontario, Canada), a phenazine compound produced by *Pseudomonas* was used as the standard since the activity of the chloroform extracted compound was at neutral pH and the active spots appeared were blue on thin layer chromatography plates, both being the characteristic properties of pyocyanin (Chang and Blackwood 1969; Knight et al. 1979).

3.2.9 GC-MS and LC-MS analysis of anti-vibrio compound

Gas chromatography/mass spectrometric analysis of anti-vibrio compound from *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 and authentic sample of pyocyanin obtained from Color Your Enzymes, Ontario, Canada was done on a Varion 1200 gas chromatography/quadrupole mass spectrometer. LC-MS analysis of these samples was carried out on a Esquire 3000 plus ESI (Bruker Daltonics) with Agilent 1100 series HPLC.

3.2.10 UV- visible -NIR spectra of purified compound in different solvents

The anti-vibrio compound extracted and purified by the method described earlier was subjected to spectrophotometric analysis. Ultraviolet and visible absorption spectra were recorded on a Shimadzu UV-102 spectrophotometer in different solvents (chloroform, methanol, dichloromethane, and 0.1 M HCl) as described by Fernandez and Pizarro (1997) for phenazines from *Pseudomonas*. Absorption spectra were recorded on a Varian Cary 500 UV-VIS-NIR spectrophotometer.

3.2.11 ^{1}H NMR spectral analysis

^{1}H NMR spectra of the anti-vibrio compounds obtained from *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 were recorded on a Bruker AMX 400 High Resolution Multinuclear FT-NMR Spectrometer operating at 400 MHz. $CDCl_3$ was used as the solvent and tetramethylsilane (TMS) was used as the internal standard.

3.2.12 *In vivo* application of anti-vibrio compound

Concentration of antagonistic compound were determined as described earlier in the chapter 2 and accordingly different concentrations of the anti-vibrio compound (100 ppm, 80 ppm, 60 ppm, 40 ppm, 20 ppm, 15 ppm, 10 ppm and 5 ppm) were prepared in sterile 15 ppt seawater. *V. harveyi* (18 hours old) was introduced into the preparations in such a way that final count of *V. harveyi* was equal to 10^6 m l^{-1}. The preparations were incubated at 28 ^{0}C for 48 hours. Aliquots (100 µl each) from each concentration at 6-hour intervals were withdrawn and inoculated into fresh medium (100 ml) and incubated at 28 ^{0}C. Presence/absence of growth in the fresh medium inoculated from different preparations was recorded.

3.3 Results

3.3.1 Heat stability and pH stability of cell-free supernatants

Response of cell-free supernatants of *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 in terms of antagonistic activity on *V. harveyi* at elevated temperatures is presented in Table 1.

There was no significant difference ($P > 0.05$) in antagonistic activity between the control and the supernatants exposed to 60 °C, 80 °C and 100 °C. However, a significant reduction in activity ($P < 0.05$) was obtained after autoclaving for 15 min at 121 °C. On exposing the culture supernatant to pH

2, 4, 6, 8 and 10, the antagonistic activity remained unaltered compared to that of the control (Table 2).

3.3.2 Bacteriostatic/cidal action of cell-free supernatants

Anti-vibrio compound produced by *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 was bactericidal in nature.

3.3.3 Shelf life of cell-free supernatants

On storage at –20 and – 4 °C the anti-vibrio activity of the culture supernatant remained for 12 months after which a slow decrease in activity was observed. On the other hand, activity was lost on storage at room temperature after four months (Fig.1).

3.3.4 Extractability in organic solvents

On extracting cell-free supernatants of *Pseudomonas* MCCB 102 and 103 with five organic solvents, antagonistic activity was observed in 1-butanol, dichloromethane and chloroform and no activity in ethyl acetate and hexane fractions as determined in terms of halo zone on the target pathogen *V. harveyi* (Table 3). Solvent extraction protocol for phenazine compounds of *Pseudomonas* given by Chang and Blackwood (1969) was followed and no antibacterial property was observed in chloroform layer when the supernatant at acidic pH. However, antibacterial property was detected in chloroform when extracted at neutral pH. The supernatant at neutral pH after chloroform extraction did not show any antibacterial property.

3.3.5 Thin layer chromatographic separation of the anti-vibrio compound and its solubility in different solvents

The blue coloured spots (dark pink in acidic condition) appeared on TLC plates alone showed anti-vibrio property (Fig. 2). In solvent system A {Butanol:Aceticacid :Water (4:1:1, v/v/v)] and C (ethyl acetate: acetic acid: water, (3:2:1, v/v/v) the active compounds appeared dark pink/purple, and

after drying they turned blue. Meanwhile in solvent system B [chloroform: methanol (1:1, v/v), it appeared crescent shaped narrow blue coloured band. Rf value of the anti-vibrio compound in different solvent systems is given in Table 4. In ninhydrin, bioactive spots remained dark (no reaction) and on exposure to iodine vapour, it appeared dark brown. Under UV illumination, the active spots remained nonfluorescent. Iron reagent and phenolite spray did not give any reaction. Active fractions were soluble in water, acetone, methanol, ethanol and 1-propanol.

3.3.6 HPLC Analysis of the anti-vibrio compound

The retention time (RT value) of anti-vibrio compound from *Pseudomonas* MCCB 102 was 31.6 min. and that of *Pseudomonas* MCCB 103 was 31.4 min (Fig.3). This was identical (within acceptable limits) with the RT of the pyocyanin standard (30.8 min.).

3.3.7 GC-MS and LC-MS analysis of the anti-vibrio compound

GC-MS analysis of the active compounds did not yield dependable data. We ascribe this to the low volatility/stability of the compound under the conditions employed for the analysis. Our findings are consistent with those reported by Watson et al. (1986). However, LC-MS analysis of the samples was particularly useful. In LC-MS analysis, the active compound from *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 and pyocyanin standard exhibited identical retention time (12.5 minutes) indicating that the similarity of the three compounds are identical. Further analysis of this peak by mass spectroscopy demonstrated a protonated molecular ion at *m/z* 211 (Fig. 4 and 5) confirming that pyocyanin (MW = 210) was the active component isolated from *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103.

3.3.8 UV- Visible spectra of purified compound in different solvents

Absorption maxima of anti-vibrio compound from both *Pseudomonas* MCCB 102 and 103 in solvents such as chloroform, 0.1 N HCl, methanol and dichloromethane are given in Table 5. From this data, it is further confirmed that the anti-vibrio compounds present in the supernatants of both organisms were same. The absorption spectrum of pyocyanine recorded in solvents such as chloroform and dichloromethane remained relatively unchanged. However, protonated pyocyanin (recorded in 0.1 mol l^{-1} HCl) exhibited a large blue shift (170 nm). On the other hand a substantial red shift (23 nm) was observed when the spectrum was recorded in a polar protic solvent such as methanol. These results were consistent with a zwitterionic structure in resonance with a semiquininiod structure for pyocyanin. It appeared that the zwitterionic form was more predominanet in polar protic solvents where it was further stabilized by hydrogen bonding. The absorption spectral data observed by us were consistent with that of pyocyanine described by Fernandez and Pizarro (1997).

3.3.9 NMR analysis of anti-vibrio compound

The structure of the anti-vibrio metabolite was further confirmed by nuclear magnetic resonance (NMR) spectroscopy. In the ^{1}H NMR spectrum (Fig. 6), peaks observed in the aromatic region (δ 7-9) exhibited a good correspondence with those reported in literature (Rao and Sureshkumar, 2000). The methyl protons appeared as a broad peak at δ 3.9. Based on these data, we concluded that the active ingredient present in the extracts from *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 was pyocyanin itself (Fig. 7).

3.3.10 *In vivo* application of anti-vibrio compound

The cidal effect of the anti-vibrio compound (pyocyanin) was studied over a period of 48 hours by exposing *V. harveyi* at a cell density of 10^{6} cells ml^{-1} to different concentrations. At 100 and 80 ppm it required 6

hours for total inactivation of the pathogen, 18 hours at 60, 40 and 20 ppm and 36 hours at 15 ppm. However, at 10 ppm and below the bacterial property was not exhibited during the exposure period of 48 hours (Table 6).

3.4 Discussion

In vitro antagonism of *Pseudomonas fluorescens* against *V. anguillarum* (Gram et al., 1999) and *Pseudomonas* I-2 against *V. harveyi* (Chythanya et al. 2002) and *Pseudomonas* MCCB 102 and 103 against a range of vibrios (Vijayan et al. 2006, Jayaprakash 2005) point to the possibility of using the molecule responsible for the antagonistic properties as viable alternatives to the conventional antibiotics. However, a detailed investigation of the nature of the anti-vibrio compounds produced by *Pseudomonas* has not been carried out. In general the antibacterial property of the antagonistic bacteria is preferably due to the production of antibiotics (Raaijmakers et al. 1997), bacteriocins (Parret et al. 2003), siderophores (Gram et al. 1999), lysozymes, proteases, hydrogen peroxide and/or alteration of pH (Sugita et al., 1996). Vijayan et al. (2006) and Jayaprakash (2005) observed that *Pseudomonas* MCCB102 and *Pseudomonas* MCCB 103 secreted antagonistic compounds into the growth media and in the present study the filter-sterilized supernatants were with heat stable pH insensitive antagonistic activity. This indicated that the active material was not a protein. The cell free supernatants were alkaline in nature; nevertheless the antagonistic activity was not due to alteration of pH (Sugita et al. 1996) as it was tested after neutralization.

The anti-vibrio compounds produced by *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 were bactericidal in nature. The active fraction appeared in blue colour (dark pink in acidic condition) in chloroform, n-butanol and dichloromethane. This suggested that the activity might be due to the blue-green phenazine compound, pyocyanin (1-hydroxy-5-N-methylphenazine) produced by *Pseudomonas* (Chang and Blackwood 1969;

Baron and Rowe 1981; Fernandez and Pizarro 1997; Norman et al. 2004). This led to the implementation of the solvent extraction protocol for phenazine pigments given by Chang and Blackwood (1969). It was observed that the antibacterial property was absent in the chloroform layer at acidic pH giving the strong indication that the activity was not due to Phenazine-1-carboxylic acid and/or Oxychlororaphine. At neutral pH the activity was obtained in chloroform layer but the supernatant after chloroform extraction showed no antibacterial property. These observations led to the assumption that the anti-vibrio compound might be pyocyanin.

It has to be pointed out that only blue coloured spots obtained after thin layer chromatography (TLC) of the concentrated supernatants of the *Pseudomonas* cultures were active and the other spots were with out antibacterial property. The inhibitory fraction was negative to ninhydrin confirming it not a bacteriocin. In the previous study it was observed that *Pseudomonas* MCCB102 produced siderophores (Vijayan et al, 2006). But, siderophores generally appear fluorescent under UV light, turn red with iron spray reagent and attain deep blue with the phenolate spray reagent (Cox and Gram, 1979). Contrary to it in the present study the active spots were non-fluorescent and negative to both iron spray reagent and phenolate spray reagents. In silica gel G -TLC, Rf value of the active spots obtained using solvent system B (1Chloroform: 1methanol,vol/vol), for *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 culture supernatants were 0.57±0.04 and 0.61 ±0.05 respectively and that of solvent system C (3 Ethyl acetate: 2Acetic acid: 1Water,vol/vol/vol) were 0.25±0.02 and 0.25±0.01 respectively. Interestingly, the Rf values were very closer to that of pyocyanin as described by Knight et al. (1979). They observed Rf value of 0.6 in solvent system B and 0.23 in solvent system C for pyocyanin.

The absorption maxima of active compound in different solvents were very close to that of the phenazine compound, pyocyanin, as described by Watson et al. (1986) and Fernandez and Pizarro (1997).

HPLC analysis of the bioactive compound from *Pseudomonas* cultures showed only a single peak having the retention time (31.6 min. for *Pseudomonas* MCCB 102 and 31.37 min. for *Pseudomonas* MCCB 103) same as that of pyocyanin standard (30.83 min) in agreement with the argument of Fernandez and Pizarro (1997).

The retention time of the active compounds for both samples (*Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103) and pyocyanin standard in liquid chromatography were the same. Further analysis of this peak by mass spectroscopy demonstrated a protonated molecular ion cluster at m/z 211/212, and identified it as pyocyanin a secondary metabolite produced by *Pseudomonas* (Watson et al. 1986, Vukomanovic et al. 1997; Norman et al. 2004). In NMR spectroscopy, the chemical shift spectra between 7 and 9 ppm showed a good correspondence between samples and the reference.

The above properties of bioactive compound prove that the active compound was the phenazine antibiotic, pyocyanin. May species of *Vibrio* are found to be pathogenic to shrimps/prawns and are reported to be resistant to antibiotics. Among these *V. harveyi* was reported as a most important aquaculture pathogen with multiple antibiotic resistance and causing mass mortalities in shrimps/prawns hatcheries (Tendencia and de la Pena 2001). However, *in vivo* tests suggested that the pyocyanin was a potential vibriocidal compound and it could inactivate *V. harveyi*, at low concentration. The antibacterial, and antifungal property of phynazine antibiotic, pyocyanin has been described against human and plant pathogens (Baron and Rowe 1981;

Kerr et al. 1999). We are now reporting its anti-vibrio property, especially against aquaculture pathogens, for the first time.

The pyocyanin as such in the cell free supernatant can be stored at 4 °C for a period of one year with out loosing its anti-vibrio property. Persistence of anti-vibrio property up to 100 °C, shelf life at 4 °C, stability in different organic solvents and varying pHs makes the molecule ideal for aquaculture application. However, more work has to be done to bring out right dosage and mode of application for the management of vibrios in shrimps/prawns hatcheries as well as in grow out systems.

Table 1 Heat stability of supernatants at different temperature

Antagonistic Cultures	Diameter of the halo zone in mm after incubating at different temperature				Control
	60°C (30 min.)	80°C (30 min.)	100°C (30 min)	121°C/15lbs (20 min.)	
Pseudomonas MCCB 102	15 ± 0.82	15 ± 0.5	14.5 ± 0.58	13 ± 0.82	15.5 ± 0.58
Pseudomonas MCCB 103	13.75±1.23	13.25±0.96	12.5±0.58	10.75 ± 0.5	13.75±1.26

Table 2 pH stability of cell-free supernatants of *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103

Antagonistic Cultures	Diameter of the halo zone in mm after incubating at different pH for two hours					Control
	pH-2	pH-4	pH-6	pH-8	pH-10	
Pseudomonas MCCB 102	12.25 ±1.71	11±1.71	12±0	11.5 ±0.96	11.5±0.58	11.5 ±0.58
Pseudomonas MCCB 103	14.5 ±1	14.75±0.5	13.5±1	13.75 ±0.5	13.75±1.26	13.75 ±1.26

Table 3 Activity of cell-free supernatant of *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 in different solvents

Antagonistic Cultures	Activity in different solvents (Diameter of the halo zone in mm)				
	n-Butanol	Dichloro-methane	Chloroform	Ethyl acetate	Hexane
Pseudomonas MCCB 102	11.5 ± 1	10.75 ± 0.5	10.75 ± 0.5	Nil	Nil
Pseudomonas MCCB 103	9.25 ± 0.96	8 ± 0	8.75 ± 0.96	Nil	Nil
Control (solvent alone)	Nil	Nil	Nil	Nil	Nil

Table 4 Rf value of antivibrio compound in different solvent systems

Antagonistic Cultures	Rf value of antivibrio compound in different solvent systems		
	Solvent system A [1-butanol-acetic acid-water (4:1:1, v/v/v]	Solvent system B[chloroform-methanol, (1:1, v/v)]	Solvent system C[ethyl acetate - acetic acid - water (3:2:1, v/v/v)]
Pseudomonas MCCB 102	0.35±0.06	0.57±0.04	0.25±0.02
Pseudomonas MCCB 103	0.37±0.005	0.61±0.05	0.25±0.01

Table 5 Absorption maxima of antivibrio compound from *Pseudomonas* MCCB102 and *Pseudomonas* MCCB103 in different solvents

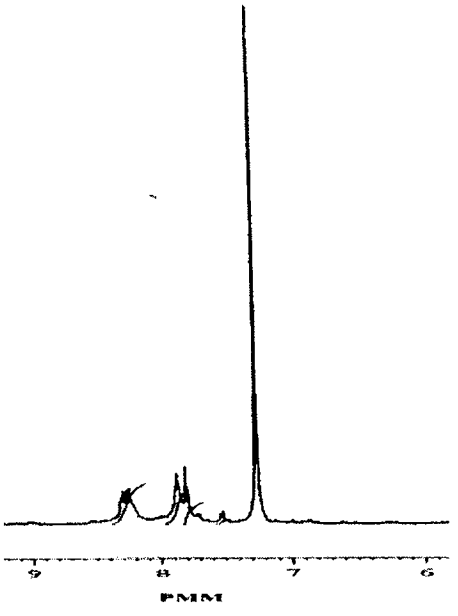
	Antivibrio compounds	
	Pseudomonas MCCB102	*Pseudomonas* MCCB103
Appearance	Blue in alkaline and Pink-purple in acidic condition	Blue in alkaline Pink/purple in acidic condition
Absorption maxima (nm)		
In Cl_3CH	693,327,309,265,242	691,327,309,264,243
In 0.1 mol l^{-1} HCl	520,387,278,243,205	522,387,278,242,203
In Methanol	717,321,239,209	717,319,239,207
In Dichloromethane	693,327,308,260,240	695,326308,238

Table 6 *In vitro* application of anti-vibrio compound

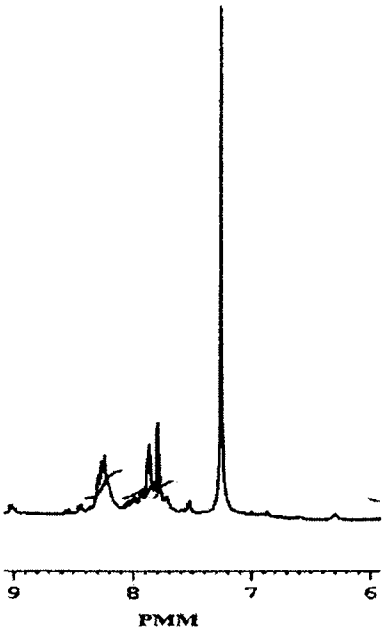
Pyocyanin concentration	Incubation period (hours)							
	6	12	18	24	30	36	48	72
100 ppm	-	-	-	-	-	-	-	-
80 ppm	-	-	-	-	-	-	-	-
60 ppm	+	+	-	-	-	-	-	-
40 ppm	+	+	-	-	-	-	-	-
20 ppm	+	+	-	-	-	-	-	-
15 ppm	+	+	+	+	+	+	-	-
10 ppm	+	+	+	+	+	+	+	+
5 ppm	+	+	+	+	+	+	+	+
Control(Without pyocyanin)	+	+	+	+	+	+	+	+

Fig.6 Nuclear Magnetic Resonance Spectra of the antivibrio compound from *Pseudomonas* MCCB 102 (A) *Pseudomonas* 103 (B) and pyocyanin standard (C).

A



B



C

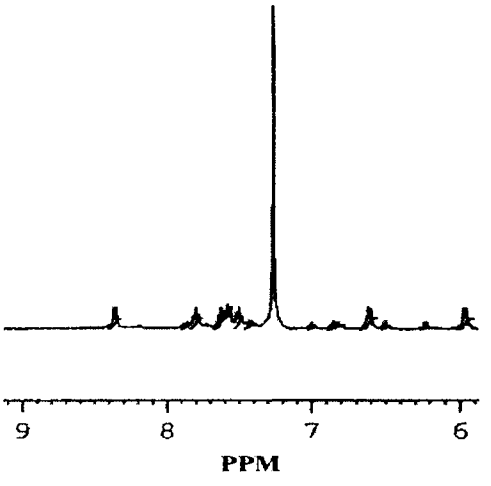
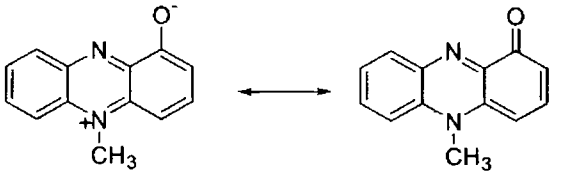


Fig.7 Structure of the antivibrio compound produced by *Pseudomonas* MCCB 102 and 103 based on UV-Vis, ^{1}H NMR and mass spectral data.



Chapter-4

Optimization of Culture Conditions and Media Designing for the Mass Production of Antagonistic Probiotic Micrococcus MCCB 104

Chapter 4

Optimization of Culture Conditions and Media Designing for the Mass Production of Antagonistic Probiotic *Micrococcus* MCCB 104

4.1 Introduction

Research on probiotics for aquaculture application has increased substantially with the demand for environment friendly alternatives in disease management strategies. One of such alternatives is the application of probiotics at different stages of culture. The identified probiotics for aquaculture belong to *Planococcus* (Austin and Billaud 1990), Lactic acid bacteria (Gatesoupe 1994), a few strains of *Vibrio* (Austin et al. 1995, Gomez-Gil et al. 2002), *Bacillus* (Rengpipat et al. 2000), *Carnobacterium* (Robertson et al 2000), *Pseudomonas* (Chythanya et al. 2002) etc. In few cases the probiotic bacterial strains have shown *in vitro* antagonism also to pathogens. As an addition to the already known probiotics, Jayaprakash et al.(2005) isolated and identified a strain of *Micrococcus* MCCB 104, antagonistic to a range of *Vibrio*, as a possible probiotic in aquaculture systems. In this context to facilitate it's mass production and commercial application, an appropriate bioprocess technology is found essential.

The conventional optimization method involves varying one parameter at a time and keeping the others constant and this often does not bring about the effect of interaction of various parameters. But the statistical experimental design techniques in fermentation process involve study of the main and interacting effect of factors and their by it can improve product yield and reduce process variability, time, coast etc (Elibol, 2004). Response surface methodology (RSM) is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a

limited number of experiments. However, it has not yet been reported for probiotics, especially in aquaculture.

This chapter describes optimization of the culture conditions such as pH, salinity and temperature and designing of media to economize the production of *Micrococcus* MCCB 104, considering both biomass and antagonistic activity against *V. harveyi,* by employing the statistical experimental design, Response Surface Methodology.

4.2 Materials and methods

4.2.1 Probiotic organism

The organism used in this study was, *Micrococcus* MCCB 104, previously described by Jayaprakash et al. (2005). This isolate forms part of the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, India.

4.2 2 Experimental design for optimization of pH, temperature and salinity for the mass production of *Micrococcus* MCCB 104

4.2.2.1 Culture medium and Inoculum preparation

The organisms were grown in ZoBell's marine broth 2216 E for optimization of culture conditions such as sodium chloride concentration, pH and temperature. A single colony of *Micrococcus* MCCB104 grown on ZoBell's agar 2216 E (Oppenheimer and ZoBell 1952) was inoculated in 100 ml ZoBell's broth and incubated at 28°C for twenty-four hours and used as the inoculum.

4.2.2.2 Shake flask experiments

Shake flask experiments were carried out as mentioned earlier in the Chapter 2.

4.2.2.3 Analysis of the samples

4.2.2.3.1 Biomass determination and antagonism assay

Biomass determination and antagonism assay were carried out as mentioned earlier in the Chapter 2.

4.2.2.4 First step optimization of pH, temperature and salinity for biomass and antagonistic activity for *Micrococcus* MCCB 104

One-dimensional screening experiments were carried out as mentioned earlier in the Chapter 2.

4.2.2.5 Second step optimization of pH, temperature and salinity using statistically designed experiments for *Micrococcus* MCCB 104

Second step optimization was carried out using central composite design of response surface methodology as mentioned earlier in the Chapter 2.

4.2.2.6 Experimental verification of the Identified optimum from the model for *Micrococcus* MCCB 104

Experiments were repeated at the optimum concentration of sodium chloride, pH and temperature obtained from the model equations for biomass and antagonistic activity for *Micrococcus* MCCB 104.

4.2.3 Experimental design for optimization of media for the mass culture of *Micrococcus* MCCB 104

4.2.3.1 Screening of C-source, N-source, amino acids and vitamins

Twenty-four C-sources were screened as mentioned earlier in the Chapter 2. Subsequently five of them (glucose, fructose, lactose, maltose and sucrose) were selected for further screening (for both biomass production and antagonistic activity) at 1% level in M-9 mineral medium (Na_2HPO_4:0.68% (w/v), KH_2PO_4:0.3%(w/v), NH_4Cl:0.1%(w/v), $MgSO_4.7H_2O$:0.049%(w/v), $CaCl_2$:0.001%(w/v)) supplemented with 2% sodium chloride (Sambrook and Russel 2001). Ammonium chloride, ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate, sodium nitrate and urea were screened as

sole N-source at 0.2% level in M-9 medium (with 1% glucose and without N-source). Twenty-four amino acids (DL- Ala, DL-2-amino-n-butyric acid, L-Arg- monohydrochloride, DL-Asp, L-Cys-hydrochloride, L-Cys, 3-(3,4-dihydroxyphenyl) DL-Ala, L-Glu, Gly, L-His-monohydochloride, L-hydroxy-Pro, L-Lue, L-Ile, DL-nor-Lue, L-Lys-monohydrochloride, L-Met, DL-Orn- monohydrochloride, L-Pro, DL-β-Phe, D-Ser, DL-Tyr, L-Trp, L-Tyr and DL-Val, Hi-Media, India) and Casamino acid (BD Biosciences) were screened as growth factors at 0.02% level and vitamins such as ascorbic acid, biotin, cyanocobalamine, folic acid, inositol, pantothenic acid, riboflavin and thiamine (Hi-Media, India) at 0.002% level in a pattern of one at a time for biomass and antagonistic activity. All incubations were done for ninety-six hours at 28° C.

4.2.3.2 Shake flask experiment

Primary screening of nutrients (one-at-a-time) and the final optimization of selected ingredients were carried out in Erlenmeyer flasks (250 ml capacity) with 100 mL mineral medium (M-9). Sugars, amino acids and vitamins were filter sterilized using cellulose acetate membrane (Millipore, India) having 0.22 μm porosity and added to sterile mineral medium. Subsequently pH was adjusted by using sterile 1N NaOH and 1N HCl, employing narrow range pH paper. All flasks through out the study were inoculated with the culture to a final concentration equivalent to 0.01 at A_{600} (10^{3} CFU m l^{-1}) in 100 ml aliquots. Incubations were done in a temperature controlled rotary shaker (Scigenics Biotech. (Pvt.) Ltd, India) at 120 rev. min^{-1}.

4.2.3.3 Analysis of the sample

4.2.3.3.1 Biomass determination and antagonism assay

Biomass determination and antagonism assay were carried out as mentioned earlier in the Chapter 2.

4.2.3.4 Experimental design and optimization of media using RSM

The medium for the production of probiotic has been first optimized by a 'one-variable-at-a-time' approach. The minimum and maximum ranges of selected variables were investigated. The medium composition that resulted in the maximum biomass production and antagonistic activity was further optimized by response surface methodology (RSM) using central composite design (CCD) and a set of fifty experiments was carried out as mentioned earlier in the Chapter 2.

4.2.3.5 Experimental verification of biomass and antagonistic compound production in newly designed media for *Micrococcus* MCCB 104

Experiments were conducted at the optimum concentrations of ingredients obtained from the model equations for both biomass and antagonistic compound production of *Micrococcus* MCCB 104. Then the experimental values were compared with predicted values obtained from the model equations.

4.3 Results

4.3.1 Optimization of sodium chloride concentration, pH and temperature for the mass production of *Micrococcus* MCCB 104

4.3.1.1 First step optimization of sodium chloride concentration, pH and temperature

One-dimensional screening results were used for the regression analysis, using biomass as X and antagonistic activity as Y as responses to varying sodium chloride concentration, pH and temperature (Table 1). On obtaining the highly significant and least significant regression values, the corresponding factor levels were tabulated in a maximum - minimum range (0-25 g l^{-1} sodium chloride, 6-7 pH and 25 to 35 ^{0}C) for the application of central composite design of response surface methodology.

4.3.1.2 Second step optimization of sodium chloride concentration, pH and temperature for maximum biomass and antagonistic activity

The coded values of the independent variables [sodium chloride concentration (X_1), pH (X_2) and temperature (X_3)] are given in Table-2 along with the experimental and predicted values of biomass and antagonistic activity. The CCD matrix was analyzed by standard analysis of variance (ANOVA) as approximate to the experimental design used. The ANOVA of the quadratic regression model demonstrates that the model is highly significant ($P< 0.0001$) for both biomass and activity (Table 3 and 4). The Model F-value is 67.84 for biomass and 45.61 for activity. In the case of biomass, A, C, A^2, B^2, C^2, AC, BC are significant model terms. Where 'A' is sodium chloride concentration, 'B' pH and 'C' temperature. When we consider activity, C, A^2, C^2, AC and BC are significant model terms.

The goodness of fit of the model was checked by coefficient of determination (R^2). $R^2 = 0.9839$ in the case of biomass and 0.9762 in the case of activity. It can be expressed in percentage also and then it is interpreted as the per cent variability in the response in the given model. As per the model, sample variation of 98.39 % for biomass and 97.62% for antagonistic activity were attributed to the independent variables and only 1.61% and 2.38%, respectively of the total variation was not explained by the model. For, biomass R is equal to 0.9919 and for activity it is 0.9881.The coefficient of variation is 1.73% and 2.06% for biomass and activity respectively. An adequate precision was obtained, 22.402 and 23.975 for biomass and activity respectively. In the case of biomass "Pred R-Squared" 0.8712 is in reasonable agreement with the "Adj R-Squared" of 0.9694 and the "Pred R-Squared" of 0.7789 is in reasonable agreement with the "Adj R-Squared" of 0.9548 in the case of activity also.

The RSM gave the following regression equations for the biomass (Y_1) and antagonistic activity (Y_2) as a function of sodium chloride concentration (X_1), pH (X_2) and temperature (X_3).

Final Equations in Terms of Coded Factors are:

$$Y_1 = +2.13 + 0.083\ X_x + 9.239E\text{-}003\ X_2 - 0.072\ X_3 - 0.12\ X_1^2 - 0.076\ X_2^2 - 0.12\ X_3^2 + 0.021\ X_1\ X_2 - 0.13\ X_1\ X_3 - 0.044\ X_2\ X_3 \quad \text{.....Eq.1}$$

$$Y_2 = +2.10 + 0.025\ X_x + 0.015\ X_2 - 0.093\ X_3 - 0.042\ X_1^2 + 6.438E\text{-}003\ X_2^2 - 0.17\ X_3^2 + 0.000X_1\ X_2 - 0.100X_1\ X_3 + 0.050\ X_2\ X_3 \quad \text{.....Eq.2}$$

The two-dimensional contour plots and its respective response surface plots on biomass show the interaction between sodium chloride concentration and temperature at the optimum pH, 6.75, (Fig.1A) and interaction between pH and temperature at optimum sodium chloride concentration, 22.13 g l^{-1}, (Fig.1B). It was also clear from the plots that biomass increased towards pH 6.5 and remained stable up to pH 7 (Fig.1 B).

The Fig. 2 shows similar plots of the interaction between sodium chloride concentration and temperature at the optimum pH, 6.42, (Fig. 2 A) and the interaction between pH and temperature at optimum sodium chloride concentration (19.94 g l^{-1}) for antagonistic activity (Fig. 2 B). Antagonistic activity increases towards pH 6.42.

Optimum pH, temperature and sodium chloride concentration were found out for maximum biomass and antagonistic activity from the regression equations (Eq.1 and Eq. 2) and were experimentally verified as shown in the

Table 5. After optimization biomass was found increased by 6.62% and activity by 21.6%.

4.3.2 Optimization of media for the mass culture of *Micrococcus* MCCB 104

4.3.2.1 First step optimization of ingredients of media for biomass production and antagonistic activity of *Micrococcus* MCCB 104

Variables such as glucose and lactose were selected as carbon sources (Table 6 and Fig. 3), ammonium chloride as nitrogen source (Fig. 4) and mineral salts solution were chosen for the study by a 'one variable-at-a-time method', and the range of sodium chloride concentration was taken from the previous experiment. The minimum and maximum limits of the variables were, glucose: 0.2-2%, lactose: 0.2-2%, sodium chloride: 1.5 - 2.5 %, ammonium chloride: 0.04%-0. 4% and mineral salts solution: 0.5 % - 2 % (v/v). Growth factors such as aminoacids (Fig. 5 and 6) and vitamins (Fig. 7) did not have any effect on biomass and activity.

4.3.2.2 Second step optimization of ingredients of media for biomass production and antagonistic activity of *Micrococcus* MCCB 104

The coded values of the independent variables (glucose: A, lactose: B, sodium chloride: C, ammonium chloride: D and mineral salts solution: E) along with the experimental and predicted values of biomass and antagonistic activity are given in Table 7. The ANOVA of the quadratic regression model demonstrated that the model was highly significant ($P< 0.0001$) for both biomass and activity (Table 8 and 9). The model F-value was 39.4 for biomass and 19.4 for antagonistic activity.

The 'lack of fit 'value was insignificant for both biomass and activity and the goodness of fit of the model was checked by coefficient of determination (R^2). R^2 was equal to 0.9645 in the case of biomass and 0.9305 in the case of activity. It can be expressed in percentage also and then it is interpreted as the percent variability in the response in the given model. As per

the model, sample variation of 96.45 % for biomass and 93.05% for antagonistic activity was attributed to the independent variables and the model did not explain only 3.55% and 6.95%, respectively of the total variation. For biomass, correlation coefficient (R) was equal to 0.9821 and for activity 0.9646. An adequate precision was obtained, 22.608 and 15.471 for biomass and activity respectively. In the case of biomass "Pred R-Squared" 0.8687 is in reasonable agreement with the "Adj R-Squared" of 0.94 and in the case of activity the "Pred R-Squared" of 0.7383 is in reasonable agreement with the "Adj R-Squared" of 0.8825.

The RSM gave the following regression equations for the biomass and antagonistic activity as a function of glucose (A), lactose (B), sodium chloride (C), ammonium chloride (D) and mineral salts solution (E).

Final Equations in Terms of Coded Factors are:

$$\text{Biomass} = +1.57 + 0.15A + 0.16B\ 0.055 + 0.16\,D + 0.10\,E - 0.12A^2 - 0.080\,B^2 - 0.17C^2 - 0.24\,D^2 - 0.20\,E^2 - 0.15\,AB + 0.026\,AC + 0.070\,AD + 0.029\,AE + 0.067\,BC + 0.064BD + 0.13BE - 0.11\,CD + 0.025CE + 0.16\,DE \quad \text{.....Eq.3}$$

$$\text{Activity} = +22.55 + 2.12\,A + 2.52\,B - 0.61C + 1.78\,D + 1.28E - 1.55\,A^2 - 0.78\,B^2 - 1.72\,C^2 - 2.96\,D^2 - 2.55\,E^2 - 2.10\,AB + 0.68AC + 0.62\,AD + 0.64AE + 1.28BC + 0.64BD + 2.19BE - 1.59CD + 0.22CE + 2.29\,DE \quad \text{.....Eq.4}$$

All linear (A, B, C, D & E) and quadratic coefficients (A^2, B^2, C^2, D^2 & E^2) are highly significant for both biomass and activity. The interaction coefficients such as AB, AD, BC, BD, BE, CD and DE are significant model terms for biomass and in the case of activity AB, BC, BE, CD and DE are the significant model terms.

Effect of interaction of varying concentrations of glucose and lactose, glucose and ammonium chloride, lactose and sodium chloride, lactose and ammonium chloride, lactose and mineral salts solution, ammonium chloride and mineral salt solution and sodium chloride and ammonium chloride on biomass production when all other parametres at optimum are presented in Fig. 8 A, B, C, D, E, F and G. Based on the data generated the optimum concentration of the factors / ingredients were glucose:17.4g l^{-1}, lactose:17 g l^{-1}, sodium chloride : 16.9 g l^{-1}, ammonium chloride :3.3 g $l^{-1}$and mineral salts solution 18.3 ml l^{-1}.

The interaction between nutrients and their effect on antagonistic activity were also studied (Fig.9). Effect of interaction of varying concentrations of ingredients as listed above on antagonistic activity when all the other parametres kept at optimum are presented in Fig. 9 A, B, C, D and E. The optimum concentrations of the glucose, lactose, sodium chloride, ammonium chloride and mineral salts solution for the antagonistic activity were the same as recorded for biomass production.

4.3.2.3 Experimental verification of biomass and antagonistic compound in newly designed media for *Micrococcus* MCCB 104

The predicted concentrations of ingredients of the media from the regression equations (Eq 1 and Eq. 2) were same for both biomass and antagonistic activity i.e., glucose (17.4), lactose (17 g l^{-1}), sodium chloride (16.9 g l^{-1}), ammonium chloride (3.3 g l^{-1}) and mineral salts solution (18.3 ml. l^{-1}). At these conditions the predicted biomass was 1.7 g l^{-1} and activity in terms of halo zone was 24.5 mm. The experimental values were 1.8 ± 0.04 g l^{-1} for biomass production and 24.33 ± 0.6 mm diameters for halo zone. More over in the above composition of the medium the biomass could be increased by 11.11% and activity by 5.78 % compared to M-9 mineral medium (Sambrook and Russell 2001).

4.4 Discussion

One-dimensional screening experiments showed that the culture conditions such as pH, temperature and sodium chloride concentration have important roles to play in the biomass production and antagonistic activity. Central composite design (CCD) of response surface methodology was used to maximize the biomass production and antagonistic activity, and to determine the interaction between parameters. The P-value ($P< 0.0001$) and model F-value for both biomass and antagonistic activty implies that the models were highly significant. Coefficient of determination (R^2) gives a measure of how much variability in the observed response value can be explained by the experimental factors and their interactions (Huang et al. 2006). Models. R^2 values were very high for models used for optimization of sodium chloride concentration, pH and temperature, and media designing.

A higher value of correlation coefficient, indicates an excellent correlation between independent variables but a relatively low value of coefficient of variation (CV) shows the reliability and improved precision of the experimets (Adinarayana et al. 2003). The purpose of statistical analysis is to determine which experimental factors generate signals, which are large in comparison to the noise. Adequate precision measures signal to noise ratioand a ratio grater than 4 is disirable (Wang and Lu 2004). An adequate precision was obtained for both biomass and antagonistic activity.

A linear effect was observed on biomass, which increased with increasing concentration of sodium chloride up to 22.13 gl^{-1}, a quite possible observation, as it was isolated from brackish water (Jayaprakash et al. 2005). Similar trend was observed for temperature on biomass, which increased, with temperature from 25 to 30 °C. The slightly acidic pH (6.42) may be required for the production of the antagonistic agent. However, the antagonistic compound is slightly alkaline in nature (Jayaprakash et al. 2005). Both the

biomass and antagonistic activity responded more or less uniformly to the combination of the three factors.

Shape of the contour plot, circular or elliptical, indicates whether the mutual interactions between the corresponding variables are significant or not. If it is circular the interactions between the variables are negligible and if it is elliptical the interaction between the variables are significant (Wang and Lu 2004). A linear effect was observed for antagonistic activity, which increased with temperature up to 28 °C, irrespective of the presence of other factors.

It has been observed that as most of the probiotics used in aquaculture have been isolated from natural environment, optimizations of physical as well as chemical parameters are invariably required for improving the efficacy of the bioprocess for the commercial production.

The interactive effect of nutritional factors on both biomass and activity were also studied. The P values are used as a tool to test significance of each coefficient and the pattern of interaction between the coefficient on both biomass and activity. The smaller the P value, the more significant is the corresponding coefficient (Rao et al. 2000). All linear and quadratic coefficients were highly significant for both biomass and activity but all interaction coefficients were not significant. Since it is hierarchiel model the insignificant coefficients were not omitted from the Eq. 3 and Eq.4, (Wang and Lu 2004).

The response surface plots are the graphical representations of the regression equations and this methodology has been used successfully for optimizing multiple variables in order to predict the best performance with minimum number of experiments considering both main and interactive effect of factors (Adinarayana et al. 2003; Beg et al. 2003; Wang and Lu 2004;

Xiong et al. 2005). Response surface plots of biomass showed similar patterns with that of antagonistic activity at the optimum conditions which indicated a strong correlation between the biomass and antagonistic activity (Liong and Shah 2005). In the newly designed medium the biomass could be increased by 11.11% and activity by 5.78 % compared to M-9 mineral medium. Moreover the validation of experiment showed that experimental values of biomass and antagonistic activity were very close to the predicted values for maximum biomass production and antagonistic activity.

Table 1 Factor levels used for regression analysis and their significance in the first step optimization

Sodium chloride concentration ($g\ l^{-1}$)	Significance F	pH	Significance F	Temperature	Significance F
0	0.000571	6	0.012945	25 ^{0}C	0.045244
5	0.00122	6.5	0.000972	30^{0}C	0.090452
10	0.028345	7	0.00000016	35^{0}C	0.002886
15	0.030275	7.5	0.00000079	40^{0}C	0.205517
20	0.031284	8	0.0000026		
25	0.031881				
30	0.35226				
35	0.068502				

Table 2 Central composite design matrix of the three variables in coded units along with the experimental and predicted values of biomass and antagonistic activity

Expt. No.	Sodium chloride (g l^{-1})	pH	Temperature (°C)	Biomass (g l^{-1})		Activity (Diameter of inhibition zone in mm)	
				Experimental	Predicted	Experimental	Predicted
1	0	6	25	1.34	1.36	19	18.9
2	25	6	25	1.67	1.67	21	21.4
3	0	7	25	1.43	1.42	18	18.2
4	25	7	25	1.79	1.79	21	20.7
5	0	6	35	1.54	1.51	18	18.1
6	25	6	35	1.42	1.41	17	16.6
7	0	7	35	1.45	1.43	20	19.4
8	25	7	35	1.43	1.39	18	17.9
9	0	6.5	30	1.56	1.60	20	20.3
10	33.52	6.5	30	1.58	1.60	20	20.2
11	12.5	5.66	30	1.57	1.57	21	20.9
12	12.5	7.34	30	1.57	1.60	21	21.4
13	12.5	6.5	21.59	1.59	1.58	18	17.7
14	12.5	6.5	38.41	1.33	1.37	14	14.6
15	12.5	6.5	30	1.77	1.77	21	21.0
16	12.5	6.5	30	1.77	1.77	21	21.0
17	12.5	6.5	30	1.77	1.77	21	21.0
18	12.5	6.5	30	1.77	1.77	21	21.0
19	12.5	6.5	30	1.77	1.77	21	21.0
20	12.5	6.5	30	1.77	1.77	21	21.0

Table 3 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass

Source	SS	DF	MS	F-value	Probability P>F
Model	0.47	9	0.052	67.84	< 0.0001
Residual (error)	0.00764	10	0.000764		
Lack of fit	0.00764	5	0.001529		
Pure error	0.000	5	0.000		
Cor total	0.47	19			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=1.73 %, R^2=0.9839, R=0.9919

Table 4 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic activity

Source	SS	DF	MS	F-value	Probability P>F
Model	0.67	9	0.075	45.61	< 0.0001
Residual (error)	1.64	10	0.16		
Lack of fit	1.64	5	0.33		
Pure error	0.000	5	0.000		
Cor total	0.69	19			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=2.06 %, R^2=0.9762, R=0.9881

Table 5 Experimental verification of identified optimum from the model

Variable	Levels before Optimi-zation	Levels after optimization		Biomass (g/L) (Cell Dry Mass, CDM)		Activity (Diameter of halo zone in mm)	
		For biomass	For activity	Initial	After Optimiza-tion	Initial	After Optimization
Sodium chloride (gl^{-1})	30	22.13	19.94	1.66 ± 0.03	1.77 ± 0.01	18.5 ± 0.68	22.5 ± 0.4
pH	7.2 ± 0.5	6.75	6.42				
Temperat-ure (°C)	28	26.34	28.06				

Table 7 Central composite design matrix of the variables (g l^{-1}) along with the experimental and predicted values of biomass and antagonistic activity

Expt. No.	Glu -cose	Lact -ose	Sodium chloride	Ammon -ium chloride	Mineral salts solution	Biomass (mg l^{-1})		Activity (Diameter of inhibition zone in mm)	
						Exper -imental	Predi -cted	Exper -imental	Predi -cted
1	0.2	0.2	1.5	0.04	0.5	0.6	0.56	12.33	10.75
2	2	0.2	1.5	0.04	0.5	0.8	0.91	15.33	15.29
3	0.2	2	1.5	0.04	0.5	0.56	0.64	10.67	11.79
4	2	2	1.5	0.04	0.5	0.5	0.40	10.88	7.928
5	0.2	0.2	2.5	0.04	0.5	0.5	0.44	11	8.357
6	2	0.2	2.5	0.04	0.5	0.8	0.89	15	15.63
7	0.2	2	2.5	0.04	0.5	0.84	0.78	16	14.51
8	2	2	2.5	0.04	0.5	0.7	0.65	14.67	13.38
9	0.2	0.2	1.5	0.4	0.5	0.6	0.51	12	10.39
10	2	0.2	1.5	0.4	0.5	1.12	1.14	16.33	17.43
11	0.2	2	1.5	0.4	0.5	0.88	0.84	14.33	13.98
12	2	2	1.5	0.4	0.5	1	0.89	15	12.62
13	0.2	0.2	2.5	0.4	0.5	0	-0.06	0	1.63
14	2	0.2	2.5	0.4	0.5	0.8	0.67	14.24	11.39
15	0.2	2	2.5	0.4	0.5	0.5	0.54	10.33	10.33
16	2	2	2.5	0.4	0.5	0.8	0.69	13.67	11.69
17	0.2	0.2	1.5	0.04	2	0	0.07	0	2.64
18	2	0.2	1.5	0.04	2	0.5	0.53	10	9.75
19	0.2	2	1.5	0.04	2	0.52	0.67	12	12.43
20	2	2	1.5	0.04	2	0.6	0.55	13	11.13
21	0.2	0.2	2.5	0.04	2	0	0.04	0	1.11
22	2	0.2	2.5	0.04	2	0.6	0.61	12	10.95
23	0.2	2	2.5	0.04	2	0.9	0.91	16.33	16.01
24	2	2	2.5	0.04	2	0.8	0.89	16.2	17.45
25	0.2	0.2	1.5	0.4	2	0.68	0.67	12.42	11.46
26	2	0.2	1.5	0.4	2	1.4	1.42	20	21.07
27	0.2	2	1.5	0.4	2	1.6	1.53	22.67	23.79
28	2	2	1.5	0.4	2	1.61	1.69	23.68	24.99
29	0.2	0.2	2.5	0.4	2	0	0.20	0	3.56
30	2	0.2	2.5	0.4	2	1.2	1.05	17.2	15.9
31	0.2	2	2.5	0.4	2	1.57	1.33	23.33	21.01
32	2	2	2.5	0.4	2	1.52	1.59	23.67	24.94
33	0	1.1	2	0.22	1.25	0.5	0.51	10	8.75
34	3.24	1.1	2	0.22	1.25	1.2	1.24	16	18.83
35	1.1	0	2	0.22	1.25	0.8	0.75	13	12.13
36	1.1	3.24	2	0.22	1.25	1.4	1.49	21.67	24.12
37	1.1	1.1	0.81	0.22	1.25	0.8	0.75	14	14.24
38	1.1	1.1	3.19	0.22	1.25	0.4	0.49	10	11.33
39	1.1	1.1	2	0	1.25	0	-0.16	0	1.54
40	1.1	1.1	2	0.65	1.25	0.4	0.61	10	10.03
41	1.1	1.1	2	0.22	0	0	0.18	0	5.07
42	1.1	1.1	2	0.22	3.03	0.8	0.66	14.67	11.18
43	1.1	1.1	2	0.22	1.25	1.46	1.57	23.33	22.55
44	1.1	1.1	2	0.22	1.25	1.62	1.57	22.33	22.55
45	1.1	1.1	2	0.22	1.25	1.45	1.57	23.33	22.55
46	1.1	1.1	2	0.22	1.25	1.6	1.57	23.67	22.55
47	1.1	1.1	2	0.22	1.25	1.56	1.57	23.67	22.55
48	1.1	1.1	2	0.22	1.25	1.6	1.57	23	22.55
49	1.1	1.1	2	0.22	1.25	1.67	1.57	20	22.55
50	1.1	1.1	2	0.22	1.25	1.6	1.57	20	22.55

Table 8 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass

Source	SS	DF	MS	F-value	Probability P>F
Model	12.63	20	0.63	39.4	< 0.0001
Residual (error)	0.46485	29	0.016029		
Lack of fit	0.42305	22	0.01923	3.220255	0.0586
Pure error	0.0418	7	0.005971		
Cor total	13.09741	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=14.6 %, R^2=0.9645,R=0.9821

Table 9 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic activity

Source	SS	DF	MS	F-value	Probability P>F
Model	2201.366	20	110.0683	19.40414	< 0.0001
Residual (error)	164.5	29	5.672412		
Lack of fit	147.66	22	6.71189	2.790245	0.0834
Pure error	16.838	7	2.405484		
Cor total	2365.87	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV=16.7 %, R^2=0.9305, R=0.9646

Chapter-5

Evaluation of Synechocystis MCCB 114 and Synechocystis MCCB 115 as Probiotics for the Management of Vibrio in Shrimp Culture Systems

Chapter 5

Evaluation of *Synechocystis* MCCB 114, 115 as Probiotics for the Management of *Vibrio* in Shrimp Culture Systems

5.1 Introduction

In recent years, there has been tremendous increase in the interest on application of microalgae as feed and as disease control agents in aquaculture. Green water culture systems became more popular and have been suggested as a tool for sustainable aquaculture. Anti-luminous *Vibrio* factors have been reported in green water systems of *Penaeus monodon* (Lio-Po et al. 2005 and Tendentia et al. 2005). However, cyanobacteria in general have not been exploited as biological control agent in aquaculture.

Cyanobacteria are rich in proteins, essential aminoacids, vitamins, β-carotene etc. (Morist et al. 2001; Kumar et al. 2003). Sundararaman et al. (1994) reported marine cyanobacterial isolates as animal feed with potential pharmacological application due to its biological activity. Later, a marine isolate of Cyanobacteria, *Synechocystis* sp. SY-4, was found to be a potential feed for rotifers (Sakamoto et al. 1996). Cyanobacteria produce many biologically active metabolites, most of them with anti-viral, immuno stimulatory and antioxidant properties (Kumar et al. 2003).

Probiotics, in the context of the aquatic environment, are defined as live microbial adjuncts having beneficial effects on the host by modifying the host-associated or ambient microbial community, ensuring improved use of feed, enhancing its nutritional value, host's response to diseases, or by improving the quality of its ambient environment (Verschuere 2000). Eventhough several

potential probiotic organisms have been isolated and identified for aquaculture, cyanobacteria (blue green algae) have not yet been suggested as candidate probiotics. However, marine cyanobacteria, in particular, have been in focus for quite some time as potential source of antagonistic compounds (Pergament and Carmeli 1994, Rao 1994, Kulik 1995, Mundt 2001). Secretion of antimicrobial substances is an important part of the competition process in the natural environment having a beneficial effect on the host by modifying the host-associated ambient microbial community (Verschuere 2000).

The cell contents of ingested blue greens may promote the growth of gut bacteria and accelerate the process of nutrient absorption. Considering the above, the present work was undertaken to screen and segregate specific cyanobacterial cultures from marine environment as putative probiotics to be used in shrimp hatcheries. This selection has been made based on their 1. *In vitro* antagonism; 2. Property to regulate the intestinal bacterial flora; and 3.Enhancement in the survival of post larvae of *P.monodon* against challenge with *V.harveyi* subsequent to feeding with the cyanobacteria.

5.2 Materials and Methods

5.2.1 Cyanobacterial cultures

Water samples were collected from the sea surface off Vypeen Island (Latitude-09-58N, Longitude-76-17E and Std. Diff. from GMT 05:30:00) using a surface water sampler, transferred aseptically to sterile glass bottles of 100 mL capacity, and transported to the laboratory in an ice chest. Six media namely BG –11, ASN-111 and MN (Rippka et al. 1979), modified Chu –11 (Cohen 1975), Allen and Nelson's (Allen and Nelson 1910) and Walne's medium (Walne 1974) were employed for the enrichment of cyanobacteria. All the above media were prepared in seawater having 30 parts per thousand (ppt) salinity. Enrichment of cyanobacteria was accomplished in sterile,

cotton- plugged 100 mL conical flasks (glass) with 50 mL media. Before inoculation, pH of the samples was checked and the pH of the media adjusted accordingly. Altogether, nine samples were inoculated 1 ml aliquots in six media and incubated at 28 ± 0.4^{0}C under 12 Hour illumination at 40 μEm^{-2}s provided by cool fluorescent lamps (Philips, India).

5.2.2 Primary screening and segregation of cyanobacteria

Altogether, 54 cyanobacterial enrichment cultures were developed in six media. Antagonistic property of the cultures on a panel of 35 isolates of *Vibrio* was tested as follows: Aliquots of 10 ml cyanobacterial cultures were centrifuged at 4^{0}C at 10,000 × g for 15 minutes. The supernatant was saved in sterile vials and stored at 4^{0}C until used. Antibacterial activity of the supernatants were determined by disc diffusion method as described earlier in Chapter-2 on the target Vibrios. The plates were incubated at 28 ± 0.4^{0}C for 24-48 hours and zones of clearing around the disc were measured using a HiAntibiotic Zone scale (HiMedia, Mumbai, India). The cyanobacterial cultures, which showed antibacterial activity on the maximum number of target isolates of *Vibrio* in the panel were selected for secondary screening (Table 1).

5.2.3 Secondary screening of cyanobacteria

Secondary screening was accomplished by evaluating the antimicrobial activity of the cell free supernatant, cells and cell lysate independently. Aliquots of 50 m l each of the cyanobacterial cultures segregated in the primary screening were centrifuged at 10,000 × g (4^{0}C) for 15 min, the supernatant used as sample 1. The pellet was washed several times in seawater, centrifuged at 10,000 × g (4^{0}C) for 15 min, resuspended in 50 ml distilled water, sonicated (VCX-500, Sonics, USA), and cell rupture confirmed under bright field microscope. The sonicated samples were centrifuged at 10,000 ×g (4^{0}C) for 15 min, supernatant used as sample 2 and

the cell debris resuspended in distilled water, as sample 3. Aliquots of the cultures were centrifuged, washed several times in seawater and the cells resuspended in seawater as sample 4. Antagonistic activity of all the samples were tested on a panel of 12 species of *Vibrio* isolated from shrimp larval rearing systems and characterized (Alsina and Blanch 1994, Table 2).

5.2.4 Effect of feeding cyanobacterial cultures on the intestinal bacterial flora of post larvae of *Penaeus monodon*

Post larvae (PL 20) of *Penaeus monodon* were brought from Matsyafed Shrimp Hatchery, Kollam, Kerala, India and maintained 50 larvae each in fibre glass containers with 25 l sea water (30ppt), in duplicate. The cyanobacterial cultures segregated during the secondary screening were centrifuged at 4^0C, at 10,000 × g for 15 minutes, supernatant discarded and the pellets were coated onto a commercial feed (Higashimaru India (P) Ltd., Cochin, Kerala, India) at the time of feeding using 'Bindex' (Matrix Vet Formulations, Hyderabad, India) at 1:1 ratio, dried in a desiccator at room temperature (28±1°C) for two hours and fed *ad libitum* to post larvae for 20 days continuously. Control tanks were maintained with normal feed. During this period thirty per cent of water used to be removed daily and replaced with fresh filtered seawater coupled with the removal of uneaten feed and faecal matter. Parameters such as pH, salinity and temperature were measured respectively using narrow range pH paper (Merck, U.K), a salinometer (Aquafouna, Japan) and a maximum minimum glass-thermometer. The chemical parameters such as NH_4^+-N and NO_2^--N were measured as described earlier (Strickland & Parson 1968) and maintained below 0.1 and 1 parts per million (ppm) respectively, through water exchange.

During the experiment, five post larvae each were sampled once in five days from each tank, disinfected using sodium hypochlorite (25 parts per million available chlorine) for 15 minutes and repeatedly washed in sterile

seawater. They were macerated in 1 ml sterile seawater diluent (25ppt) using glass homogenizers, serially diluted and inoculated onto ZoBell's marine agar 2216E plates (HiMedia, Mumbai, India). Water samples from each tank were also plated after serial dilution in the same way. Viable counts were determined after seven days of incubation from those plates, which showed well-distributed colonies between 100 to 300. Colonies were isolated at random on to ZoBell's agar slants at random. They were identified to genera following Oliver (1982) and Alsina and Blanch (1994), and generic diversity indices (Shannon Index) determined (Pielio 1966).

5.2.5 Effect of feeding cyanobacterial cultures on the survival of post larvae of *Penaeus monodon* following challenge with *V. harveyi*

After 20 days of feeding, the percentage survival of larvae was determined and they were challenged with *V.harveyi* MCCB 111 cultured on ZoBell's marine agar 2216E plates supplemented with prawn muscle extract (Singh and Philip 1993) after confirming their luminescence. The culture was harvested in sterile seawater, aliquots of 0.1 mL (10^9cells) suspension coated onto 1g feed using 'Bindex' (Matrix Vet Formulations, Hyderabad, India) and fed afresh at a dosage of 0.5 g/50 larvae twice a day, at 6 AM and 6 PM. From the following day feeding with the diet, coated with the cyanobacterial cultures was resumed. The tanks were observed for mortality. After 10 days the post larvae which survived the first challenge were re-challenged with *V.harveyi* in the same pattern and again observed for mortality. All the above experiments were repeated three times. The data were analyzed statistically using ANOVA and the relative percentage survival (RPS) calculated using the equation:

RPS = [1- (mortality in the group administered with cyanobacteria) / (mortality in the group without cyanobacterial administration) × 100] (Gram et al. 1999).

Based on the results two cyanobacterial cultures which were found associated with statistically significant higher percentage survival during the challenge were segregated for further investigation.

5.2.6 Heterotrophic bacteria associated with cyanobacteria

To isolate heterotrophic bacteria associated with the culture supernatant of the segregated cyanobacteria, 2 ml aliquots of the cultures were centrifuged at 10,000 ×g (4^0C) for 15 min. and the supernatant diluted serially in sterile seawater diluent (30 ppt) and plated on to ZoBell's marine agar 2216E (sample-1). To isolate the cyanobacterial cell associated heterotrophic bacteria, the pellets were washed gently several times in seawater, serially diluted and plated as above (sample 2). Colonies were isolated from both above at random on to ZoBell's marine agar 2216 E slants, and identified to genera (Oliver 1982, Alsina and Blanch 1994).

5.2.7 Identification of the segregated cyanobacteria

The segregated cultures were identified to genera based on micro-morphology as described by Herdman et al. (2001). The cultures were centrifuged at 10,000 × g, washed several times in artificial seawater (ASW) having 30 ppt, resuspended in 2.5% gluteraldehyde prepared in ASW, incubated overnight at 4°C, centrifuged and washed several times in ASW, post-fixed in OsO_4 (Electron microscopy sciences, USA) prepared in ASW at 1:1 ratio for an hour, centrifuged and repeatedly washed in phosphate buffered saline (pH 7). They were graded in acetone, dried in critical point drying apparatus, gold- coated and viewed under Scanning Electron Microscope (H600, Hitachi Ltd, Japan). A portion of the cells after dehydration was infiltrated and embedded in epoxy resin (Electron microscopy sciences, USA) and ultra-thin sections prepared, stained with uranyl acetate and lead citrate

(Electron microscopy sciences, USA) and viewed under a Transmission Electron Microscope (CM-10, Philips Electron Microscope).

5.2.8 Growth phase versus antagonistic activity of the cyanobacterial cultures

To determine the phase of growth of the cyanobacterial cultures at which maximum antibacterial property got exhibited, the cultures were inoculated in the above media and incubated until stationary phase attained. During this period, aliquots of 2-4 mL from each culture were centrifuged at 4^0 C at 10,000 ×g for 15 minutes once in every three days and the antivibrio activity of the supernatant determined by standard disc diffusion using selected isolates of *Vibrio* as described earlier in Chapter-2. The media and the stage/phase at which each culture exhibited maximum activity were recorded.

5.2.9 Biochemical analysis

Cells were harvested by centrifugation at 10,000 × g for15 min at 4 °C. The cell pellet was collected and washed repeatedly in saline (0.85% NaCl), dried in a vacuum desiccator at room temperature (28±0.5^{0}C) to constant weight. The dried cells were powdered using glass homogenizers and used for further analysis.

5.2.9.1 Total proteins, carbohydrates and lipids

The cyanobacterial dry cell mass was digested in 1 mol l^{-1} NaOH (100 µg dried powder/ml) in a boiling water bath for 20 minutes, cooled to room temperature, centrifuged at 10,000 × g for 10 min at 4 °C and 1 ml supernatants used for protein analysis (six samples were used for each culture). Total protein was determined by Bradford method using Bovine serum albumin (BSA) as the standard (Bradford 1976), total carbohydrates by Anthrone method (Hedge 1962) and total lipids by Sulpho-phospho-vanillin method (Barnes and Blachstock 1973).

5.2.9.2 Pigments

Chlorophyll content was determined following APHA (1995), carotenoids as described by Anon (1998), water soluble pigments (C-phycocyanin, Allophycocyanin and phycoerythrin) following Becker (1994).

5.3 Results

5.3.1 Primary screening and segregation

Out of fifty-four cyanobacterial cultures enricheded and screened, fifty-two showed antagonism to at least one isolate of *Vibrio* (Table 1). From this assemblage, seven cyanobacterial cultures numbered C-44, C-45, C-48 C-50, C-51, C-53, and C-54 could be segregated in such a way that antibacterial activity could be obtained against all the isolates in the panel of 35 *Vibrio*.

5.3.2 Secondary screening

Whenever antagonistic activity in the culture supernatant was detected (sample 1), it could be demonstrated in the cell lysate also (sample 2 and 3). However, at times the activity was observed only in the cell lysate, not in the culture supernatant (Table 2).

5.3.3 Effect of feeding cyanobacteria on the intestinal bacterial flora of *P.monodon* post larvae

Total heterotrophic bacteria in the larval rearing water and in the intestine of post larvae while feeding with the experimental diets (cyanobacterial cultures incorporated feeds) and commercial feed (control) were found out (Table 3), and identified as *Pseudomonas, Aeromonas, Vibrio, Alteromonas, Bacillus, Micrococcus* and *Staphylococcus*. However, heterotrophic bacterial flora in the intestine of post larvae fed on the

experimental diets with cyanobacterial cultures C-45, C-51 and C-54 exhibited comparatively higher generic diversity index and absence of *Vibrio* (Table 3).

5.3.4 Effect of feeding cyanobacteria on overall survival of post larvae of *Penaeus monodon* on challenge with *V. harveyi.*

A significant difference (P< 0.001) in the per cent survival of batches of post larvae fed on different cyanobacterial cultures was clearly observed. The least significant difference (LSD) at the 0.1% level was calculated to identify the significant treatments. This LSD was calculated to be 1.1051. Administration of cyanobacteria C-45, C-48, C-50, C-51, C-53 and C-54 resulted in significantly higher survival rate in the test group of animals compared to the control. However, on repeated challenge with *V.harveyi* the RPS of the batches of larvae fed on C-51 and C-54 was alone significantly higher compared to that of all others (Table 4). Based on the observations, the cultures C-51 and C-54 were segregated as the putative probionts for shrimp larval rearing systems.

5.3.5 Heterotrophic bacteria associated with cyanobacteria

Heterotrophic bacteria isolated from the cyanobacterial cultures C-51 and C-54 were identified to genera such as *Bacillus*, *Micrococcus*, *Moraxella*, *Planococcus* and *Pseudomonas.*

5.3.6 Identification of the segregated cyanobacteria

The cyanobacterial cultures C-51 and C-54 segregated on the basis of the above characteristics were found to be of single morphological type under the light and electron microscope and identified as species of *Synechocystis* based on the morphological features (Fig. 1, 2 and 3). The electron micrographs show the morphological difference of the two cultures,one with pili (*Synechocystis* C-51) and the other without (*Synechocystis* C-54).

5.3.7 Growth phase versus antagonistic activity of the cyanobacterial cultures

Synechocystis C-51 exhibited antagonistic activity in Walne's medium during log phase attaining maximum at the end of the phase. However, maximum antagonistic activity of *Synechocystis* C-54 was during stationary phase in ASN-111. For generating biomass under the experimental conditions medium Chu –111 and medium BG -11 were found comparatively more suitable, for C-51 and C-54 respectively.

5.3.8 Biochemical analysis

Total proteins, carbohydrates and lipids of C-51 were 34.74 ± 2.92, 22.82 ± 5.76 and 33.18 ± 12.23, and those of C-54 were 43. 43 ± 7.43, 20.57 ± 2.27 and 28.25_± 2.19 mg for 100 mg dry weight respectively (Fig. 4). Chlorophyll a and carotenoid content of C-51 were 6.9 ± 1.83 and 0.85 ± 0.13 µg and those of C-54, 3.99 ± 0.64 and 0.4±0.08 µg mg^{-1} dry weight respectively. Allophycocyanin and C-phycocyanin of C-51 were 4.48 ± 1.62 and 1.26 ± 0.39 mg, and those of C-54, 2.14 ± 0.55 and 0.5 ± 0.08 mg for 100 mg dry weight respectively. Phycoerythrin was absent in both the cultures of *Synechocystis*.

5.4 Discussion

Over the years application of probiotics in aquaculture has gained an impressive momentum focused at sustainability in production process. Different groups of microorganisms such as *Lactobacillus*, *Bacillus*, *Pseudomonas*, *Micrococcus*, certain strains of *Vibrio*, yeast, microalgae etc. have been reported as putative probiotics. (Gomez-Gil et al. 2000; Verschuere 2000; Irianto and Austin 2002). Several of them have been with antagonistic properties and are designated as 'biocontrol agents' (Verschuere 2000). It is in this context the present work was undertaken to screen cyanobacteria enriched from marine environment to be used as candidate probiotics in shrimp larval

production systems. More than 96% of the cyanobacterial culture brought under the study exhibited *in vitro* antagonism to at least one *Vibrio* isolate used.

The antivibrio property of the cyanobacteria investigated here was not extracellular alone but intracellular too strengthening the argument of Chetsumon et al. (1995) that bioactive molecules initially got accumulated intracellularly. If so, the cells might be used as probiotics to regulate the intestinal bacterial flora favorable to shrimp post larvae through antagonism.

Two of the enriched cyanobacterial cultures two were segregated based on three criteria of merits observed on administering along with diet and identified as *Synechocystis*. These cultures showed antagonism against known aquaculture pathogens such as *V.harveyi* and *V.parahaemolyticus* (Karunasagar et al. 1994; Jayaprakash et al 2005). It is postulated that the resistance of post larvae of *P.monodon* fed with the *Synechocystis* C-51 and C-54 to repeated challenges with *V.harveyi* may be due to the antagonistic probiotic effect. Moreover high protein content of *Synechocystis* C-51 and C-54 and the presence of carotenoids make the cultures ideal feed additives, which would enhance the weight gain and colouration of the shrimp post larvae. During the study post larvae fed with *Synechocystis* were found to be visibly more pigmented than the control animals. Besides, as suggested by Becker (1994), phycobiliproteins such as C-Phychocyanin and Allophycocyanin from different species of cyanobacteria have growth-promoting activity and immunostimulatory properties as well.

Apart from applying the cells coated onto the diet, the *Synechocystis* C-51 and C-54 can be bioaccumulated in rotifers (Sakamoto et al. 1996) and fed to crustacean larvae. Additionally, the cultures as such or the washed cells separately could be added directly to the larval rearing system.

Heterotrophic bacteria such as *Bacillus*, *Micrococcus*, *Moraxella*, *Planococcus* and *Pseudomonas* were found intimately associated with *Synechocystis* C-51 and C-54 in the autotrophic mineral-base medium not supplemented with any carbon source. Obviously the heterotrophs have been thriving on the extracellular products (ECP) of the cyanobacteria. Interestingly it was observed that the associated flora had been reported as part of normal intestinal bacterial flora of shrimp larvae (Singh et al. 1995; Singh et al.1998) and several of them had already been accepted as putative probiotic bacterial genera. This suggested that it would be better not to purify further the *Synechocystis* C-51 and C-54 from the associated heterotrophs as the cultures were destined to be used as probiotic preparations. This prompted to use *Synechocystis* C-51 and C-54 along with its heterotrophic bacterial flora for the feeding experiments. Significantly when the cyanobacterial cultures were fed to shrimp larvae and the intestinal bacterial flora evaluated, the generic diversity index of the intestinal heterotrophic bacterial flora had gone up with the concomitant reduction in *Vibrio* population, culminating in its absence in post larvae especially fed with *Synechocystis* C-51 and C-54. This is in accordance with the observations of Rico-Mora et al. (1998) who reported that a bacterial strain associated with *Skeletonema costatum* had prevented the establishment of *V. alginolyticus* in crustacean larvae. While the exact role-played by the associated heterotrophic bacterial flora of *Synechocystis* C-51 and C-54 could not be elucidated, the *Synechocystis* cultures as a whole unequivocally enhanced the survival of shrimp larvae against the challenge with *V. harveyi.*

In conclusion, the investigation suggests that the *Synechocystis* C-51 and C-54 could be considered as candidate probiotic organisms suitable for shrimp culture. These cyanobacterial cultures form part of the microbial culture collection of the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682016, Kerala, India and are catalogued as MCCB 114 and 115.

Table1 Preliminary screening of cyanobacterial cultures for antagonistic properties to vibrios and their spectrum of activity.

Cb No	Pathogenic Vibrios											Spec -trum
1	-											1
2	-											1
3	708(++)											1
4	1148(+)											1
5	684(+++)											1
6	1101b(+)											1
7	594b(+)											1
8	1142(+++)											1
9	708(++)	646(+++)										2
10	536(++)	1101b(++)										2
11	536(++)	1101b(++)										2
12	708(++)	684a(++)										2
13	708(++)	1442(+)										2
14	684a(++)	781(+)										2
15	708(++)	684a(++)										2
16	708(++)	684a(++)										2
17	702(++)	684a(++)										2
18	684a(++)	618a(+)										2
19	536(+++)	618a(+)										2
20	684a(++)	646(++)										2
21	708(++)	1142(++)										2
22	684a(++)	712(++)										2
23	708(++)	1101b(++)	646(+)									3
24	597(++)	932(+)	645(+)									3
25	708(++)	717(++)	781(+)									3
26	708(++)	629(++)	718aa(++)									3
27	702(++)	708(++)	536(+)									3
28	1142(++)	684(++)	932(+)									3
29	932(+)	712(+)	646(++)									3
30	708(++)	1101(++)	618a(+)	646(+++)								4
31	702(++)	708(++)	1142(++)	646(+++)								4
32	702(++)	708(++)	1101b(++)	646(++)								4
33	684a(+++)	618a(+)	1008a(+)	645(++)								4
34	708(++)	536(++)	1101b(++)	113(++)								4
35	702(++)	708(++)	717(++)	712(+)								4
36	702(++)	708(++)	684a(++)	718aa(++)								4
37	717(++)	684a(++)	536(++)	932(+)								4
38	708(+)	1108d(++)	1101b(++)	646(++)	645(+)							5
39	702(++)	708(++)	781(++)	646(+++)	645(+)							5
40	1142(++)	597(++)	721(++)	625(+)	11(+)							5
41	684a(++)	718aa(++)	1101b(++)	781(+)	932(+)							5
42	708(++)	684a(+++)	712(++)	646(++)	113(+)							5
43	702(++)	1142(++)	536(+++)	113(++)	1555(+)							5
44	1142(++)	684a(++)	1101b(++)	594(+)	598c_2(++)	645(++)						6
45	702(++)	708(+)	536(++)	721(++)	718aa(++)	781(++)						6
46	702(++)	708(++)	1142(++)	684a(++)	629b(+)	1008a(++)						6
47	702(++)	708(++)	1142(++)	629b(++)	536(++)	718aa(++)	594(+)	646(+++)				8
48	702(++)	708(++)	1142(++)	684a(++)	701(++)	712(+)	1008a(+)	646(+++)				8
49	702(++)	708(++)	717(++)	1142(++)	684a(++)	932(+)	712(++)	1008a(++)	646(++)			9
50	702(++)	708(++)	1142(++)	684a(++)	932(+)	712(+++)	618a(+)	1008a(++)	646(++)			9
51	702(++)	708(+)	717(++)	684a(++)	536(+)	1104a(++)	618a(+)	112(+)	113(++)	1555(+)		10
52	702(++)	708(++)	1142(++)	684a(++)	1101b(++)	932(+)	639b_1(++)	712(+++)	618a(+)	646(++)		10
53	702(+++ 536(++)	717(+++) 718aa(++)	1142(+++) 1142(+++)	684a(+++) 1104a(++)	684a(++) 932(+)	629b(+++) 598c_2(+)	727(+++) 594b(+)	880c(+++) 1128a_2 (+++)	1108(+++) 1131(++)	597(+++) 1555(+)		20
54	702(+++) 625(++) 1101b(++)	708(+++) 1142b (+++) 781(+)	717(+++) 1104a(+) 618a(+)	1142(++) 932(+) 646(+)	684a(+++) 598c_2(++) 644b(+)	629b(+++) 594b(+) 1555(+)	727(+++) 1561(+++)	880c(+++) 1128a_2 (+++)	1108(+++) 639b1(+)	597(+++) 719(+)	536(++)	27

Cb: Cyanobacterial cultures

(-): No Activity, (+): 5mm<D<8mm, (++): D≤ 10mm, (+++): D>10mm.D: Diameter of the inhibition zone in millimete^{2}rs including the diameter of the disc (5mm).

Table 2 Secondary screening of the segregated cyanobacterial cultures based on the diameter of the halo zone and spectrum of activity.

Cb: No:	*Vibrio* Species	Supernatant (Sample 1)	Cell lysate (Sample 2)	Cell debris (Sample 3)	Live cells (Sample 4)
C-44	*V.cholerae*	- - -	- - -	++/++/++	- - -
	V.mediterranei	++/++/++	+++/+++/+++	+++/+++/+++	++/++/++
C-45	*V.cholerae*	- - -	- - -	+++/+++/+++	- - -
	V.orientalis	- - -	- - -	+++/+++/+++	- - -
	V.splendidus	- - -	- - -	+++/+++/+++	- - -
C-48	*V.mediterranei*	- - -	- - -	++/++/++	- - -
C-50	*V.alginolyticus*	++/++/++	++/++/++	++/++/++	++/++/++
	V.cincinatensis	++/++/++	++/++/++	++/++/++	++/++/++
	V.cholerae	++/++/++	++/++/++	++/++/++	++/++/++
	V.orientalis	++/++/++	++/++/++	++/++/++	++/++/++
	V.parohaemo-lyticus	++/++/++	++/++/++	++/++/++	++/++/++
C-51	*V.cincinatensis*	- - -	+++/+++/+++	+++/+++/+++	+/++/++
	V.fulvialis	- - -	+++/+++/+++	- - -	- - -
	V.harveyi	- - -	++/++/++	++/++/++	- - -
	V.orientalis	++/++/++	++/++/++	+++/+++/+++	+++/+++/+++
	V.parohaemolyticus	+++/+++/+++	++/++/++	+++/+++/+++	++/++/++
	V.Proteolyticus	- - -	++/++/++	+++/+++/+++	+++/+++/+++
C-53	*V.orientalis*	+++/+++/+++	- - -	+++/+++/+++	+++/+++/+++
C-54	*V.cincinatensis*	++/++/++	++/++/++	++/++/++	- - -
	V.harveyi	- - -	++/++/++	++/++/++	- - -
	V.marinus	- - -	- - -	++/++/++	- - -
	V.mimicus	- - -	++/++/++	++/++/++	- - -
	V.orientalis	- - -	++/++/++	++/++/++	- - -
	V.parohaemolyticus	- - -	++/++/++	++/++/++	- - -
	V.vulnificus	- - -	- - -	++/++/++	- - -

Cb: Cyanobacterial cultures

(-): No Activity, (+): 5mm<D<8mm, (++): D≤ 10mm, (+++): D>10mm.D: Diameter of the inhibition zone in millimeters including the diameter of the disc (5mm).

Table 3 Bacterial populations and Shannon Index of Microbial Diversity on administering the Cyanobacteria Coated feed in larval rearing tanks.

Cyanobacterial Feed	TPC count (CFU mL^{-1}) in water	TPC count (CFU mL^{-1}) in water	Shannon Index	
			Water	Larvae
C-44	95×10^4 - 123×10^4	60×10^4- 456×10^4	0.64-0.69	0.64-0.64
C-45	129.6×10^4- 130.6×10^4	32×10^4- 261×10^4	0.32-1.24	1.09-1.6
C-48	79.6×10^4 - 121.5×10^4	73×10^4 - 148×10^4	0.59-1.03	0.59-1.33
C-50	109.2×10^4- 129.6×10^4	76.7×10^4- 296×10^4	0.69-1.04	0.637-0.69
C-51	129.7×10^4- 195.9×10^4	10×10^4 – 127×10^4	0.69-0.693	0.637-0.69
C-53	155.1×10^4- 164×10^4	61×10^4 – 203×10^4	0.69-0.693	0.637-0.69
C-54	164×10^4 - 181×10^4	66×10^4 – 220×10^4	1.011-1.561	1.561-1.6
Control (Normal feed)	110.1×10^4 - 146.9×10^4	31×10^4- 624×10^4	0.867-1.039	0.450-0.95

Table 4 Survival of *P. monodon* larvae fed with cyanobacteria and relative percentage survival (R.P.S) after challenge with *V. harveyi*

	Control	Experimental diets with cyanobacterial cultures						
		C-44	C-45	C-48	C-50	C-51	C53	C-54
Average percentage survival	76.6 ± 0.3%	83.86 ± 0.26%	90.55 ± 0.21%	89.25 ± 0.22%	91.5 ± 0.2%	96.77 ± 0.13%	75.5 ±0.3%	98.94 ± 0.07%
R.P.S after 1st callenge with *V. harveyi*	-	23.5	29.4	23.5	47.1	82.4	5.9	70.6
R.P.S after 2nd callenge with *V. harveyi*	-	18.75	37	0	48	59.3	18.75	74.1

Chapter-6

Conclusions

Chapter 6
Conclusions

In prawn larval rearing systems vibriosis has been designated as a systemic bacterial infection caused by several species of *Vibrio* such as *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. fluvialis*, *V. neris*, *V .penaeicida* etc. and have been reported to cause serious infections and consequent low shrimp production. One of the options adopted to tide over the situation has been administration of antibiotics. May be due to their continuous administration the associated *Vibrio* were found exhibiting multiple antibiotic resistance and several of them were more virulent. The abuse of antimicrobials can result in the development of resistant strains of bacteria and the resistant factor can be transferred to other diverse strains too including human pathogens. As an alternate management strategy bacterial cultures or their products as probiotics with antagonistic properties have been proposed, and subsequently several applications have been observed. Probiotics, in the context of the aquatic environment, are defined as live microbial adjuncts having beneficial effects on the host by modifying the host-associated or ambient microbial community, ensuring the improved use of feed or enhancing its nutritional value, enhancing the host's response towards diseases, or by improving the quality of its ambient environment.

Present work is aimed at development of an appropriate bioprocess technology for mass production of the already identified aquaculture probiotics such as *Pseudomonas* MCCB 102, *Pseudomonas* MCCB 103 and *Micrococcus* MCCB 104. Under the work element, culture conditions and media ingredients were optimized for probiotics with the help of statistically designed experiments. The conventional optimization method involves

varying one parameter at a time and keeping the others constant and this often does not bring about the effect of interaction of various parameters. But the statistical experimental design techniques in fermentation process involve study of the main and interacting effect of factors and their by it can improve product yield and reduce process variability, time, coast etc. response surface methodology (RSM) is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. This technique was used for designing media, optimization of culture conditions and for the production of enzymes, antibiotics, bacteriocins, organic acid, solvents, polysaccharides etc. However, it has not yet been reported for probiotics, especially in aquaculture.

Culture conditions of the probiotics (*Pseudomonas* MCCB 102, *Pseudomonas* MCCB 103 and *Micrococci* MCCB 104) such as salinity, pH and temperature in ZoBell's broth were standarardized to economize the production, considering both biomass and antagonistic activity against *V. harveyi.* This was accomplished *by* employing statistically designed experiments with the help of computer software Design Expert (version 6.0.9, Stat Ease, Minneapolis, M N). A full factorial central composite design of response surface methodology (RSM) was used for the above study.

Subsequently new media were designed by employing the same statistical modeling technique for enhanced production of antivibrio molecule and biomass. Twenty-four C-sources were screened as sole source of carbon for growth. Subsequently seven of them were selected for both *Pseudomonas* MCCB 102 and 103 based on their role as sole source of carbon for further screening at 1% level in a mineral medium supplemented with 1% sodium chloride, the concentration of which was obtained from the above experiment. Five C-sources were selected for *Micrococcus* MCCB 104 and screened at 1% level in a mineral medium supplemented with 2% sodium chloride. Different

N-sources were also screened in the same mineral based medium with 10 gl^{-1} glucose and without N-source. Growth factors such as amonoacids and vitamins were screened in a pattern of one at a time for biomass and antagonistic activity. Variables such as glucose, glycerol, urea, sodium chloride and mineral salt solution were chosen for *Pseudomonas* MCCB 102 and mannitol, glycerol, sodium chloride, urea and mineral salts solution were chosen for *Pseudomonas* MCCB 103. Other growth factors did not have any effect on biomass and activity. Optimum concentrations of the selected ingredients were found out using RSM. Two media were developed for *Pseudomonas* MCCB 102 and 103, one for maximum biomass production and the other for maximum antagonistic compound production. Optimum concentration of the ingredients for the maximum biomass production of *Pseudomonas* MCCB 102 were glucose:19.8 g l^{-1}, glycerol : 4.1 g l^{-1}, sodium chloride : 14.5 g l^{-1}, urea :2.4 g $l^{-1}$and mineral salts solution 15.5 ml $l^{-1}$and that of antagonistic compound production were glucose: 2 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5 g l^{-1}, urea :2.1 g $l^{-1}$and mineral salts solution 17.4 ml l^{-1}.However, optimum concentration of the ingredients for the maximum biomass production of *Pseudomonas* MCCB 103 were mannitol: 20 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5 g l^{-1}, urea :3.3 g $l^{-1}$and mineral salts solution 20 ml l^{-1} and that of antagonistic compound production were mannitol: 2 g l^{-1}, glycerol : 20 g l^{-1}, sodium chloride : 5.1 g l^{-1}, urea :3.6 g $l^{-1}$and mineral salts solution 20 ml l^{-1}. In the case of *Micrococcus* MCCB 104, good correlation was obtained between biomass and antagonistic activity hence concentrations of ingredients were same for both biomass and antagonistic activity i.e., glucose (17.4), lactose (17 g l^{-1}), sodium chloride (16.9 g l^{-1}), ammonium chloride (3.3 g l^{-1}) and mineral salts solution (18.3 ml. l^{-1}).

Antagonism is not uncommon in aquatic environment but the ecological importance and nature of the antagonistic compounds are not well

investigated. A bulk of literature is available on the occurrence of bacterial strains showing *in vitro* antagonism towards known pathogens in aquaculture. These antagonistic bacteria bring forth inhibition by altering composition of microbial flora by releasing chemical substances that have bactericidal or bacteriostatic effect on other microbial communities or by competition for nutrients or available energy. The probiotics used for the present study showed antagonism against a range of vibrios. Subsequently the phenomenon of antagonism became a subject of research of this group, as investigations on the nature of the inhibitory compounds and their role in eliminating pathogens were found inevitable for their application.

In vitro antagonism of *Pseudomonas* against a range of vibrios, point to the possibility of using the molecule responsible for the antagonistic properties as viable alternatives to the conventional antibiotics. However, a detailed investigation of the nature of the anti-vibrio compounds produced by *Pseudomonas* had not been carried out. In general the antibacterial properties of antagonistic bacteria are preferably due to the production of antibiotics, bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide and/or alteration of pH. It was observed that *Pseudomonas* MCCB102 and *Pseudomonas* MCCB 103 secreted antagonistic compounds into the growth medium and in the present study the filter-sterilized supernatants were heat stable, pH insensitive and the mode of action of the compound was bactericidal. The antagonistic principles produced by both the organisms are thermostable capable to withstand 100°C and shelf life of the compound was suitable for application (More than 12 months at 4°C).

The active fraction was soluble in chloroform, could be purified by TLC and the absorption maxima determined. HPLC analysis of the bioactive compound showed only a single peak having retention time same as that of pyocyanin standard. The identity of the molecule was confirmed by mass

spectroscopy and NMR analysis. 20 ppm of pyocyanin can eliminate *V. harveyi* (10^6 cfu/ml) completely within 18 hours. Moreover newly designed media increased antivibrio compound concentration up to 100 ppm so that the cell free supernatant as such can be used therapeutically.

Even though several potential probiotic organisms have been isolated and identified for aquaculture, cyanobacteria (blue green algae) have not yet been identified as candidate probiotics. However, marine cyanobacteria, in particular, have been in focus for quite some time as potential sources of antagonistic compounds. Secretion of antimicrobial substances is an important part of the competition process in the natural environment having a beneficial effect on the host by modifying the host-associated ambient microbial community. Moreover cyanobacteria are a good source of nutrients and the cell contents of ingested blue greens may promote the growth of gut bacteria and accelerate the process of nutrient absorption.

Two cultures of *Synechocystis* spp. (C-51and C-54) were isolated from marine environment by an enrichment technique and investigated as possible probiotics for shrimp larvae. They were primarily screened from a collection of fifty-four enrichment cultures based on their extracellular antagonistic activity against a panel of target vibrios, and seven of them were then evaluated, based on their intracellular antagonistic properties. On feeding shrimp larvae with the cultures (C-51 and C-54), a comparatively higher generic diversity index was obtained with less or no *Vibrio* in their digestive system. On repeated challenge with *V.harveyi* the relative percentage survival of the larvae was significantly higher for both of the cyanobacterial cultures. The electron micrographs show the morphological difference of the two cultures, the *Synecocystis* sp. C-51 with pili and *Synecocystis* sp. C-54 without. Total proteins, lipids, carbohydrates, chlorophyll, carotinoids, phycobiliproteins etc were found out. The cyanobacterial cultures have a high

protein and carotinoid content, making them suitable as feed additives. Heterotrophic bacteria such as *Bacillus*, *Micrococcus*, *Moraxella*, *Planococcus* and *Pseudomonas* were found to be intimately associated with the segregated cyanobacteria, which could also contribute to the modification of intestinal flora of the shrimps. The study suggested that the *Synechocystis* spp. might be used as probiotics to carry out two functions: firstly, controlling *Vibrio* through antagonism and secondly, enhancement of generic diversity of the intestinal flora, regulating its composition favorable to shrimp larvae. Besides applying coated on to diet they may be bio-accumulated or/ and added to the rearing water for larval consumption.

Concisely the objectives accomplished suggest that optimization of culture conditions and newly designed media significantly enhance biomass and antagonistic compound production of *Pseudomonas* MCCB 102, *Pseudomonas* MCCB 103 and *Micrococcus* MCCB 104. The antagonistic compounds produced by *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 were isolated, purified and characterized as pyocyanin based on LC-MS and ^{1}HNMR analysis. Persistence of antagonistic property up to 100 °C, shelf life at 4 °C, stability in different organic solvents and varying pHs makes the molecule ideal for aquaculture application. However, more work has to be done in this direction for its application in shrimps/prawns hatcheries as well as in grow out systems. The study also suggested that the *Synechocystis* spp. might be used as probiotics to carry out two functions: control of *Vibrio* through antagonism and enhancement of generic diversity of the intestinal microbial flora by regulating its composition favorable to shrimp larvae.

References

References

Abraham TJ, Manley R (1995) Luminous and non-luminous *Vibrio harveyi* associated with shell disease in cultured *Penaeus indicus*. J Aqua Trop 10:273-276

Abraham TJ, Manley R, Palaniappan R, Dhevendaran K (1997) Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. J Aqua Trop 12:1-8

Adinarayana KP, Ellaiah B, Srinivasulu R, Devi B, G A (2003) Response surface methodological approach to optimize the nutritional parameters for neomycin production by *Streptomyces marinensis* under solid state fermentation. Proc Biochem 38:1565-1572

Aguirre-Guzman G, Filipe AV (2000) Infectious disease in shrimp species with aquaculture potential. Recent Res Devl Microbiol 4:333-348

Aguerre-Guzman G, Vazquez-Juarez R, Ascencis F (2001) Difference in the susceptibility of American white shrimp larval substages (*Litopenaeus vannamei*) to four *Vibrio* sp. J Invertibr Pathol 78:215-219

Alapide-Tendencia EV, Dureza LA (1997) Isolation of *Vibrio* spp. from *Penaeus monodon* (Fabricus) with red disease syndrome. Aquaculture 154:107-114

Allen EJ, Nelson EW (1910) On the artificial cultures of marine planktonic organisms. J Mar Biol Ass 8:421

Alsina M, Blanch AR (1994) A set of keys for biochemical identification of environmental *Vibrio* species. J Appl Bacteriol 76:79-85

Anjaiah V, Abeysinghe S, Koedam N, Cornelis P (1998) Biocontrol of Plant Diseases by *Fluorescent Pseudomonas* In: Pandey A (ed) Advances in Biotechnology. Educational Publishers & Distributors New Delhi, p 183-194

Anon (1998) GIS based information system for critical coastal habitats in India. In: Manual on methodology for biological parameters. Department of ocean development, Government of India, Channai, India

APHA (1995) Standard methods for the examination of water and waste water, American Public Health Association, Washington D. C

Arias CR, Macian MC, Anzar R, Garay E, Pujalte MJ (1999) Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. J Appl Microbiol 86:125-134

Austin B, Baudet E, Stobie M (1992) Inhibition of bacterial fish pathogens by *Tetraselmis suezica*. J Fish Dis 15:55-61

Austin B, Billaud AC (1990) Inhibition of bacterial fish pathogens, *Serratia liquefaciens*, by an antibiotic-producing isolate of *Planococcus* recovered from sea water. J Fish Dis 13:553-556

Austin B, Day JG (1990) Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture 90:389-392

Austin B, Stuckey LF, Robertson PAW, Effendi I, Griffith DRW (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio angulliarum* and *Vibrio ordalli*. J Fish Dis 18:93-96

Balcazar JL, de Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Muzquiz JL (2006) The role of probiotics in aquaculture. Vet Microbiol 114:173-186

Barnes H, Blachstock J (1973) Estimation of lipids in marine animals and tissues: Detailed investigation of the sulphophosphovanillin method for 'total lipids'. Mar Biol Ecol 2:103-118

Baron SS, Rowe JJ (1981) Antibiotic action of pyocyanin. Antimicrob Agents Ch 20:814-820

Baticados MCL, Lavilla - Pitogo CR, Cruz- Lancierda ER, De la Pena LD, Sunaz NA (1990) Studies on the chemical control of luminous bacteria *V. harveyi* and *V. splendidus* isolated from diseased *P. monodon* larvae and rearing water. Dis Aquat Org 9:133-139

Baticados MCL, Paclibare JO (1992) The use of chemotheraputic agents in aquaculture in the Philippines. In: Shariff M, Subasinghe RP, Arthur JR (eds) Fish health section, Diseases in Asian Aquaculture. Asian Fisheries Society, Manila, Philippines, p 531-546

Becker EW (1994) Measurement of algal growth. In: J. Baddiley NHC, I .J. Higgins and W. G. Potter (ed.), Microalgae: biotechnology and microbiology. Cambridge University Press, U.K., p 59-60

Beg QK, Sahai V, Gupta R (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Proc Biochem 39:203-209

Bender J, Phillips P (2004) Microbial mats for multiple applications in aquaculture and bioremediation. Bioresource Technol 94:229-238

Bly JE, Quiniou SMA, Lawson LA, Clem LW (1997) Inhibition of *Saprolegnia* pathogenic for fish by *Pseudomonas fluorescens*. J Fish Dis 20:35-40

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantity of proteins utilizing the principle of protein dye binding. Anal. Biochem. 72:248-254

Brodhagen M, Henkels MD, Loper JE (2004) Positive autoregulation and signeling properties of pyoluteorin, an antibiotic produced by the biological control organism *Pseudomonas fluorescens* Pf-5. Appl Environ Microbiol 70:1758-1766

Brunt J, Austin B (2005) Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 28:693–701

Cahill MM (1990) Bacterial flora of Fishes: a review. Microbial Ecol 19:21-41

Canizares-Villanueva A, Ontiveros-Arredondo C (1993) Comportamicnto cinetico de un cultivo mixto de la microalga Marina *Teteraselmis chuii*, 7 dos bacterias. Rev Invest Mar 14:86-91

Chabrillon M, Arijo S, Diaz-Rosales P, Balebona MC, Morinigo MA (2006) Interference of *Listonella anguillarum* with potential probiotic microorganisms isolated from farmed gilthead seabream (*Sparus aurata*, L.). Aquac Res 37:78-86

Chabrillon M, Rico RM, Balebona MC, Morinigo MA (2005) Adhesion to sole, *Solea senegalensis* Kaup mucus of microorganisms isolated from fish and their interaction with *Photobacterium damselae* subsp. piscicida. J Fish Dis 28:229-237

Chang C-I, Liu W-Y (2002) An evaluation of two probiotic bacterial strains, *Enterococcus faccium* SF 68 and *Bacillus toyoi*, for reducing edwardsiellosis in cultured European eel, *Anguilla angulla* L. J Fish Dis 25:311-315

Chang PC, Blackwood AC (1969) Simultaneous production of three phenazine pigments by *Pseudomonas aeruginosa* Mac 436. Can J Microbiol 15:439-444

Chen SN, Huang SL, Kuo GH (1992) Studies on the epizootiology and pathogenicity of bacterial infections in cultured giant tiger prawn, *P. monodon* in Thaiwan. In: Fulks W, Main KL (eds) Diseases of cultured penaeid shrimp in Asia and the United States. The Oceanic Insitute, Hawaii, p 195-205

Chetsumon A, Maeda I, Umeda F, Yagi K, Miura Y, Mizoyuchi (1995) Continuous antibiotic production by an immobilized cyanobacterium in a seaweed-type bioreactor. J Appl Phycol 7:135-139

Chu M, Mierzwa R, Xu L, He L, Terracciano J, Patel M, Zhao W, Black TA, Chan T-M (2002) Structure of Such 419560, a novel α- pyrone produced by *Pseudomonas fluorescens*. J Antibiot 55:215-218

Chythanya R, Karunasagar I, Karunasagar I (2002) Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. Aquaculture 208:1-10

Clark CJ, Langley D, Bushel ME (1995) Oxygen limitation can induce secondary metabolite formation—investigations with miniature electrodes in shaker and bioreactor cultures. Microbiol UK 141:663–669

Cohen Y (1975) Dynamics of prokariotic photosynthetic communities of the solar lake, Hebrew University

Costa R, Mermoud I, Koblavi S, Morlet B, Haffner P, Berthe B, Legroumellec M, Grimont P (1998) Isolation and characterization of bacteria associated with a *Penaeus stylirostris* disease (Syndrome 93) in New Caledonia. Aquaculture 164:297-309

Cox CD, Graham R (1979) Isolation of iron-binding compound from *Pseudomonas aeruginosa*. J Bacteriol 137:357-364

Dahod SK (1999) Raw material selection and medium development for industrial fermentation process. In: Demain AL, Davis JE (eds) Manual of industrial microbiology and biotechnology. American Society for Microbiology Press, Wasington D C, p 213-265

Dalmin G, Kathiresan K, Purushothaman A (2001) Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem. Indian J Exp Biol 39:939-942

Demain AL (1986) Control of secondary metabolism in Actinomycetes, Biological, Biochemical and Biomedical Aspects of Actinomycetes. In: Szabo G, Biro S, Goodfellow M (eds) Proc. 6 international symposium on Actinomycetes Biology. Budapest: Academiav, Kiado, p 215

DeSchrijver R, Ollevier F (2000) Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effect of dietary administration of *Vibrio proteolyticus*. Aquaculture 186:107-116

Doull JC, Vining LC (1990) Physiology of antibiotic production in actinomyces and some control mechanisms Biotech Adv 8:141–158

Eleonor V, Alapide-Tendencia EV, Dureza LA (1997) Isolation of *Vibrio* spp. from *Penaeus monodon* (Fabricius) with red disease syndrome. Aquaculture 154:107-114

Elibol M (2004) Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3 (2) with response surface methodology. Proc Biochem 39:1057-1062

Essar WD, Eberly L, Hadero A, Crawfords IP (1990) Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: Interchangeability of the two anthranilate synthases and evolutionary implications. J Bacteriol 172:884-900

Fernandez RO, Pizarro RA (1997) High-performance liqid chromatography analysis of *Pseudomonas aeruginosa* phenazines. J Chromatogr A 771:99-104

Fuller R (1989) A review: probiotics in man and animals. J. Appl. Bacteriology 66:365-378.

Gatesope FJ (1991) *Bacillus sp.* spores as food addictive for the rotifer *Brachionus plicatilis*: improvement of their bacterial environment and their dietary value for larval turbot, *Scophthalmus* L. In: Kaushik S (ed) Proceedings of the 4th international symposium on Fish nutrition and feeding. Institute National de la Recherche Agronomique, Paris, France

Gatesoupe FJ (1994) Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus,* against pathogenic *Vibrio*. Aquat. Living Resour 10:239-246

Gomez-Gil B, Roque A, Velasco-Blanco (2002) Culture of *Vibrio alginolyticus* C7b, a potential probiotic bacterium with the micro-algae *Chaetoceros muelleri.* Aquaculture 211:43-48

Gomez-Gil B, Roque A, Turnbull J (2000) The use and selection of probiotic bacteria for use in culture of larval aquatic organisms. Aquaculture 191:259-270

Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas flurescens* AH2, a possible probiotic treatment of fish. Appl Environ. Microbiol. 65:969-973

Guerra NP, Pastrana L (2002) Nisin and Pediocin production on mussel-processing waste supplemented with glucose and five nitrogen sources. Lett Appl Microbiol 34: 114-118

Hameed ASS (1993) A study of the aerobic heterotrophic bacterial flora of hatchery-reared eggs, larvae and post-larvae of *Penaeus indicus.* Aquaculture 117:195-204

Hameed ASS (1994) Studies on the chemical control of *Vibrio campelli*-like bacterium affecting *Penaeus monodon* larvae. Aquaculture 27:1-9

Hameed ASS, Rahaman KH, Alagan A, Yoganandhan K (2003) Antibiotic resistance in bacteria isolated from hatchery-reared larvae and post-larvae of Aquaculture 217:39–48

Hays A, Hobbs G, Smith CP, Oliver SG, Butler PR (1997) Environmental signals triggering methylenomycin production by *Streptomyces coelicolor*A3 (2). J Bacteriol 179:5511–5515

Hedge JE (1962) Analysis and preparation of sugars. In: Whistler RL, Wolfrom ML (eds) Methods in carbohydrate chemistry. Academic Press, New York, p 335-343.

Herdman M, Castenholz RW, Itman I, Waterbury JB, Rippka R (2001) Phylum BX cyanobacteria, subsection 1. In: Garrity GM, Boone DR, Castenholz RW

(eds) Bergey's manual of systematic bacteriology, Vol 1. Williams and Wilkins, Baltimore, MD, p 493-514

Hisbi D, Vandenberghe J, Robles R, Verdonck L, Swings J, Sorgeloos P (2000) Characterization of *Vibrio* and related bacteria associated with shrimp *Penaeus monodon* larvae in Indonesia. Asian Fish Sci 13:57-64

Hong HA, Duc LH, Cutting SM (2005) The use of bacterial spore formers as probiotics. FEMS Microbiology Reviews 29: 813 -832

Huang L, Lu Z, Yuan Y, Lu F, Bie X (2006) Optimization of a protective medium for enhancing the viability of freeze-dried *Lactobacillus delbrueckii* sub. sp. *bulgaricus* based on response surface methodology. J Ind Microbiol Biot 33:55-61

Hubert E, Lobos O, Brevis P, Padilla C (1998) Note: Purification and characterization of the bacteriocin PsVP -10 produced by *Pseudomonas sp.* J Appl Microbiol 84:910-913

Imada C, Simidu U, Taga N (1985) Isolation and characterization of marine bacteria producing alkaline protease inhibitor. Bull Jpn Soc Sci Fish 51:799-803

Irianto A, Austin B (2002) Review: Probiotics in aquaculture. J Fish Dis 25:633-642

James PDA, Edwards C, Dawson M (1991) The effect of temperature and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* growth in a chemostat. J Gen Microbiol 137:1715–1720

Jayaprakash NS (2005) Antibody based diagnostics for detection of vibrios and their biological control using antagonistic bacteria in *Macrobrachium rosenbergii* larval rearing system. . Ph. D Thesis, Cochin University of Science and Technology, p.67-89

Jayaprakash NS, Pai SS, Anas A, Preetha R, Philip R, Singh ISB (2005) A marine *Micrococcus* MCCB104 antagonistic to vibrios in prawn larval rearing systems. Dis Aquat Org 68:39-45

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

Joborn A, Olsson CJ, Kjelleberg S (1997) Colonization in the fish intestinal tract and production of inhibitory substance in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain KI. J Fish Dis 20:383-392

Kaiser D, Onken U, Sattler I, Zeeck A (1994) Influence of increasing the dissolved oxygen concentration on the formation of secondary metabolite by manamycin producing *Streptomyces parvulus*. Appl Microbiol Biotechnol 41:309-312

Kanner D, Gerber NN, Bartha R (1978) Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa*. J Bacteriol 134:690-692

Karunasagar I, Otta SK, Karunasagar I (1996) Biofilm formation by *Vibrio harveyi* on surfaces. Aquaculture 140:241-245

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

Kerr JK, Taylor GW, Rutman A, Hoiby N, Cole, PJ, Wilson R (1999) *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. J Clin Pathol 52:385-387

Knight M, Hartman PE, Hartman Z, Young VM (1979) A new method of preparation of pyocyanin and demonstration of an unusual bacterial sensitivity. Anal Biochem 95:19-23

Kogure K (1998) Bioenergetics of marine bacteria Curr Opin Biotechnol 9 278–282

Kulik MM (1995) The potential for using cyanobacteria and algae in the biological control of plant pathogenic bacteria and fungi. . Eur J Plant Pathol 101:585-599

Kumar K, Lakshmnan A, Kannaiyan S (2003) Bioregulatory and therapeutic effects of blue green algae. Indian J Microbiol 43:9-16

Kumar RS, Ayyadurai N, Pandiaraja P, Reddy AV, Venkateswarlu Y, Prakash O, Sakthivel N (2005) Characterization of antifungal metabolite produced by a new strain of *Pseudomonas aeruginosa* PUPa 3 that exhibits broad spectrum antifungal activity and biofertilizing traits. J Appl Microbiol 98:145-154

Lavilla - Pitogo CR (1995) Bacterial disease of penaeid shrimp: an Asian view. In: Shariff M , Arthur JR, Subasinghe RP (eds) Disease in Asian Aquaculture II, Fish Health section,. Asian fisheries Society, Manila, Phillippines., p 107 - 121. I

Lavilla-Pitogo CR, Baticados MCL, Cruz-Lacierda ER, de la Pena LD (1990) Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture 91:1-13

Leal-Sanchez MV, Jimenez-Diaz R, Maldonado-Barragan A, Garrido-Fernandez A, Ruiz Barba JL (2002) Optimization of Bacteriocin Production by Batch Fermentation of *Lactobacillus plantarum* LPCO10. Appl Environ Microbiol 68: 4465-4471

Lemos ML, Toranzo AE, Barja JL (1985) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweed. Microbial Ecol 11:149-163

Lightner DV, Bell TA, Redman RM, Mohney LL, Natividad JM, Rukyani A, and Poernomoa. (1992) A review of some major diseases of economic significance in Penaeid Prawns/Shrimps of America and Indo Pacific. In Shariff M, Subasinghe RP and. Arthur JR (eds) Diseases in Asian Aquaculture I . Fish Health Section, Asian Fisheries Society, Manila, Philippines. . p 57

Lightner DV (1996) Diseases of cultured penaeid shrimp. In: Mcvey. JP (ed) Handbook of Mariculture, Crustacean Aquaculture. CRC Press, Boca Raton, FL, p 393–486

Lightner DV (1998) Disease diagnosis and control in North American marine aquaculture In: Sindermann CJ, Lightner DV (eds) Aquaculture and Fisheries Science Vol 17. Elsevier, Amsterdam, p 42-47

Liong MT, Shah NP (2005) Optimization of cholestrol removal by probiotics in the presence of prebiotics by using a response surface method. Appl Environ Microbiol 71:1745-1753

Lio-Po GD, Lean˜o EM, Pen˜aranda MMD, Villa-Franco AU, Sombito CD, Guanzon Jr NG (2005) Anti-luminous *Vibrio* factors associated with the green water grow-out culture of the tiger shrimp *Penaeus monodon*. Aquaculture 250 1– 7

Lizarraga-Partida M, Montoya-Rodriguez L, Gentrop-Funes V (1997) The use of bacterial counts in two mexican shrimp hatcheries Cienc Mar 23:129-140

Long RA, Azam F (2001) Antagonistic interactions among marine pelagic bacteria Appl Environ Microbiol 67:4975-4983

Macey BM, Coyne VE (2005) Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. Aquaculture 245:249-261

Martain JF, Demain AL (1980) Control of antibiotic synthesis. Microbiol Review 44: 230-231

Marwick JD, Plillip CW, Burgess JG (1999) Bioprocess intensification for the production of novel marine bacterial antibiotic through bioreactor operation and design. Mar Biotechnol 1:495-507

McCarthy SA, Johnson RM, Kakimoto D, Sakata T (1985) Effects of various agents on the pigment (violacein) and antibiotic production of *Alteromonas leutoviolacea*. Bull Jpn Soc Sci Fish 51:1115–1121

Mermoud I, Costa R, Ferre O, Goarant C, Haffner P (1998) 'Syndrome 93' in New Caledonia outdoor rearing ponds of *Penaeus stylirostris*: history and description of three outbreaks. Aquaculture 164:323-335

Messenger AJ, Turner JM (1983) Effect of growth conditions on phenazine production by *Pseudomonas phenazinium*. J Gen Microbiol 129:1013–1018

Mohney KL, Lightner DV, Bell TA (1994) An epizootic of vibriosis in Ecudorian pond-reared *Pennaeus vannamei* Boone (Crustacea : Decapoda). Journal of World Aquaculture Society 25 116-125

Moriarty DJW (1997) The role of microorganisms in aquaculture ponds. Aquaculture 151:333-349

Moriarty DJW (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164:351-358

Morist A, Montesinos JL, Cusido JA, Godia F (2001) Recovery and treatment of *Spirulina platensis* cultured in a continuous photobioreactor to be used as food. Proc Biochem 37:535-547

Mundt S, Kreitlow S, Nowotny A, Effmert U (2001) Biochemical and pharmacological investigation of selected cyanobacteria. Int J Hyg Environ Health 203:327-334

Myogga H, Yoshimizu M, Tazmia K, Ezuma Y (1995) Purification of an antimicrobial substance produced by *Alteromonas* sp. and its viricidal activity against fish viruses. Fish Pathol 30:15–32.

Nair S, Tsukamoto K, Shimidu U (1985) Distribution of bacteriolytic bacteria in the coastal marine environments of Japan. Bull Jpn Soc Sci Fish 51:1469-1473

Nikoskelainen S, Ouwehand AC, Bylund G, Salmimen S, Lilius E-M (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). Fish Shellfish Immun 15:443-452

Nithimathachoke C, Pratanpipat P, Thongdaeng K, Withyachumnarkul B, Nash G (1995) Luminous bacterial infection and pond reared *Penaeus monodon*. Asian Shrimp News 23:1-4

Nogami K, Maeda M (1992) Bacteria as biocontrol agents for rearing larvae of the crab *Portunus trituberculatus*. Can J Fish Aquat Sci 49:2373-2376

Norman RS, Moeller P, McDonald TJ, Morris PJ (2004) Effect of pyocyanin on a crude-oil-degrading microbial community. Appl Environ Microbiol 70:4004-4011

Nykanen A, Vesanen S, Katho H (1998) Synergistic antimicrobial effect of nisin whey permeate and lactic acid in microbes isolated from fish. Lett Appl Microbiol 27:345-348

Okami Y, Kusasawa S, Hirose, Y (1980) A new glucanase production by a marine *Bacillus*. J Antibiot 44:1191-1192.

Olafsen JA (2001) Interactions between fish larvae and bacteria in marine aquaculture Aquaculture 200:223-247

Oliver JD (1982) Taxonomic scheme for the identification of marine bacteria. Deep Sea Res 297:95-798

Olsson JC, Joborn A, Westerdahl A, Blomberg L, Kjelleberg S, Conway PL (1998) Survival, persistence and proliferation of *Vibrio anguillarum* in juvinile turbot, *Scophthalmus maximus* (L), intestine and faeces. J Fish Dis 21:1-9

Olsson JC, Westerdahl A, Conway PL, Kjelleberg S (1992) Intestinal colonisation potential of turbot (*Scophthalmus maximus* L) and dab (*Limanda limanda)*-associated bacteria with inhibitory effect against *Vibrio anguillarum*. Appl Environ Microbiol 58:551-556

Oppenheimer CH, ZoBell CE (1952) The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. J Mar Res 11:10-18

O'Sullivani DJ, O'Gara F (1992.) Traits of flurescent *Pseudomonas Spp.* involved in suppression of plant root pathogens. J Appl Bacteriol 56:662-676

Otta SK, Karunasager I, Karunasagar I (1999) Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon*. J Aqua Trop 14:309-318

Owens l, Muir P, Sutton D, Wingfield M (1992) The pathology of microbial diseases in tropical Australien Crustacea. In: Shariff M, Subasinghe RP, Arthur JR (eds) Diseases in Asian Aquaculture I, Fish Health Section. Asian Fisheries Society, Mannila, Philippines

Palmer DA, Bender CL (1993) Effect of environmental and nutritional factors on production of the polyketide phytotoxin Coronatine by *Pseudomonas syringae* pv *glycia*. Appl Environ Microbiol 59:1619-1626

Parret AHA, Schoofs G, Proost P, De Mot R (2003) Plant lectin-like bacteriocin from a rhizosphere-colonizing *Pseudomonas* isolate. J Bacteriol 185:897-908

Pergament I, Carmeli S (1994) Schizotrin A, a novel antimicrobial peptide from a cyanobacterium. In: Chandler RF, Jurgens TM, Jurgens AR (eds) Abstracts from the international congress on natural products research, Halifax-Nova-Scotia, Canada

Pielio E (1966) The measurement of diversity in different types of biological collections. J Theoret Biol 13:131-144

Pizzutto M, Hirst RG (1995) Classification of isolates of *Vibrio harveyi* Virulent to *P. monodon* larvae by protein profile analysis and M13 DNA fingerprinting Dis Aquat Org 21:61-68

Prescott LM, Harley JP, Klein DA (2005) Microbiology, 6th Edition. McGraw-Hill, New York, p 101-102

Pybus V, Loutit MW, Lamont IL, Tagg JR (1994) Growth inhibition of the salmon pathogen *Vibrio ordalii* by a siderophore produced by *Vibrio anguillarum strain VL 4335*. J Fish Dis 17:311-324

Raaijmakers JM, David MW, Thomasshow LS (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environment. Appl Environ Microbiol 63:881-887

Raida MK, Larsen JL, Nielsen ME, Buchmann K (2003) Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri*

challenge following oraladministration of *Bacillus subtiles* and *B. licheniformis* (Bio Plus 2B). J Fish Dis 26:495-498

Rao CSVR (1994) Antimicrobial activity of cyanobacteria. Indian J Mar Sci 23:55-56

Rao JK, Kim C-H, Rhee S-K (2000) Statistical Optimization of medium for the production of recombinant hirudin from *Saccharomyces cervisiae* using response surface methodology. Proc Biochem 35:639-647

Rao YM, Sureshkumar GK (2000) Oxidative-stress-induced production of pyocyanin by *Xanthomonas campestris* and its effect on the indicator target organism, *Escherichia coli*. J Ind Microbiol Biotechnol 25:266-272

Rengipat S, Phianphak W, Piyatiratitivorakul S, Menasaveta P (1998) Effect of probiotic bacteria on black tiger Shrimp *Penaeus monodon* survival and growth. Aquaculture 167:301-313

Rengipat S, Rukpratanporn S, Piyatiratitivorakul S, Menasaveta P (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiot bacterium (*Bacillus* S11). Aquaculture 191:271-288

Rico-Mora R, Voltolina D, Villaescusa-Celaya JA (1998) Biological control of *Vibrio alginolyticus* in *Skelitonema costatum* (Bacillariophyceae). Aquacult eng 19:1-6

RingØ E, Gatesope F-J (1998) Lactic acid bacteria in fish: A review. Aquaculture 160:177-203

Rippka R, Deruelles J, Waterbury JB, Herman M, Stanierr RY (1979) Generic assignments, strains, histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1-61

Riquelme C, Araya R, Vergara N, Rojas A, Guaita M, Candia M (1997) Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* with inhibitory effects against pathogenic Vibrios. Aquaculture 154:17-26

Riquelme C, Hayashida G, Araya R, Uchida A, Satomi M, Ishida Y (1996) Isolation of a native bacterial strain from the scallop *Argopecten purpuratus* with inhibitory effects against pathogenic vibrios. J Shell Fish Res15:369-374

Robertson PAW, O'Dowd C, Burrells C, Williams P, Austin B (2000) Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Aquaculture 185:235-243

Sakamoto K, Okimasu E, Amemura A (1996) Isolation of a microalgae, *Synechocystis* sp. SY-4, potentially useful as a rotifer feed. J Fermentation Bioeng 82:157-160

Sambrook J, Russell DW (2001) Liquid media for *E.coli*. In: Molecular cloning, A laboratory manual, Vol 3. Cold Spring Harbor Laboratroy Press, Cold Spring Harbor, New York, p A2-2

Sen R, Swaminathan T (1997) Application of response-surface methodology to evaluate the optimum conditions for the enhanced production of surfactin. Appl Microbiol Biotechnol 47:358-363

Shan H, Obbard JP (2001) Ammonia removal from prawn aquaculture water using immobilized nitrifying bacteria. Appl Microbiol Biotechnol 57:791-798

Singh ISB, Lakshmanaperumalaswamy P, Chandramohan D (1989) Bateriology of eggs and larvae of Penaeus indicus in hatchery. In: Nair NB (ed) Proc Kerala Science Congress, Thiruvananthapuram, Kerala, India, p 95 -107

Singh ISB, Lakshmanaperumalaswamy P, Chandramohan D (1995) Heterotrophic bacteria associated with eggs and larvae of *P.indicus* hatchery system Proceedings of first international conference of the culture of prawns/shrimps, Ilolo city, Phillippines

Singh ISB, Lakshmanaperumalaswamy P, Chandramohan D (1998) Bacterial flora of pond reared *P.indicus* (mine Edwards). J.Aqua.Trop. 13:133-142

Singh ISB, Philip R (1993) Compartive efficiency of three media in isolating heterotrophic bacteria from larvae and larval rearing water of *Macrobrachium rosenbergii*. Indian J. Microbiol 33:67-69

Singh MP, Kong F, Janso JE, Arias DA, Suarez PA, Bernan VS, Petersen PJ, Weiss WJ, Carter G, Gareenstein M (2003) Novel α-pyrones produced by a marine *Pseudomonas* sp. F92S91: Taxonomy and biological activities. J Antibiot 56:1033-1044

Skjermo J, Vadstein O (1999) Techniques for microbiological control in the intestine rearing of marine larvae. Aquaculture 177:333-343

Slininger PJ, Jackson MA (1992) Nutritional factors regulating growth and accumulation of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79 Appl Microbiol Biotech 37 388–392

Smith P, Davey S (1993) Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a flurescent pseudomonad. J Fish Dis 16:521-524

Spanggaard B, Huber I, Nielsen J, Sick EB, Pipper CB, Martinassen T, Slierendrecht WJ, Gram L (2001) The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Environ Microbiol 3:755-765

Spizek J, Tichy P (1995) Some aspects of overproduction of secondary metabolites. Floia Microbiol 40 43–50

Stanbury PF, Whitaker A, Hall SJ (1997) Principles of fermentation, Vol 1. Adithya books private limited, New Delhi, p.97-98

Strickland JDH, Parson TR (1968) A practical hand book of seawater analysis. Fish Res Bd Can Bull 167:311-312

Strobel RJ, Sullivan GR (1999) Experimental design for improvement of fermentation. In: Demain AL, Davies JE (eds) Manual of industrial microbiology and biotechnology. American Society for Microbiology Press, Washington D C, p 80-93

Sudheesh PS, Xu H-S (2001) Pathogenicity of *V.parahaemolyticus* in tiger prawn *P. monodon* Fabricus: possible role of extracellular protease. Auaculture 196:37-46

Sugita H, Shibuya K, Shimooka H, Deguchi Y (1996) Antibacterial activities of intestinal bacteria in fresh water cultued fish. Aquaculture 145:195-203

Suminto A, Hirayama K (1997) Application of a growth promoting bacteria for stable mass culture of three marine micro-algae. Hydrobiologia 358:223-230

Sunayanto A, Mariam A (1986) Occurance of pathogenic bacteria causing luminance in penaeid larvae in Indonasian hatcheries. Bull Brackishwater Aquacult Dev Cent 8:64-70

Sundararaman M, Averal HI, Akbarsha MA, Subramanian (1994) Bio-activity of marine cyanobacteria in the animal-based systems: Modulation of food intake, body weight and some haematological characters. Ann. Appl. Biology 125:195-206

Tendencia EA, dela Pen˜a LD (2001) Antibiotic resistance of bacteria from shrimp ponds. Aquaculture 195:193-204.

Tendencia EA, dela Pen˜a MR (2003) Investigation on some components of the greenwater culture system which makes it effective in the initial control of luminous bacteria. Aquaculture 218:115–119.

Tendencia EA, dela Pen˜a MR, Choresca Jr CH (2005) Efficiency of *Chlorella* sp. and *Tilapia hornorum* in controlling the growth of luminous bacteria in a simulated shrimp culture environment. Aquaculture 249 55– 62

Tepsic K, Gundecimeran N, Frisvad JC (1997) Growth and mycotoxin production by *Aspergillus fumigatus* strains isolated from a saltern. FEMS Microbiol Lett 157:9–12

Than PP, Carmelo SDC, Takeshi Y, Taizo S (2004) Extracellular protease production of bacteriolytic bacteria isolated from marine environments. Fisheries Sci 70:659–666

Thomashow LS, Weller DM, Bonsall RF, Pierson III LS (1990) Production of the antibiotic phenazine-1-carboxylic acid by flurescent *Pseudomonas* species in the rhizosphere of wheat. . Appl Environ Microbiol 56:908-912

Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. Microbiol Mol Biol R 68:403-431

Toranzo AE, Romalde JL, Bandin I, Santos Y, Barja JL (1992) Evaluation of the sensitivity of bacterial fish pathogens to different antimicrobial compounds. In: Michel C, Lightner DV (eds) Chemotherapy in Aquaculture: from Theory to Reality. Office International des Epizootics (OIE), Paris, p 315-325

Toranzo AE, Torres JL (1996) *In vitro* inhibition of *Vibrio harveyi* by *Pseudomonas* species isolated from aquatic environment. UPV J Nat Sci 1:130-138

Turner JM, Messenger AM (1986) Occurrence, physiology and biochemistry of phenazine pigment production. Adv Microbiol Physiol 27:211–275

Vandenberghea J, Thompsona FL, Gomez-Gilb B, Swingsa J (2003) Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems Aquaculture 219:9 –20

van-Pee KH (1996) Biosynthesis of halogenated metabolite by bacteria. Ann Rev Microbiol 50:375-399

Vaseeharan B, Ramasamy P (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT 23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett Appl Microbiol 36:83-87

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol R 64:655-671

Vidaver AK (1967) Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic *Pseudomonas*: Effect of carbon sources. Appl Microbiol 15:1523-1524

Vijayan KK, Singh ISB, Jayaprakash NS, Alavandi SV, Somnath Pai S, Preetha R, Rajan JJS, Santiago TC (2006) A brackishwater isolate of *Pseudomonas* PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. Aquaculture 251:192-200

Villamil L, Figueras A, Toranzo AE, Planas M, Novoa B (2003) Isolation of a highly pathogenic Vibrio pelagius strain associated with mass mortalities of turbot, Scophthalmus maximus (L.), larvae. J Fish Dis 26:293-303

Vine NG, Leukes WD, Kaiser H (2004) In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. FEMS Microbiol Lett 231:145-152

Vukomanovic DV, Zoutman DE, Stone JA, Marks GS, Brien JF, Nakatsu K (1997) Electronspray mass-spectrometric, spectrophotometric and electrochemical methods do not provide evidence for the binding of nitric oxide by pyocyanin at pH 7. Biochem J 322:25-29

Walne PR (1974) Culture of bivalve mollusks, Vol. Fishing News (Book's) Ltd., Surry

Wang Y-X, Lu Z-X (2004) Statistical optimization of media for extracellular polysaccharide by *Pholiota squarrosa* (Pers. Ex Fr.) Quel. AS 5.245 under submerged cultivation. Biochem Eng J 20:39-47

Watson D, MacDermot J, Wilson R, Cole PJ, Taylor GW (1986) Purification and structural analysis of pyocyanin and 1-hydroxyphenazine. Eur J Biochem159:309-313

Weruster-Botz D (2000) Experimental design for fermentation media development statistical design or global random search?. J Biosci Bioeng 90: 473-483

Westerdahl A, Olsson JC, Kjelleberg S, Conway PL (1991) Isolation and characterization of turbot (*Scophthalmus maximus*) associated bacteria with inhibitory effects againsyt *V. anguillarum*. Appl Environ Microbiol 57:2223-2228

Whistler CA, Stockwell VO, Loper JE (2000) Lon Protease influenses antibiotic production and UV tolerance of *Pseudomonas fluorescens*. Appl Environ Microbiol 66:2718-2725

Wright AE (1998) Isolation of marine natural products. In: Cannell RJP (ed) Natural products isolation, Vol 1. Humana Press, New Jersey, p 365-408

Wright PC, Stevenson C, McEvoy E, Burgess JG (1999) Opportunities for marine bioprocess intensification using novel bioreactor design: Frequency of barotolerance in microorganisms obtained from surface waters J Biotech 70:343 – 349

Xiong C, Shouwer C, Ming S, Ziniu Y (2005) Medium optimization by response surface methodology for poly-α-glutamic acid production using dairy manure as the basis of a solid substrate. Appl Microbiol Biotechnol 69: 390-396

Yamaski S, Furuya N, Matsuyama N (1998) Effect of specific ions in agar on antibiotic production by Burkholderia glumae. J Faculty Agric Kyusitu Univ 42:309-314.

Yang JD, Wang NS (1992) Oxygen mass transfer enhancement via fermenter headspace pressurization. Biotech Prog 8:224–251

Zhang J, Marcin C, Shifflett MA, Brix T, Salmon P, Greasham R, Buckland B, Chartrain M (1996) Development of a defined medium fermentation process for physostigimine production by *Streptomyces grisiofuscus*. Appl Microbiol Biotechnol 44:568-575

Annexure 1

Publications emerging out of the Thesis

In Press

1.Preetha R, Jayaprakash NS, Philip R, Singh ISB. Optimization of carbon and nitrogen sources and growth factors for the production of an aquaculture probiotic (*Pseudomonas* MCCB 103) using response surface methodology. (Journal of Applied Microbiology)

Communicated

1. Preetha R, Jayaprakash NS, Philip R, Singh ISB.Optimization of culture conditions for the production of an aquaculture probiotic *Micrococcus* MCCB 104 using response surface methodology (Communicated to Journal of Industrial Microbiology).

2. Preetha R, Jayaprakash NS, Singh ISB. *Synechocystis* MCCB 114 and 115 as putative probionts for shrimp larvae. (Communicated to Diseases of Aquatic Organisms)

To be communicated

1. Preetha R, Vijayan KK, Alavandi SV, Santiago TC, Singh ISB. Optimization of culture conditions for the production of an aquaculture probiotic *Psudomonas* MCCB 102 using response surface methodology.

2. Preetha R, Vijayan KK, Alavandi SV, Santiago TC, Singh ISB. Optimization of carbon and nitrogen sources and growth factors for the production of an aquaculture probiotic *Psudomonas* MCCB 102 using response surface methodology

3. Preetha R, Jayaprakash NS, Philip R, Singh ISB. Optimization of culture conditions for the production of an aquaculture probiotic *Psudomonas* MCCB 103 using response surface methodology.

4. Preetha R, Jayaprakash NS, Philip R, Singh ISB.Optimization of carbon and nitrogen sources and growth factors for the production of an aquaculture probiotic *Micrococcus* MCCB 104 using response surface methodology.

5. Preetha R, Prathapan S, Vijayan, KK, Jayaprakash NS, Philip R, Singh ISB. Characterization of an antivibrio compound produced by potential antagonistic probiotics in aquaculture, *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103.

Annexure 2

Patents emerging out of the Thesis

1.Bioprocess technology for the mass production of an aquaculture probiotic *Micrococcus* MCCB 102.

2.Bioprocess technology for the mass production of an aquaculture probiotic *Pseudomonas* MCCB 102.

3.Bioprocess technology for the mass production of an aquaculture probiotic *Pseudomonas* MCCB 103.