A MARINE ACTINOMYCETE *NOCARDIOPSIS* MCCB 110 AS SOURCE OF NOVEL DRUGS TO MANAGE VIBRIOSIS

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In partial fulfillment of the requirements for the degree of

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in

Marine Biotechnology

Under the Faculty of Marine Sciences

Department of Marine Biology, Microbiology and Biochemistry

by

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A Marine Actinomycete *Nocardiopsis* MCCB 110 as Source of Novel Drugs to Manage Vibriosis

Ph.D. Thesis under the Faculty of Marine Sciences

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Certificate

This is to certify that the research work presented in this thesis entitled "**A MARINE ACTINOMYCETE *NOCARDIOPSIS* MCCB 110 AS SOURCE OF NOVEL DRUGS TO MANAGE VIBRIOSIS"** is based on the original work done by Mr. Sunish K.S under our guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi 682016, in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the work presented in this thesis entitled "**A MARINE ACTINOMYCETE *NOCARDIOPSIS* MCCB 110 AS SOURCE OF NOVEL DRUGS TO MANAGE VIBRIOSIS"** is based on the original work done by me under the guidance of Prof. A. Mohandas (Emeritus Professor), Prof. I. S. Bright Singh, Co-ordinator, National Centre for Aquatic Animal Health and Dr. Rosamma Philip, Assistant Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Kochi- 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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Sunish K. S

Contents

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Chapter

1

GENERAL INTRODUCTION

Contents

Aquaculture is a form of agriculture that involves the propagation, cultivation and marketing of aquatic plants and animals in a controlled environment (Swann, 1992). After growing steadily, particularly in the last four decades, aquaculture is for the first time set to contribute half of the fish consumed by the human population worldwide. Given the projected population growth over the next two decades, it is estimated that at least an additional 40 million tonnes of aquatic food will be required by 2030 to maintain the current per capita consumption (FAO, 2006). Capture fisheries and aquaculture supplied the world with about 110 million tonnes of food fish in 2006. Of this total, aquaculture accounted for 47 percent (FAO, 2009). Globally, penaeid shrimp culture ranks sixth in terms of quantity and second in terms of value amongst all taxonomic groups of aquatic animals cultivated (FAO, 2006). In places where warm-water aquaculture was possible black tiger shrimp, *Penaeus monodon* became the preferred variety of shrimp

cultivar owing to its fast growth, seed availability and importantly due to high prices it fetches (Pechmanee, 1997). World shrimp production is dominated by *P.monodon*, which accounted for more than 50 % of the production in 1999 (FAO, 2000). In the last few years the whiteleg shrimp, *Litopenaeus vannamei*, has replaced *P.monodon* in many countries. Indian shrimp culture is dominated by *P.monodon* with the East Coast accounting for 70% of the production (Hein, 2002). Intensive culture, apart from other problems, results in enhanced susceptibility of the cultured species to diseases (Jory, 1997), which in fact have become the biggest constraint in shrimp aquaculture (FAO, 2003).

1.1 Shrimp culture practice

Crustacean aquaculture is considered a high value activity worldwide and tends to have higher monetary value, and annual world production is over 8 million metric tonnes (FAO 2000). Of this figure over half is made up of shrimp and prawns and the proportion of this production coming from farms has increased rapidly since 1980s. Much of the world production still comes from extensive culture.

In India, commercial shrimp farming started gaining roots only during the mid eighties. The potential area available in the coastal region of the country for shrimp farming is estimated between 1.2 million to 1.4 million hectares. Presently, an area of about 1, 57,000 ha are under farming with an average production of about 1, 00,000 metric tonnes of shrimp per year. The average productivity has been estimated at 660kg per hectare per year. Cultured shrimps contribute a major portion of national income through high export earnings (Anon, 2002). In 2003-2004, cultured shrimps contributed

Rs 3348 crores out of the total shrimp exports of Rs 4013 crores which was about 83 % of total shrimp exports (Ravichandran, 2005). It also provides direct employment to about 0.3 million people and the ancillary units provide employment to 0.6- 0.7 million (Aquaculture Authority News, 2002).

1.2 Shrimp diseases

The rapid expansion of the shrimp farming industry is plagued by diseases affecting shrimp survival and growth. During the past two decades, the worldwide shrimp aquaculture has been greatly puzzled by diseases mostly due to bacteria (especially the luminous *Vibrio harveyi*) and viruses. Most diseases occur as a result of environmental deterioration and stress associated with the intensification of shrimp farming. The high density of animals in hatchery tanks and ponds is conducive to the spread of pathogens, and the aquatic environment, with regular applications of protein-rich feed, is ideal for the growth of bacteria. Many shrimp farms have been particularly affected by outbreaks associated with vibriosis- causing bacteria like *Vibrio harveyi*, *Vibrio damsel*, *Vibrio alginolyticus* etc. (Song *et al*., 1993 and Lee *et al*., 1996). *P.monodon* larvae are also susceptible to host of diseases, chief among them being vibriosis caused by various *Vibrio* spp., including *V.harveyi* (Lightner, 1996; Alapide-Tendencia & Dureza, 1997; Aguerre-Guzman *et al*., 2001; Austin *et al*., 2005). These diseases particularly hamper larval production and lead to profitability problems due to stock mortalities. They also lead to overexploitation of broodstock. Consequently, the control of disease became a priority at the world level if shrimp production is to be ecologically and economically sustainable. To a greater extent, the durability of production is dependent on the equilibrium between i) the environment

quality ii) the prevention of disease by diagnosis and epidemiological surveys of the pathogen and iii) the health status of the shrimp (Bachere, 2000).

1.2.1 Vibriosis

The Dutch Microbiologist, Dr. Martinus Bejerinck reported the first non-pathogenic *Vibrio* species i.e. *V. fischeri, V.splendidus* and *Photobacterium phosphoreum* from aquatic environment (Thompson *et al*., 2004). Vibrios are abundantly isolated from estuaries, marine waters and sediment and aquaculture settings globally where they occur either as free-living or associated with organisms such as corals (Rosenberg & Ben-Haim, 2002), fish (Muroga, 2001; Toranzo *et al*., 2005), molluscs (Torkildsen *et al*., 2005), sea grass, sponges, shrimp (Gomez Gil *et al*., 1998; Vandenberghe *et al*., 2003; Jayaprakash *et al*., 2006a) and zooplankton (Heidelberg *et al*., 2002). The external surfaces of marine organisms particularly those of zooplankton harbour large numbers of vibrios (Heidelberg *et al*., 2002). To date, all vibrios are chitinolytic utilizing it both as a carbon and nitrogen source (Heidelberg *et al*., 2002) and they play a significant role in the mineralization of chitin in aquatic systems (Lipp *et al*., 2002). Many *Vibrio* species are ubiquitous in aquaculture settings associated with all cultured species (fish, molluscs, crustaceans) (Ramesh *et al*., 1986; Alapide- Tendencia & Dureza, 1997; Verdonck *et al*., 1997; Vandenberghe *et al*., 1998; Thompson *et al*., 2001; Vandenberghe *et al*., 2003; Jayaprakash *et al*., 2006a).

Vibriosis is a major disease caused by *Vibrio* spp. afflicting all varieties of shrimps in culture at all stages (Lightner 1988; Singh *et al*., 1989; Singh, 1990; Singh *et al*., 1998; Jayaprakash *et al*., 2006b). The species identified from diseased and healthy *P.monodon* samples are *V.aestuarianus, V.alginolyticus,*

V.campbelli, V.cholerae, V.costicloa, V.damsela, V.fischeri, V.fluvialis, V.furnissii, V.haloplanktis, V.harveyi, V.hollise, V.ichtyoenterii, V.logei, V.mediterranei, V.metschnikovii, V.natriegens, V.nigripulchritudo, V.parahaemolyticus, V.pelagicus, V.penaeicida, V.protelyticus, V.splendidus, V.tuhiashii, and *V.vulnificus* (Lightner,1996; Lavilla-Pitogo *et al*., 1990; Song *et al*., 1993; Alapide-Tendencia & Dureza 1997; Goarant *et al*.,1998; Vandenberghe *et al*., 1998; Sung *et al*., 1999; Sung *et al*., 2001). Other (reported) names for this disease are penaeid bacterial septicaemia, penaeid vibriosis, luminescent vibriosis and red-leg disease. Some species and strains of *Vibrio* cause the shrimp to be luminescent. Mortality ranges from insignificant to 100%, particularly in larvae, post larvae and juvenile shrimp. The effect and severity are related to *Vibrio* species and doses, water, feed, shrimp quality, and aquaculture management (Lightner, 1996). In gross observations, this infection is evidenced as black or brown cuticular lesions, necrosis, opacity of musculature, black lymphoid organ, and melanisation of appendages.

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

coastal waters and estuaries harbour high densities of this organism (Nair *et al*., 1979; Chari & Dubey, 2006).

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

Gross observations, wet mounts, histology and bacterial cultures are the main diagnostic methods of vibriosis. Large number of bacteria in the haemolymph is observed in wet mounts (Lightner, 1996). Necrosis and inflammation of different organs (lymphoid organ, gills, heart, hepatopancreas, etc.) are easily studied with histology methods. Isolated *Vibrio* sp from shrimp will grow on a variety of nutrient agar media like TCBS and ZoBell's agar.

Use of antibiotics has been the method of choice amongst shrimp culturist to protect their crop from luminous disease. Early studies pertaining to the control of bacterial pathogens in shrimp aquaculture were directed

towards the selection of appropriate antibiotics (Baticados *et al*., 1990; Hameed & Rao, 1994). Antibiotics used in aquaculture are amoxicillin, benzylpencillin, cotrimazine, enrofloxacin, florfenicol, flumequine, oxolinicacid, oxytetracycline, sarafloxacin, trimethoprim, sulphadiazine (Alderman & Hastings, 1998; Roque *et al*., 2001). However, the use of antimicrobials has been largely prophylactic in aquaculture (Baticados & Paclibare, 1994; Cabello, 2006) and their growing use has been a cause of concern (Teuber, 2001). Unsurprisingly, haphazard use of antibiotics has resulted in the emergence of *V.harveyi* and other *Vibrio* spp. strains with multiple antibiotic resistances (Holmstrom *et al*., 2003).

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

The large scale resistance to antibiotics observed in bacteria isolated from aquaculture systems together with human and environmental concerns pertaining to the spread of resistance to human pathogens and persistence of residues in tissues has invited legislations restricting their use in many countries (Holmstrom *et al*., 2003). The European Union, United States of America and Japan, importers of shrimp, have banned shrimps produced in tropical countries because of the presence of certain antibiotics and set maximum residual limits (MRL) in the meat of shrimp being imported (Alderman & Hastings, 1998, Pakshirajan, 2002). Many antibiotics have been banned from use in shrimp culture in India too (Pakshirajan, 2002). This has necessitated a search for alternative treatment and management strategies to manage the occurrence and spread of *V.harveyi* in shrimp culture systems in hatcheries as well as in farms. Such strategies include the use of immunostimulants, vaccines, probiotics and phage therapy. These, however, are in their infancy in aquaculture and it may require some more time before they become the norm. Adoption of immunostimulants, vaccines and probiotics coupled with good water quality management strategies to manage diseases of animals in culture for more than a decade has yielded encouraging results (Grugel & Wallmann, 2004).

1.3 Actinomycetes

Actinomycetes are among the most fascinating microorganisms. Their developmental lifecycle, including morphological and physiological differentiation and the rich repertoire of secondary metabolites, about 70 - 80 % of bioactive secondary metabolites are being produced by actinomycetes, have resulted in a large research community studying these microbes. Actinomycetales, commonly referred to as Actinomycetes, is an order of

bacteria in the class Actinobacteria. The suborders within Actinomycetales are Actinomycineae, Micrococcineae, Corynebacterineae, Micromonosporineae, Propionibacterineae, Pseudonocardineae, Streptomycineae, Streptosporangineae, Frankineae and Glycomycineae.

1.3.1 General characteristics

Actinomycetes are a group of diverse bacteria. They have in common that they all are Gram-positive and have a high content of guanine plus cytosine in their DNA (>55 mol %). Most are aerobic and neutrophilic. The name actinomycete derives from the Greek "actys" (ray) and "mykes" (fungus) and the actinomycetes were initially regarded as minute fungi because of their mycelium-like growth. The attention paid to this group rose notably after the discovery of streptomycin by Waksman and Schatz in 1943 (Erikson, 1949) and as the number of studies and the scientific data accumulated, they were finally recognized as bacteria. Their morphology, however, varies among the different genera, from cocci and pleomorphic rods to branched filaments that break down into spherical cells or aerial mycelium with long chains of spores. The spores are formed as a result of nutrient depletion and can survive prolonged desiccation until nutrients are again available (McCarthy & Williams, 1992). This ability to sporulate is important for their survival in the environment.

Actinomycetes are ubiquitous in soils, where they usually are present in numbers of 10^5-10^6 colony-forming units per gram of soil. They play an important role in the recycling of organic carbon and are able to degrade complex polymers (Goodfellow & Williams, 1983). Many strains have the ability to solubilise lignin and degrade lignin-related compounds by producing

cellulose- and hemicellulose - degrading enzymes and extracellular peroxidases (Ball *et al*., 1989; Pasti *et al*., 1990; Mason *et al*., 2001). They also occur in other environments rich in organic matter such as composts, in both the mesophilic and thermophilic phases (Steger, 2006), and sewage sludge, where, notably the mycolic acid-containing actinomycetes are associated with the extensive and undesirable formation of stable foams and scum (Seong *et al*., 1999; Goodfellow *et al*., 1996).

In general the optimal conditions for their growth are temperatures of 25-30°C (50°C for the thermoactinomycetes) and neutral pH, but many species have been isolated from extreme environments. For example, the psychrophilic *Arthrobacter ardleyensis* was isolated from sediment from an Antarctic lake and is able to grow at temperatures as low as 0°C (Chen *et al*., 2005) and *Nocardiopsis alkaliphila* was isolated from desert soil in Egypt and grows at pH 9.5-10 (Hozzein *et al*., 2004). A number of mycolate actinomycetes were isolated from the deep-sea bed, an environment with high pressure, low temperatures, lack of light and variable concentration of oxygen and salinity (Colquhoun *et al*., 1998).

Some actinomycetes are important as human pathogens. *Mycobacterium tuberculosis* is the etiologic agent of tuberculosis, a disease that, according to data from the World Health Organization in 2004, caused 1.7 million deaths. Another *Mycobacterium, M. leprae,* causes leprosy and *Corynebacterium diphteriae,* diphtheria. Although they are not frequent in clinical practice, other genera have the potential to cause serious human and animal infections, *e.g. Nocardia, Rhodococcus, Gordonia* and *Actinomadura* (McNeil & Brown, 1994). Some actinomycetes are also phytopathogenic and belong mainly to the genera *Corynebacterium* and *Streptomyces* (Young *et al.,* 1992; Locci, 1994,).

The actinomycete genera that are considered human pathogens include the genera *Actinomadura*, *Cellulomonas*, *Corynebacterium*, *Dermatophilus*, *Mycobacterium*, *Nocardia*, *Nocardiopsis*, *Rhodococcus*, and *Streptomyces.*

Probably most of the interest in this group of microorganisms lies in their ability to produce secondary metabolites. Two thirds of the microbial antibiotics known today are produced by actinomycetes (Berdy, 1995), mainly by *Streptomyces* species, although the number of rare actinomycetes (non-*Streptomyces*) is increasing due to the application of new selective isolation methods (Lazzarini *et al.,* 2000).

Although heavily studied over the past three decades, actinomycetes continue to prove themselves as reliable sources of novel bioactive compounds. Among the well-characterized pharmaceutically relevant microorganisms, actinomycetes remain major sources of novel, therapeutically relevant natural products (Jensen and Fenical, 2000). The majority of these compounds demonstrate one or more bioactivities many of them developed into drugs for treatment of wide range of diseases in human, veterinary and agriculture sectors (Bernan *et al*., 1997). Actinomycetes also produce secondary metabolites that show bioactivities other than antibiotics, such as enzyme inhibitors, immunosuppressors, phytotoxins and pesticides (Bèrdy, 1995; Park *et al*., 2002; Imada, 2005). They are responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005), notably antibiotics (Berdy, 2005, Strohl, 2004), antitumor agents (Cragg *et al*., 2005) immunosuppressive agents (Mann, 2001) and enzymes (Oldfield *et al*., 1998; Pecznska *et al*., 1988).

1.3.2 Bioactive metabolites

By the end of 2002 over 22000 bioactive secondary metabolites (including antibiotics) were published in the scientific and patent literature (Berdy, 2005).

1.3.2.1 Approximate number of bioactive microbial natural products (2002).

Source	Antibiotics	"Other bioactive" metabolites	Total bioactive metabolites
Bacteria	2900	900	3800
Actinomycetales	8700	1400	10100
Fungi	4900	3700	8600
Total	16500	6000	22500

1.3.2.2 Number of actinomycetes producing bioactive microbial metabolites

Streptomycetaceae:

Streptomyces 8000	*Streptoverticillium* 258
Kitasatosporia 37	*Chainia* 30
Microellobosporia 11	*Nocardioides* 9

Micromonosporaceae:

Micromonospora 740	*Actinoplanes* 248
Dactylosporangium 58	*Ampullariella* 9
Glycomyces 2	*Catenuloplanes* 3
Catellatospora 1	

Pseudonocardiaceae:

Saccharopolyspora 131	*Amycalotopsis*/*Nocardia* 120/357
Kibdellosporangium 34	*Pseudonocardia* 27
Amycolata 12	*Saccharomonospora* 2
Actinopolyspora 1	

Streptosporangiaceae:

Streptosporangium 79	*Streptoalloteichus* 48
Spirillospora 11	*Planobispora* 10
Kutzneria 4	*Planomonospora* 2

Thermomonosporaceae:

Actinomadura 345	*Saccharothrix* 68
Actinosynnema 51	*Nocardiopsis* 41
Thermomonospora 19	*Micropolyspora*/*Faenia* 13/3
Thermopolyspora 1	*Thermoactinopolyspora* 1

Mycobacteriaceae:

Nocardia (357)	*Mycobacterium* 57
Arthrobacter 25	*Brevibacterium* 17
Proactinomyces 14	*Rhodococcus* 13

Other (unclassified) species:

Actinosporangium 30	*Microellobosporia* 11
Westerdykella 6	*Kitasatoa* 5
Sebekia 3	*Elaktomyces* 3
Waksmania 3	*Alkalomyces* 1
Erythrosporangium 1	*Streptoplanospora* 1
Salinospora 1	

1.4 Marine microbes as source of bioactive compounds

More than 70% of our planet's surface is covered by oceans and life on earth originated from the sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than in the tropical rainforests (Haefner, 2003). As a great promising source for new natural products which have not been observed from terrestrial microorganisms, marine bacteria are being developed for the discovery of bioactive substances with new types of structure, with growing intensive interest. Marine microorganisms continue to provide pharmacologically important secondary metabolites which are unique and novel chemical compounds and are continuously explored for drug discovery. Apart from microbes all other marine sources have also provided valuable chemical diversity (Kelecom, 2002).

With the traditional sources of actinomycetes diversity having been expended it is essential to look in to new environments for unique actinomycetes that might provide new pharmaceutical products. The ocean sediment is an untapped source of unique microorganisms whose singular evolutionary environment could possibly have resulted in the development of novel secondary metabolites to be used for pharmaceuticals. As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Fenical *et al*., 1999). Thus, it is crucial that

new groups of actinomycetes from unexplored or underexploited habitats be pursued as sources of novel bioactive secondary metabolites.

Marine actinomycetes are widely distributed throughout the world's oceans. They are found in inter-tidal zones as well as in deep ocean sediment. Actinomycetes are defined as marine if they require sea water for growth. The first actinomycetes to be isolated from oceanic sediment were not considered to be marine organisms. Scientists believed that they came from the spores of terrestrial bacteria that had simply blown into the ocean and had remained dormant (Goodfellow and Haynes 1984). However, further investigation showed that many actinomycetes isolated from ocean sediment require seawater for growth. Marine actinomycetes evolved in a habitat different from that of terrestrial actinomycetes, with different stresses and obstacles. Because they have been subject to the demanding marine environment, to gain selective advantage and to cope with competitors it is hypothesized that these bacteria have developed unique metabolic and physiological capabilities not found in terrestrial actinomycetes. This aspect of their development makes them an excellent candidate for synthesizing novel secondary metabolites. It is now clear that specific populations of marine adapted actinomycetes not only exist but add significant new diversity within a broad range of actinomycete taxa (Mincer *et al*., 2002; Stach *et al*., 2003).

Early evidence supporting the existence of marine actinomycetes came from the description of *Rhodococcus marinonascene*, the first marine actinomycete species to be characterized (Helmke *et al*., 1984). Further support has come from the discovery that some strains display specific marine adaptations (Jensen *et al*., 1991), whereas others appear to be metabolically active in marine sediments (Moran *et al*., 1995). Recent data from culture-

dependent studies have shown that indigenous marine actinomycetes indeed exist in the oceans. These include members of the genera *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium marinum, Williamsia maris and Verrucosispora* (Bull *et al*., 2005; Jensen *et al*.,2005; Magarvey *et al*.,2004; Jensen *et al*.,2005 ; Stach *et al*., 2004). Among these, the most exciting finding is the discovery of the first obligate new marine actinomycete genus, *Salinispora* (formerly known as *Salinospora*), and the demonstration of the widespread populations of this genus in ocean sediments (Mincer *et al*., 2002; Mincer *et al*., 2005).

1.4.1 Novel metabolites produced by marine actinomycetes

Natural products remain to be the most propitious source of antibiotics (Bull and Stach, 2007). There are approximately 32,500 natural products reported from microbial sources (Antibase data base) including about 1000 derived from marine microbes (Singh and Pelaez, 2008). Several antibiotics were derived from marine actinomycetes (Baltz, 2008) and marine derived antibiotics are more efficient at fighting microbial infections because the terrestrial bacteria have not developed any resistance against them (Donia and Hamman, 2003).

Although the exploitation of marine actinomycetes as a source for discovery of novel secondary metabolites is at an early stage, numerous novel metabolites have been isolated in the past few years. Table shows some examples of novel secondary metabolites isolated from marine actinomycetes from 2003 to 2005.

Novel metabolites produced by marine actinomycetes during the period 2003–2005

Compound	Source	Activity	Refs
Abyssomicins	*Verrucosispora sp.*	Antibacterial	(Riedlinger *et al*., 2004)
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer	(Mitchell *et al*., 2004)
Bonactin	*Streptomyces* sp.	Antibacterial; antifungal	(Schumacher *et al*., 2003)
Caprolactones	*Streptomyces* sp	Anticancer	(Stritzke *et al*., 2004)
Chandrananimycins	Actinomadura sp.	Antialgal; antibacterial; anticancer; antifungal	(Maskey *et al*., 2003a)
Chinikomycins	*Streptomyces* sp.	Anticancer	(Li *et al*., 2005)
Chloro-dihydroquinones	Novel actinomycete	Antibacterial; anticancer	(Soria-Mercado *et al*., 2005)
Diazepinomicin(ECO-4601)	*Micromonosproa* sp.	Antibacterial; anticancer; anti-inflammatory	(Charan *et al*., 2004)
3,6-disubstituted indoles	*Streptomyces* sp.	Anticancer	(Sanchez Lopez *et al*., 2003)
Frigocyclinone	*Streptomyces griseus*	Antibacterial	(Bruntner *et al*., 2005)
Glaciapyrroles	*Streptomyces* sp.	Antibacterial	(Macherla *et al*., 2005)
Gutingimycin	*Streptomyces* sp.	Antibacterial	(Maskey *et al*., 2004a)
Helquinoline	*Janibacter limosus*	Antibacterial	(Asolkar *et al*., 2004)
Himalomycins	*Streptomyces* sp.	Antibacterial	(Maskey *et al*., 2003b)
IB-00208	*Actinomadura* sp.	Anticancer	(Rodriguez *et al*., 2003)
Komodoquinone A	*Streptomyces* sp.	Neuritogenic activity	(Itoh *et al*., 2003)
Lajollamycin	*Streptomyces nodosus*	Antibacterial	(Manam *et al*., 2005)
Marinomycins	'*Marinispora*'	Antibacterial; anticancer	(Kwon *et al*., 2006)
Mechercharmycins	*Thermoactinomyces* sp.	Anticancer	(Kanoh *et al*., 2005)
MKN-349A	*Nocardiopsis* sp.	Unknown biological activity	(Shin *et al*., 2003)
SalinosporamideA (NPI-0052)	*Salinispora tropica*	Anticancer	(Feling *et al*., 2003)
Sporolides	*Salinispora tropica*	Unknown biological activity	(Buchanan *et al*., 2005)
Trioxacarcins	*Streptomyces sp.*	Antibacterial; anticancer; antimalarial	(Maskey *et al*., 2004b)

Marine bacteria seem to be a promising source as producers of drug candidates, e.g. thiocoraline, a new anticancer drug produced by a marine *Micromonospora* strain (Romero *et al*., 1997) which is under preclinical assessment, or the antiviral drug cyclomarin A produced by a marine *Streptomyces* strain (Renner *et al*., 1999). A large selection of antibiotics originally isolated from terrestrial streptomycetes was found in marine *Streptomyces* strains, such as the well known actinomycins, belonging to the phenoxazine-depsipeptide group, the quinoxaline-depsipeptide echinomycin, macrolides from the antimycin A, bafilomycin and sporaviridin group, the pentaenepolyene macrolides filipin and lagosin, the pentaene antibiotic lipomycin, and the PKS type-2 antibiotics tetracenomycin D and chromomycin A3. Further antibiotics isolated from marine streptomycetes were enterocin, actiphenol, the macrolactam antibiotics maltophilin and ikarugamycin, the phenazine antibiotics saphenic acid and 1,6-dihydroxyphenazine, pyridindolol, elaiomycin, and the diketopiperazine 1-N-methyl-albonoursin. Indeed, the marine environment is a virtually untapped source of actinomycetes diversity and therefore, of new metabolites (Lam, 2006).

1.5 *Nocardiopsis*

The genus *Nocardiopsis* was described by J. Meyer (Meyer, 1976) to comprise actinomycetes with fragmenting mycelium and cell wall chemotype III C (with the meso isomer of diaminopimelic acid and no characteristic sugars in whole-cell hydrolysates) (Lechevalier & Lechevalier, 1970). Subsequently, the genus description was amended by Grund and Kroppenstedt (Grund E. and Kroppenstedt R.M. 1990), and only the organisms with the cell wall chemotype III C, phospholipid type P III (phosphatidylcholine and phosphatidyl-methylethanolamine as characteristic phospholipids)

(Lechevalier *et al*., 1977) , menaquinone MK-10 with variable degrees of saturation as the major isoprenoid quinones (Collins *et al*., 1977), fatty acids of the 3d type (Kroppenstedt, 1985), and a DNA G+C content ranging between 64 and 71 mol% (Grund & Kroppenstedt, 1990) were proposed to be included in this genus. The taxonomic coherence of this generic grouping was confirmed by 16S rRNA gene sequence analysis (Evtushenko, *et al*., 2000; Rainey *et al*., 1996). In a recent review of the phylogenetic structure of the actinomycetes, the genus *Nocardiopsis* was shown to represent a distinct lineage within the radiation of the order *Actinomycetales* (Embley *et al*, 1994). A combination of phylogenetic position and morphologic and chemotaxonomic properties supported the creation of the family Nocardiopsiaceae, which includes the genus *Nocardiopsis* (Rainey *et al*, 1996). The genus *Nocardiopsis* currently comprises 18 species with validly published names (Meyer, 1976; Grund & Kroppenstedt, 1990; Yassin *et al*., 1993, 1997; Al-Tai & Ruan., 1994; Evtushenko *et al*., 2000; Chun *et al*., 2000; Peltola *et al*., 2001; Al-Zarban *et al*., 2002; Ka¨mpfer *et al*., 2002; Schippers *et al*., 2002; Li *et al*., 2003; Hozzein *et al*., 2004; Sabry *et al*., 2004).

Nocardiopsis strains are distributed ubiquitously in the environment (Kroppenstedt & Evtushenko, 2002). They are frequently isolated from habitats with moderate to high salt concentrations such as saline soil or marine sediments (Al-Zarban *et al*., 2002; Al-Tai & Ruan, 1994; Evtushenko *et al*., 2000) and salterns (Chun *et al*., 2000). Although *Nocardiopsis* species are infrequently encountered in clinical practice, *N. dassonvillei* is a potential cause of human infections, including conjunctivitis (Liegard & Landrieu, 1991), mycetomas (Ajello *et al*., 1987), skin infections (Philip & Roberts, 1984; Singh *et al*., 1991), and extrinsic alveolitis (Bernatchez & Lebreux, 1991).

Like other actinomycetes, *Nocardiopsis* spp. are capable of producing metabolites with biological activities. In the last two decades several new species of *Nocardiopsis* have been described and many interesting substances, such as enzymes and antibiotics have been discovered which includes, Dopsisamine a novel antibiotic (Takahashi *et al*., 1986), Nocamycin a new antineoplastic antibiotic (Brazhnikova *et al*., 1977), Lucentamycins, a cytotoxic peptide (Cho *et al*., 2007), 3-Trehaloseamine, a disaccharide antibiotic (Dolak, *et al*., 1980), Apoptolidin, a new apoptosis inducer (Kim *et al*., 1997) , Portmicin, a new antibiotic (Kusakabe *et al*., 1987) etc.

1.6 Crustacean defence system

Successful shrimp production requires the use of effective disease prevention strategies and a good understanding of the basic immune functions. Several factors such as water quality, disease, toxins, trace nutrients (astaxanthin, vitamins and minerals), probiotics, immune stimulants (β-glucan, peptidoglycan and lipopolysaccharides) and genetical make up have been shown to influence the immune mechanisms in *P.monodon* (Supamattaya *et al*., 2006). Invertebrates do not possess an adaptive/specific immune system based on a multitude of highly specific antibodies and antigen receptors equivalent to that of vertebrates, though few aspects of specific immunity (inducibility) appear to be present in some cases.

Non-specific or the innate immune mechanisms in *P.monodon* involves the fixed and the mobile defence mechanisms. The fixed non-specific immune mechanism involves the structural barriers which act as the first line of defence against pathogens. These include: hard cuticle, tegumental glands, epithelial immunity, branchial podocytes, autotomy of appendages,

regeneration of appendages and rapid wound healing to prevent loss of haemolymph.

The mobile non-specific immune system has two main components, the humoral and cellular systems both of which are activated upon immune challenge (Liu *et al*., 2009). The cellular component involves those mediated by haemocytes and the humoral component involves those mediated by cell free haemolymph. The cellular and humoral immune mechanisms of the shrimp function synergistically to protect the shrimp and eliminate foreign particles and pathogens.

The cellular immune response in *P.monodon* involves a number of different cell types including haemocytes and the fixed phagocytes (Supamattaya *et al*., 2006). *P.monodon* haemocytes can be divided into granular, semigranular and hyaline (or agranular) haemocytes (van de Braak, 2002a)

The various cellular immune response include phagocytosis (Bachere *et al*., 1995; Itami *et al*.,1998; van de Braak *et al*., 2002b; He *et al*., 2004), apoptosis (Sahtout *et al*., 2001; Wang *et al*., 2008), encapsulation (Sung *et al*., 2003; Bian and Egusa, 1981), nodule formation (Bian and Egusa, 1981; Nash *et al*., 1998), melanisation (Bian and Egusa, 1981; Nash *et al*., 1998) and cell adhesion (Sritunyalucksana *et al*., 2001; Lin *et al*., 2006).

The humoral response in shrimp is favoured by the different biological compounds in the haemolymph that inhibit or eliminate foreign bodies and pathogens. These include pro Phenol Oxidase (pro PO) activating system (Hernandez- Lopez *et al*., 1996; Vargas- Albores *et al*., 1996); the clotting cascade (Yeh *et al*., 1998; 1999) soluble pattern recognition proteins (PRPs) (He *et al*., 2004; Vargas- Albores and Yepiz- Plascencia, 2000) lectins (Luo *et*

al., 2006; Rittidach *et al*., 2007; Yang *et al*., 2007; Ma *et al*.,2008), anti-LPS factors (ALF) (Somboonwiwat et al., 2005; de la Vega et al., 2008), peptidoglycan - binding proteins, lipopolysaccharides (LPS) - binding proteins, beta 1,3-glucan-binding proteins (Romo-Figueroa *et al*., 2004; Jimenez-Vega *et al*., 2002; Cheng *et al*., 2005) etc.; the enzymes involved in the antioxidant defense mechanism - superoxide dismutase, peroxidase, catalase, nitric oxide synthase (Rameshthangam and Ramasamy, 2006; Jiang *et al*., 2006, Mathew *et al*., 2007); defensive enzymes like lysozyme (Sotelo-Mundo *et al*., 2003), acid phosphatase (Jiang *et al*., 2004; Joseph and Philip, 2007), other molecules like haemocyanin (Adachi *et al*., 2003); reactive oxygen intermediates (ROI) (Munoz *et al*., 2000; Wang *et al*., 2006); reactive nitrogen intermediates (RNI) (Jiang *et al*., 2006); alpha 2 macroglobulin (Gollas-Galvan *et al*., 2003; Rattanachai *et al*., 2004a) and antimicrobial peptides (Destoumieux *et al*., 1997; Bachere *et al*., 2000; Munoz *et al*., 2002; Hu *et al*., 2006). Studies on the response of shrimps to pathogens at the gene and molecular level reveal that more immune related mechanisms are involved in pathogen exclusion.

1.6.1 Haemocytes

The circulating haemocytes play extremely important roles not only by direct sequestration and killing of infectious agents but also synthesis and exocytosis of a battery of bioactive molecules including reactive oxygen metabolites and microbial proteins (Smith and Chilsolm, 1992; Smith and Chilsolm, 2001). They are involved in cellular responses, including clotting, nonself recognition, phagocytosis, melanisation, encapsulation, cytotoxicity and cell to cell communication.

Of the three types of haemocytes, hyaline cells in most decapods crustaceans are characterised by the absence of granules, although some cytoplasmic inclusion bodies have been reported by electron microscopic observations (Martin and Graves, 1985) and are capable of phagocytosis (Smith and Soderhall, 1983). The percentage population of hyaline cells vary when different species of crustaceans are compared. In penaeid shrimp *P.paulensis* it accounts for 41% of total circulating haemocytes whereas, in *Macrobrachium rosenbergii* it is only 17 %.

The semigranular cells, which contain small granules and display some phagocytic capacities, are specialized in particle encapsulation (Persson *et al*., 1987). Semigranular cells can respond to microbial polysaccharides such as lipopolysaccharides and β 1, 3-glucan by degranulation process (Johansson and Soderhall, 1989). The granular haemocytes are filled with large granules. They do not show phagocytic activity and they will respond to the microbial polysaccharides directly unless they are pre-treated with some haemolymph proteins called pattern recognition proteins (PRP). The main function of these granular haemocytes is to store prophenoloxidase activating system (proPO system), which plays a key role in the defence reaction of crustaceans. The granular cells can be triggered to undergo exocytosis and subsequent release of proPO system from the granules by two endogenous proteins which are associated with the proPO system, a serine protease and the β 1, 3-glucan binding protein if previously treated with β 1, 3-glucan. Importantly, in the majority of species studied, antibacterial proteins and opsonins are contained within or derived from the granular cells, although there may be some contributions made by semigranular cells in a few taxa. Certainly, full immune

reactivity is always achieved through co-operation and interaction between haemocyte types or their products.

1.6.2 Cellular immune response

1.6.2.1 Phagocytosis

The ability to ingest and kill microorganisms is a key component in the host defence. Phagocytosis is the most common of the cellular defence reactions and together with humoral components constitutes the first line of defence. Phagocytic cells are found throughout the animal kingdom, serving nutritive function in lower invertebrates and more specialized functions like defence against microbial infections in higher phyla. Even though phagocytosis is considered as an important cellular defence mechanism, little is known about this process in most crustaceans.

Phagocytosis is comparatively inefficient in the absence of opsonins, the co-factors that coat microorganisms and enhance the ability of phagocytes to engulf them (opsonisation). Studies in fresh water crayfish and lobster have revealed the presence of some opsonins in the haemolymph, which enhances phagocytosis (Tyson and Jenkin, 1974). But the factors, which act as an opsonins in crustacean haemolymph is yet to be isolated.

1.6.2.2 Nodule formation

When the body cavity is invaded by a large number of microorganisms, nodule formation or cell clumping occurs in several invertebrates, including crustaceans. These microorganisms entrapped in several layers of haemocytes, get melanised. Such aggregates have been observed in the gill vasculature of penaeid shrimp *Sicyonia ingentis* (Martin *et al*., 1993). However, in other

crustaceans haemocyte agglutinations (nodule) have been reported to be dispersed throughout the body as well as in the antennal gland, the heart and the gill (Bauchau, 1981 and Johnson *et al*., 1981). Nodule formation is not an isolated event but occurs in conjunction with phagocytosis and other immune responses to affect a highly efficient clearance mechanism capable of dealing with pathogens. Mode of killing within the nodules is unknown but may involve melanin production and its toxic precursors, lysozyme or release of other enzymes.

1.6.2.3 Encapsulation

In addition to nodule formation and phagocytosis invertebrate blood cells are capable of immobilizing parasites that are too large to be ingested by a single blood cell by surrounding them with multicellular sheaths. Considerable confusion exists regarding the types of blood cells involved in encapsulation. Also very little is known about the initiation process of an encapsulation reaction. In crustaceans the only cells to react to foreign molecules like β -1, 3-glucan from fungi or lipo-polysaccharides (LPS) from bacteria are the semigranular cells. This cell is also the first one to react to foreign particles and to encapsulate any invading pathogens. Some opsonin factors present in the haemolymph can also mediate the encapsulation process.

1.6.3 Humoral immune response

In many invertebrates' species, several kinds of immune- related humoral activities have been reported. Several of these described factors originate and/or reside in the haemocytes and are released during the immune response. These factors are primarily non-self recognition factors that include a variety of defensive enzymes, lectins, lipoproteins, antimicrobial peptides and reactive oxygen intermediates.

1.6.3.1 The pro phenoloxidase system

The best studied enzymatic system of crustaceans is Phenoloxidase cascade. This enzyme is a part of complex system of proteinases, pattern recognition proteins and proteinase inhibitors constituting the so called prophenoloxidase (proPO) activating system. This is located in the granular and semigranular haemocytes of decapods which is activated by the signature carbohydrate constituents of microbial cell walls, through pattern recognition binding molecules.

It is proposed to be non-self recognition system because conversion of prophenoloxidase to active enzyme can be brought about by miniscule amounts of molecules such as LPS, peptidoglycan and β -1, 3-glucan of microbial cell wall. Several components of this system have been isolated and their structure determined. Phenoloxidase (monophenyl L-dopa: oxygen oxidoreductase) catalyses the oxidation of phenols to quinones followed by several intermediate steps that led to the production of melanin, a brown pigment. During the formation of melanin, toxic metabolites are formed which have microbicidal activities.

The proPO is an inactive zymogen stored in the granular haemocytes, which degranulate and release the inactive enzyme into haemolymph. According to the amino acid sequence, proPO belongs to a family of copper containing proteins including haemocyanin and tyrosinases. The activation of proPO is by a proteolytic cleavage mediated by serine protease (proPO activating enzyme, ppA) which itself is seen in an inactive form in the haemolymph. Microbial polysaccharides, like LPS or β -1, 3-glucan can mediate the activation of this inactive serine protease to active form, which in

turn activate the inactive proPO into active phenoloxidase. Phenoloxidase then oxidises the phenolic group containing amino acids (tyrosine) into semiquinones, which have microbicidal action, and these semiquinones are polymerized into melanin. Melanisation is involved in the process of tanning of cuticle during the post-molt period in wound healing and in defence reactions (encapsulation of invading microorganisms). This pigment can be recognised as dark brown spots in the cuticle of shrimps that have been injured.

Together with the activation of proPO, another important component of proPO system gets activated. That is a 76 KDa protein that mediate and enhance cell adhesion and degranulation. This is a multifunctional immune factor, which also promotes encapsulation and function as a phagocytosis - stimulating opsonins (when released together with the molecules of the proPO system). Molecular characterisations of this 76 KDa protein were done and it revealed that they belong to the family of peroxidises.

The prophenoloxidase system also needs factors that regulate the inappropriate activation and amplification of the response, as unregulated melanisation and protease activities would be disastrous to the animal. This control is partially achieved by synthesising the enzyme as an inactive zymogen that requires proteolytic cleavage in order to become active. To avoid excessive or premature activation of proPO system protease inhibitors like serine proteinase inhibitors are found active in crustaceans. Many protease inhibitors like serpins and α-macroglobulins have been reported from arthropods, which regulate the unnecessary activation of proPO system (Kanost, 1999). Activity of phenoloxidase has been reported for many crustaceans including brown shrimp *Farfantepenaeus californiensis*, tiger

shrimp, Penaeus *monodon*, white shrimp, *Litopenaeus vannamei*, SaoPaulo shrimp, *Farfantepenaeus paulensis and* Blue shrimp *Litopenaeus stylirostris.*

1.6.3.2 Lectins

Lectins / agglutinins are non-enzyme proteins or glycoproteins without catalytic activity that binds to specific carbohydrates expressed on different cell surfaces. They exist in almost all living organisms. Lectins have been regarded as potential molecules involved in immune recognition and phagocytosis of microorganisms through opsonisation. These types of carbohydrate binding proteins, which recognize surface structures common for different pathogens, represent a primitive immune response and called pattern recognition proteins (PRP). Some lectins act as opsonins and bind to foreign particles that facilitate their removal by phagocytosis (Marques and Barracco, 2000). The PRPs recognise targets such as lipopolysaccharides (LPS) or peptidoglycan from bacteria, and β -1, 3-glucans or mannans from fungi. Several PRPs recognizing β -1, 3-glucans have been found in arthropods. Soderhall *et al.* (1998) isolated a β –glucan binding protein (BGBP) from plasma of cockroach *Balberus cannifer*. Lectin activity has been identified in the haemolymph of several penaeid shrimp (Vargas- Albores *et al*., 1993). In penaeid shrimp *P.monodon*, Ratanapo and Chulivatnatol (1992) reported the agglutination of pathogenic *Vibrio vulnificus* by purified lectin called monodin.

1.6.3.3 Antimicrobial peptides

Antimicrobial peptides are widespread in the living kingdom, and a large number of these molecules have been isolated from vertebrates and invertebrates. The production of antimicrobial peptides represents a first line of defence mechanism of innate immunity that is widespread in nature. In

crustacean haemolymph, antimicrobial activities have been demonstrated but, only a few molecules have been characterised. Three antimicrobial peptides have been isolated and characterised from *P.vannamei* and named penaeidins and recent results show that these peptides are ubiquitous in crustaceans. These peptides are often broad spectrum in nature and probably act against many infectious agents. They showed activity against the shrimp fungal pathogen *Fusarium oxysporum* and also to some Gram-positive bacteria (Destomieux *et al*., 1997 & 2000). Haemocytes of horseshoe crab, *Limulus polyphemus*, contain a family of arthropod peptide antibiotic, named tachyplesins or polyphemus, and an antibacterial protein named anti-LPS factor (Muta *et al*., 1987; Miyata *et.al*., 1989).

They are classified into three distinct groups based on amino acid sequences, secondary structure and functional similarities (Bachere, 2003). The first and large group is composed of peptides stabilised by intramolecular disulphide bonds, and the other two groups are linear peptides and polypeptides characterised by (1) α –helical structure or (2) a high content of proline residues and / or a high percentage of glycine residues. The haemocytes are found to be the site of production and storage of these peptides. Degranulation of the haemocytes by stress or pathogenic invasion can lead to the release of these peptides into the haemolymph. In most cases, anti-microbial peptides were shown to disrupt microbial membrane by a pore forming action or by a detergent effect.

1.6.3.4 Reactive Oxygen Intermediates (ROI)

Another important defence reaction of haemocytes is the production of a series of microbicidal substances that either inhibit microbial activities or completely digest the microorganisms. This response termed as the respiratory

burst, is an aerobic process, which generates highly reactive oxygen species such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl ions (OH^-) and singlet oxygen ($^1O_2$) (Reactive Oxygen Intermediates or ROI). In *P.monodon*, production of ROI has been induced by immunostimulants like β –glucan and zymosan, which confers, enhanced protection against bacterial or viral infections (Song and Hsieh, 1994). Respiratory burst has also been reported in *L.vannamei* and *L.stylirostris* (Moullac *et al*., 1998). In addition, the activity of superoxide dismutase, an enzyme scavenging superoxide anion, has been measured in shrimps *Palaemnotes argentinus (*Kosower and Kosower, 1978) and *L.vannamei* (Campa-Cordora *et al*., 2002).

1.7 Strategies for control of diseases in shrimp

A large proportion of crustacean aquaculture is dependent on wild caught brood stock that may be netted from the wild with pre-existing bacterial or viral infections. Aquaculture practices themselves may further exacerbate the problem because stock animals are kept under stressful conditions of overcrowding, elevated water temperature and poor water quality (Lee and Wickins, 1992). In these stressful environments diseases associated with opportunistic bacteria, such as *Vibrio spp*. or *Pseudomonas spp*. (Sindermann and Lightner, 1988) can be prevalent. Good husbandry practices may play a vital role in preventing disease occurrence, but additional forms of protection are essential to prevent epidemics. The application of antibiotics or other chemicals to ponds is expensive and undesirable as it risks contamination of both the environment and the final product (Collier and Pinn, 1988 and Grant and Briggs, 1998), as well as causing mortality or impaired growth in juvenile stock (Stuck *et al*., 2001 and Swastika *et al*., 1992). The repeated application of antibiotics, in the long term, is also encouraging the spread of drug resistant

pathogens (Brown, 1989; Aoki, 1992; Karunasagar *et al*., 1994) and this practice atleast in Europe is phased out. Moreover, chemical disinfection may be incompatible with geographic location of the farm and the physical requirements of the stock. As Bachere *et al*. (1995) have discussed, there is a very great need to maximize the immunocompetence of the stock whilst minimizing the use of therapeutic chemicals. Selective breeding programme and the use of genetically modified strains are still a long way for providing an ethically acceptable and commercially viable means of reducing the problem posed by epidemics. It is not surprising , therefore, that there has been a growing interest in finding ways to protect the stock prophylactically in a manner conceptually equivalent to the use of vaccines now routine for humans, agricultural livestock and more recently, farmed fish. Nowadays, the application of immunostimulants and probiotics as prophylactic tools is gaining momentum in crustacean aquaculture systems.

1.7.1 Immunostimulants and probiotics in shrimp aquaculture

In many countries diseases are a major constraint to aquaculture production. Especially, in the shrimp farming production sector, infectious diseases are considered the most limiting factor for further development. Since shrimp farmers still rely on wild animals for the production of seed stock, genetic selection of resistant domesticated shrimp stock is still not feasible. In addition, epidemiological surveys and knowledge of factors that determine the health status of shrimp is scarce, adequate measures to control diseases other than management practices are not available yet. However, such measures to prevent and control diseases are essential for further development for a sustainable shrimp culture sector. Disease control depends on a complex of three factors; diagnosis, treatment and preventive measures (Sindermann and

Lightner, 1988). Treatment measures like the use of chemotherapeutics, has led to the evolution of resistant strains of pathogens and questions of safety (Esiobu and Ike, 2002). In shrimp culture, new and more often difficult pathogens frequently emerge to replace the solved pathogen problem of yesterday. Therefore, preventive measures should improve the control of diseases. Prevention may include environmental manipulation, usage of immunostimulants and probiotics. An immunostimulant is a chemical, drug, stressor or action that enhances the non specific defence mechanism or immune response, rendering the animal more resistant to diseases (Anderson, 1992). Several reports have been published about experiments to enhance the invertebrate defence mechanisms using immunostimulants. In shrimp, several microbial compounds have been reported as the main stimulants of cellular functions, such as β–glucans, lipopolysaccharides (integral component of the outer membrane of Gram-negative bacteria) and peptidoglycans (integral cell wall component of Gram-positive bacteria). These compounds have been researched to evaluate the usefulness of their supplementation against vibrios and WSSV infection (Itami *et al*., 1998). However, most of these studies have delivered these compounds as heat-killed bacteria or cell wall of bacteria and yeast (Song and Hsieh, 1994). Enhancement of the defence system in the practice of shrimp culture is most feasible by oral administration.

The concept of biological control for health maintenance has received widespread attention during the last few years. Thus, the research into the use of probiotics bacteria, live microbial supplement, for aquatic animals is increasing with the demand for environment - friendly sustainable aquaculture (Gatesoupe, 1999). Probiotics are microbial dietary supplements of benefit to the host. Probiotics generally include bacteria, cyanobacteria, microalgae, fungi etc. It is

the "effective micro biota", which includes photosynthetic bacteria, *Lactobacillus*, *Nitrobacteria*, Denitrifying bacteria, *Bifidobacterium* and Yeast. Several bacteria have been used in the larval culture of aquatic organisms. Garriques and Arevalo (1995) reported that the use of *V.alginolyticus* as a probiotic agent might increase survival and growth of *Penaeus vannamei* post larvae. Maeda and Nogami (1989) have reported the use of bacterial strains possessing vibriostatic activity to control vibriosis in prawns thereby enhancing growth. By applying these bacteria in aquaculture a biological equilibrium between competing beneficial and deleterious microorganisms was produced and results showed that the population of *Vibrio* spp. was decreased. The application of immunostimulants and probiotics will certainly continue to play an important role in disease control in intensive shrimp culture. Only a few reports could be obtained on the use of actinomycetes as immunostimulants and probiotics in shrimp aquaculture (Mathew, 2003 and Lakshmi, 2008).

The present work is aimed at the utilization of marine actinomycetes as a source of novel drugs for management of vibriosis. A detailed study of the immunological response of *Penaeus monodon* administered with actinomycetes diets is also dealt with. The present study was undertaken with the following objectives.

1) Selection of the organism, its characterization and identification.

2) Immunomodulatory property of *Nocardiopsis* MCCB110 in *Penaeus monodon.*

3) Isolation, purification and partial characterization of the bioactive compound produced by *Nocardiopsis* MCCB 110.

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Chapter

2

SELECTION OF THE ORGANISM, ITS CHARACTERIZATION AND IDENTIFICATION

Contents

2.1 Introduction

2.1.1 Molecular characterization

Definitive characterization of microorganisms is a crucial step in the screening for natural products. The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. The most powerful approaches to taxonomy are through the study of nucleic acids. Because these are either direct gene products or the genes themselves and comparisons of nucleic acids yield considerable information about true relatedness. Comparison of the bacterial 16S rRNA gene sequence has emerged as the preferred genetic technique.

Molecular systematics, which includes both classification and identification, has its origin in the early nucleic acid hybridization studies, but has achieved a new status following the introduction of nucleic acid sequencing techniques (O'Donnell *et al.*, 1993). Significance of phylogenetic

studies based on 16S rRNA gene sequences is increasing in the systematics of bacteria and actinomycetes (Yokota, 1997). Sequences of 16S ribosomal DNA have provided actinomycetologists with a phylogenetic tree that allows the investigation of evolution of actinomycetes and also provides the basis for identification. Analysis of the 16S rRNA gene begins by isolating DNA (Hapwood, 1985) and amplifying the gene coding for 16S rRNA using the polymerase chain reaction. The rRNA molecules typically consist of highly conserved sequences interspersed with regions of more variable sequences (Gutel *et al*., 1985). Here, a single pair of primers is used to amplify the DNA encoding a variable region of the 16S rRNA gene (Borrell *et al*., 1997). The purified DNA fragments are directly sequenced. The sequencing reactions are performed using DNA sequencer in order to determine the order in which the bases are arranged within the length of sample (Xu Li – Hua *et al.,* 1999) and a computer is then used for studying the sequence for identification using phylogenetic analysis procedures.

2.1.2 Cell wall fatty acid analysis

Large molecules and polymers of cells and cell envelopes have contributed significantly to an improved classification of the order Actinomycetales embracing a large group of Gram-positive bacteria of immense physiological, biochemical and morphological diversity, which are well known for their capacity to produce antibiotics and other biologically active substances (Goodfellow *et al*., 1984).

Cell wall fatty acid analysis identifies microorganisms based on gas chromatographic (GC) analysis of extracted microbial fatty acid methyl esters (FAMEs). Microbial fatty acid profiles are unique from one species to another,

and this has allowed for the creation of very large microbial libraries. More than 300 fatty acids and related compounds are found in bacteria. The wealth of information contained in these compounds can be used for identification of the organisms at both generic and species level. FAME analysis involves the following steps, Harvesting- Removal of cells from culture media; Saponification- lysis of the cells to liberate fatty acids from the cellular lipids; Methylation- formation of fatty acid methyl esters (FAMEs); Extraction - transfer of the FAMEs from the aqueous phase to the organic phase; Base wash - aqueous wash of the organic extract prior to chromatographic analysis (Kuykendall, *et al*., 1988).

2.2 Materials and methods

2.2.1 Selection of the organism

Six isolates of actinomycetes (MCCB105, MCCB106, MCCB107, MCCB108, MCCB109, & MCCB110) maintained in the Microbiology Laboratory, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, were screened for their potential to generate antibacterial compounds against *Vibrio* harveyi (Mathew, 2003). In this process the most potent isolate (MCCB110) was segregated for further study and used as the source of antibacterial compounds.

2.2.2 Identification of the segregated actinomycete by 16S rRNA gene sequencing

The 16S rRNA gene sequencing involves the following steps: Extraction of DNA, PCR amplification, cloning onto pGEM-T Easy vector, Plasmid extraction, Purification and sequencing.

2.2.3 Extraction of total DNA

Selected culture was grown in seed medium (starch 1%, yeast extract 0.4%, peptone 0.2%, seawater 30 ppt, pH 7) at 28ºC for 48 h and 1 ml cell suspension was centrifuged at 8000 rpm for 10 min at 4^0C. The pellet collected was resuspended in 500 µl TNE buffer (Tris-HCl 10 mM, pH 8.0, EDTA 1 mM, NaCl, 0.15 mM) and centrifuged again at 8000 rpm for 10 min at 4^0C. Subsequently, the pellet was resuspended in 500 µl Lysis buffer (Tris-HCl 0.05 mM, pH 8.0, EDTA 0.05 mM, NaCl, 0.1 mM, SDS 2%, PVP 0.2% and 0.1% mercaptoethanol) (Lee *et al*., 2003) and 10 µl Proteinase K was added and incubated initially for 1 hour at 37^0C and then for 2 hours at 55^0C. Further extraction was carried out by phenol-chloroform method (Sambrook and Russell, 2001). The sample was deproteinated by adding an equal volume of phenol (tris-equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and aqueous layers were separated by centrifugation at 15000 rpm for 15 min at 4^0C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once more. Following this, an equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15000 rpm for 15 min at 4^0C to separate the aqueous phase which was transferred to a fresh tube. Then the DNA was precipitated by incubation at -20^0C overnight after adding equal volume of ice-cold isopropanol and 0.1% (v/v) 3 M sodium acetate. The precipitated DNA was collected by centrifugation at 15000 rpm for 15 min at 4^0C and the pellet was washed in ice-cold 70% ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes were left open until the pellet dried. The DNA pellet was dissolved in 100 µl MilliQ (Millipore) grade water. DNA concentration and purity were assessed spectrophotometrically by comparing

absorbance at 260 nm and 280 nm as well as on running on 0.8 % agarose gels.

2. 2.4 Amplification of the extracted DNA

The 16S rRNA gene was amplified after Reddy *et al*. (2000) using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16S 2 (ACG GCT ACC TTG TTA CGA CTT). The amplification reaction was carried out by using a DNA thermal cycler (Eppendorf). Reaction mixture (final volume 25 µl) contained 2.5 µl 10X buffer, 1.5 µl 25mM $MgCl_2$, 1.0 µl of 10pmol of each oligonucleotide primer, 1.0 µl of DNA template, 2 µl of 2.5mM each deoxynucleoside triphosphate and 1 µl of Taq polymerase. Amplification profile consisted of initial denaturation at 95^0C for 5 min, followed by 34 cycles of denaturation at 94^0C for 20s, annealing at 58^0C for 30s and extension at 68^0C for 2 min. A final extension of 68^0C for 10 min was also performed. The PCR products were analyzed by electrophoresis on 1% agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

2.2.5 Cloning onto pGEM-T Easy vector

Fresh PCR product of 16S rRNA was used for cloning into the pGEM-T Easy vector (Promega, USA). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2X), 0.5 µl of the vector (50 ng/ µl), 3 µl of PCR product and 1 µl of T4 DNA ligase (3U/ µl). The ligation mix was incubated overnight at 4^0C. The entire ligated mix was used to transform *Escherichia coli* JM 109 competent cells prepared using calcium chloride method. The ligation mix was added to 10 ml glass tube previously placed in ice to which 50 µl of competent cells were added and incubated on ice for 20 min, a heat shock at 42^0C was given for 90sec, immediately the tubes

were placed on ice for 2 min and then 600 µl of SOC media was added and incubated for 2 hr at 37^0C in an incubator shaker at 250 rpm. The transformation mixture (200 µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100 mM), and X-gal (80 µg/ml). The plates were incubated at 37^0C overnight. The clones were selected using the blue/white screening. The white colonies were selected and streaked to purify on LB-Amp+X-gal+IPTG plates and incubated overnight at 37^0C. To confirm the insert, colony PCR of the white colonies were carried out using the vector primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'- GATTTAGGTGACACTATAG-53'). White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25 µL) containing 2.5 µl 10X PCR buffer, 2.0 µl of 2.5 mM dNTPs, 1 µl of 10 pmol / µl of T7 and SP6 primers, 0.5 U of taq polymerase and the remaining volume was made up with Milli Q. The thermal cycling conditions were as follows: 1x 95^0C for 5 min; 35 x (94^0C for 15s, 57^0C for 20 s, 72^0C for 60 s); 1 x 72^0C for 10 min following which the temperature was brought down to 4^0C.

2.2.6 Plasmid extraction & purification and sequencing

Plasmids from the positive clones were extracted using the 'GenElute HP' plasmid miniprep kit (Sigma). An overnight recombinant *E.coli* culture was harvested with centrifugation and subjected to a modified alkaline-SDS lysis procedure followed by adsorption of the plasmid DNA onto silica (column) in the presence of high salts. Contaminants were removed by spin wash step. Finally, the bound plasmid DNA is eluted in 5 mM Tris-HCl pH 8.0. Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Microsynth AG, Switzerland. The primers used were T7 and SP6.

2.2.7 16S rRNA gene sequence similarity & phylogenetic analysis

Sequenced DNA data were compiled and analyzed, and matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990) from the NCBI website (www.ncbi.nlm.nih.gov). The sequences were multiple aligned using the programme Clustal W (Thompson *et al*., 1994). Genetic distances were obtained by using Kimura's 2-parameter model (Kimura 1980), and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei 1987) using the software MEGA4 (Tamura *et al*., 2007).

2.2.8 Analysis of cell wall fatty acids by gas chromatography

Cultures were grown as confluent patches on trypticase soya broth agar at 28ºC for 4 d. Vegetative growth was scrapped off from the surface (100-200mg) and fatty acid methyl esters (FAME) were prepared by a modified sample preparation (Miller *et al*., 1985). Analysis of FAME was carried out by capillary gas chromatograph using an Agilent Technology model 6890N Network GC system/MIDI system (MIDI Inc, Newark, DE, USA) equipped with a phenyl methyl silicon column (0.2x25m). Chromatography conditions were recommended by the manufacturer. Individual FAME was identified using the Microbial Identification Software (MIS, MIDI Inc).

2.3 Results

2.3.1 Identification of the segregated actinomycete by 16S rRNA gene sequencing

The isolate referred above (MCCB 110) was subjected for molecular characterization through amplification and sequencing 16S rRNA gene. An amplification product of 1.5 kb length was produced as shown in the Fig 1.

The PCR products after purification and cloning have been subjected for sequencing. Subsequent to amplification and cloning, several levels of screening were carried out for the confirmation of the insert. The initial screening was by colony PCR using T7 and SP6 vector primers which produced a product of 1.7 kb. The clones with no insert or with non-targeted inserts were discarded after the colony PCR and the rest were taken for plasmid extraction. Plasmids extracted using the GenElute HP' plasmid kit was checked for purity in agarose gel (Fig 2).

Alignment of the total nucleotide sequence (Fig 3) of 1495 bp (accession number: EU008081) of the 16S rRNA gene of MCCB110 followed by matching with reported 16S rRNA gene sequences in the GenBank showed a high similarity (99%) to *Nocardiopsis* sp. Phylogenetic analyses based on a dataset consisting of 1495 bp showed that the novel isolate falls into a distinct clade with another *Nocardiopsis* species, *Nocardiopsis* sp (AN10) (Fig. 4).

2.3.2 Cell wall fatty acid analysis

The cell wall fatty acid composition, determined by gas chromatography on a culture grown on Trypticase soy agar, included: iso-$C_{14:0}$ (1.90%), iso-$C_{15:0}$ (1%), anteiso-$C_{15:0}$ (4.5 %), iso-$C_{16:0}$ (35.94%), iso-$C_{17:0}$ (3.23%), anteiso-$C_{17:0}$ (10.75 %), 10 methyl-$C_{17:0}$ (4.65%), iso-$C_{18:0}$ (6.61%), ω8c$C_{17:1}$(3.95%), and, ω9c$C_{18:1}$(13.03%) (Table1). Based on the fatty acid profile the organism was identified as *Nocardiopsis* sp.

2.4 Discussion

The genus *Nocardiopsis* under the family actinomycetales includes aerobic, Gram positive, spore forming actinomycetes that produce a branched vegetative

mycelium and aerial hyphae (Meyer, 1976). They form dirty white aerial mycelium, becoming light-yellowish grey in ageing cultures. No endo- or exopigments are produced. Hyphae of the aerial mycelium are straight to flexuous. In older cultures, hyphae of aerial mycelium disintegrate into spore-like structures. *Nocardiopsis* strains are distributed ubiquitously in the environment (Kroppenstedt & Evtushenko, 2002). Like other actinomycetes, *Nocardiopsis* spp. are capable of producing metabolites with biological activities (Kroppenstedt, 1992; Dolak *et al*., 1981; Mordarska *et al*., 1985; Tsujibo *et al*., 1990).

Several methods such as determination of cell wall chemotype (Lechevalier *et al*., 1970; Lechevalier *et al*., 1989; Meyer *et al*., 1989), menaquinone composition (Fischer *et al*., 1983; Kroppenstedt, 1985), phospholipid pattern (Lechevalier *et al*., 1989; Lechevalier *et al*., 1977) and cell wall fatty acid profile (Grund *et al*.,1990; Kroppenstedt, 1985 ; McNabb *et al*., 1997) have been developed to identify *Nocardiopsis*. In addition, 16S rRNA sequence data have also been proved invaluable in its systematics.

The rRNA is the most conserved gene in all cells. Portions of the rDNA sequence from distantly-related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. The DNA sequence of the16S rRNA gene has been determined for an extremely large number of species. In fact, there is no other gene that has been as well characterized in as many species. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny, and to estimate rates of species divergence among bacteria.

Actinomycetes are in general slow-growing and/or difficult to identify. Thus, they were an important group of organisms in early important studies establishing the usefulness of 16S rRNA gene sequencing for clinical microbiology (Boddinghaus *et al*., 1990a; Boddinghaus *et al*., 1990b; Bottger, 1989; Edwards *et al*., 1989; Kirschner *et al*., 1993; Rogall *et al*., 1990a; Rogall *et al*., 1990b; Teske *et al*., 1991). More recently, there have been several additional studies comparing the identification of actinomycetes by 16S rRNA gene sequence and phenotypic methods (Cloud *et al*., 2002; Cook *et al*., 2003; Hall *et al*., 2003; Kirschner *et al*., 1998; Patel *et al*., 1998; Springer *et al*., 1995; Turenne *et al*., 2001). In all of these studies, the accuracy of 16S rRNA gene sequencing in the identification to the species level was judged to be superior overall to phenotypic methods. Overall, by providing for the accurate identification of species in the database and the taxonomic placement if not complete identification of novel species, 16S rRNA gene sequence analysis of actinomycetes seems to be the most accurate method available. Hence, the 16S rRNA sequences offer a reliable and straightforward tool for their identification.

16S rRNA gene sequences allow bacterial identification that is more robust, reproducible, and accurate than that obtained by phenotypic testing. The test results are less subjective. 16S rRNA gene sequencing traditionally played a limited role in the identification of microorganisms in clinical microbiology laboratories, mainly due to high costs, requirements for great technical skill, and the lack of user-friendly comparative sequencing analysis software and validated databases. However, the availability of improved DNA sequencing techniques, vastly increased databases and more readily available kits and software, makes this technology a competitive alternative to routine

microbial identification techniques for several groups of organisms, such as actinomycetes.

The usefulness of 16S rRNA gene sequencing has been greatly enhanced through the establishment of large public domain databases (Maidak *et al*., 1996), which allow the comparison of a sequence with all other deposited eubacterial 16S rRNA gene sequences (Maidak *et al*., 1996). Thus, molecular methods may provide quick and accurate identification independent of standard culture methods and the amount of isolate available.

The fatty acid pattern of this strain was composed of iso/anteiso-branched fatty acids. Smaller amounts of 10-methyl-branched and unbranched fatty acids were also found. The high amount of ante-iso - fatty acids in combination with 10-methyl-branched fatty acids (fatty acid type 3d) is diagnostic for species of the genus *Nocardiopsis* (Kroppenstedt, 1985). It allowed the identification of this isolate at the genus level. Cell wall fatty acid content, however, depends on culture conditions, extraction, and chromatography techniques (McNabb *et al*., 1997) and requires large quantities of bacterial material. 16S rRNA gene sequencing allowed the definitive identification of this isolate, with a 99 % sequence similarity with that reported for *Nocardiopsis sp.*

A cell wall fatty acid pattern, including a major percentage of branched-chain fatty acids with high percentages of C17:0, anteiso-C18:0, and C18:0 10-methyl fatty acids, was reported as characteristic of the *Nocardiopsis* genus (Fischer *et al*., 1983; Kroppenstedt, 1985; McNabb *et al*., 1997). 16S rRNA gene sequencing allowed the definitive identification of this isolate, with a 99 % sequence similarity with that reported for *Nocardiopsis sp*. Accordingly, the organism was designated as *Nocardiopsis* MCCB 110.

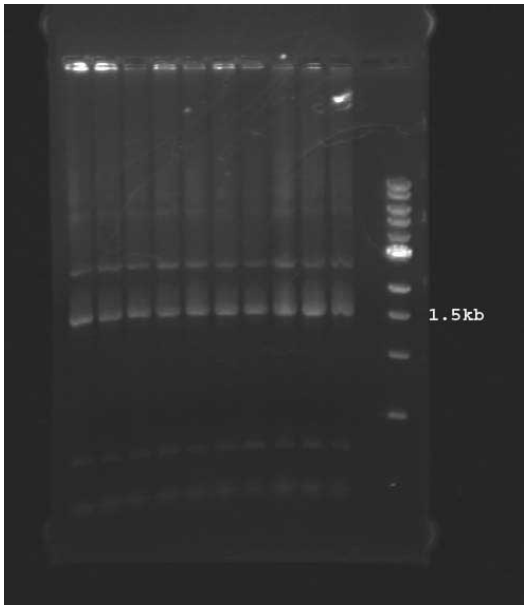


Fig.1. Amplification of 16S rRNA gene.

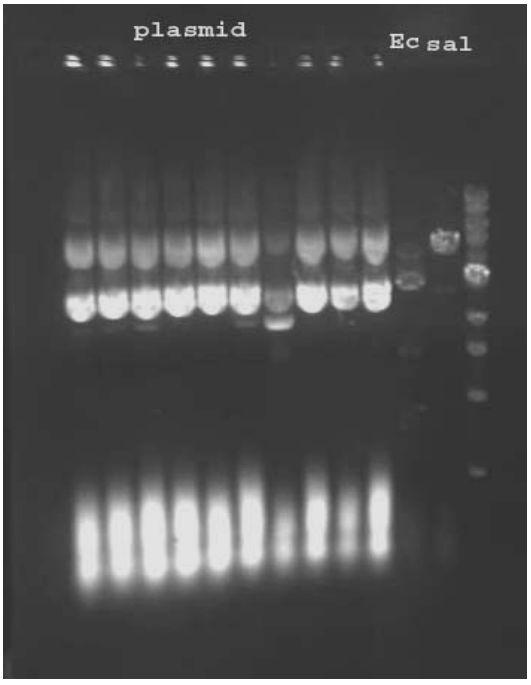


Fig.2. Plasmids extracted using the GenElute HP' plasmid kit in agarose gel.

```
   1 gagtttgatc ctggctcagg acgaacgctg gcggcgtgct taacacatgc aagtcgagcg
  61 gtaaggccct tcggggtaca cgagcggcga acgggtgagt aacacgtgag caacctgccc
 121 ctgactctgg ggtaagcggt ggaaacgccg tctaataccg gatacgacct tccacctcat
 181 ggtggagggt ggaaagtttt ttcggtcagg gatgggctcg cggcctatca gcttgttggt
 241 ggggtaacgg cctaccaagg cgattacggg tagccggcct gagagggcga ccggccacac
 301 tgggactgag acacggccca gactcctgcg ggaggcagca gtggggaata ttgcgcaatg
 361 ggcgaaagcc tgacgcggcg acgccgcgtg ggggatgacg gccttcgggt tgtaaacctc
 421 ttttaccacc aacgcaggct ccgggttctc tcggggttga cggtaggtgg ggaataagga
 481 ccggctaact acgtgccagc agccgcggta atacgtaggg tccgagcgtt gtccggaatc
 541 attgggcgta aagagctcgt aggcggcgtg tcgcgtctgc tgtgaaagac cggggcttaa
 601 ctccggttcc gcagtggata cgggcatgct agaggtaggt aggggagact ggaattcctg
 661 gtgtagcggt gaaatgcgca gatatcagga ggaacaccgg tggcgaaggc gggtctctgg
 721 gccttacctg acgctgagga gcgaaagcat ggggagcgaa caggattaga taccctggta
 781 gtccatgccg taaacgttgg gcgctaggtg tggggacttt ccacggtttc cgcgccgtag
 841 ctaacgcatt aagcgccccg cctggggagt acggccgcaa ggctaaaact caaaggaatt
 901 gacgggggcc cgcacaagcg gcggagcatg ttgcttaatt cgacgcaacg cgaagaacct
 961 taccaaggtt tgacatcacc cgtggacctg tagagataca gggtcattta gttggtgggt
1021 gacaggtggt gcatggctgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgcaa
1081 cgagcgcaac ccttgttcca tgttgccagc acgtaatggt ggggactcat gggagactgc
1141 cggggtcaac tcggaggaag gtggggacga cgtcaagtca tcatgccccc tatgtcttgg
1201 gctgcaaaca tgctacaatg gccggtacaa tgggcgtgcg ataccgtaag gtggagcgaa
1261 tcccttaaag ccggtctcag ttcggattgg ggtctgcgac tcgaccccat gaaggtggag
1321 tcgctagtaa tcgcggatca gcaacgccgc ggtgaatacg ttcccgggcc ttgtacacac
1381 cgcccgtcac gtcatgaaag tcggcaacac ccgaaacttg cggcctaacc cttcggggag
1441 ggagtgagtg aaggtggggc tggcgattgt gacgaagtcg taacaaggta gccgt
```

Fig.3. 16S rRNA gene sequence of *Nocardiopsis* MCCB110.

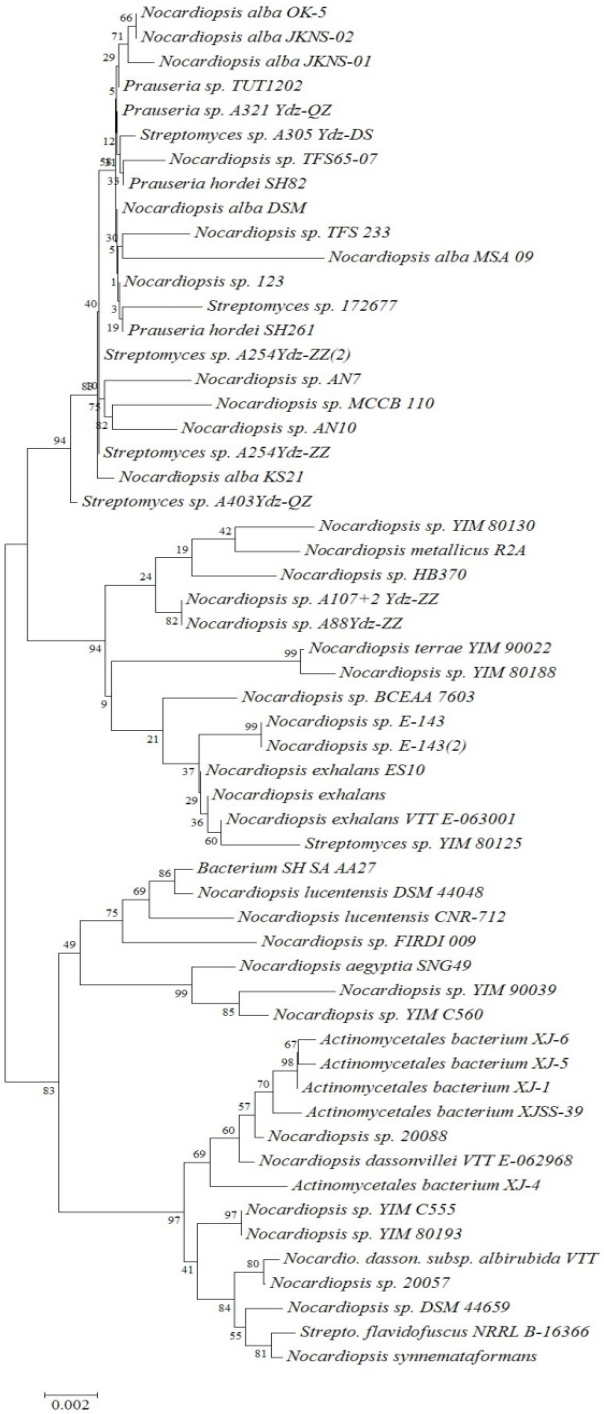


Fig.4. Phylogenetic analysis of 16S rRNA gene sequence of *Nocardiopsis* MCCB110.

Table.1. Whole-cell fatty acid composition of *Nocardiopsis* MCCB110.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent		
0.364	8090	0.216	----	4.403		----		
1.577	3.94E+8	0.024	----	7.049	SOLVENT PEAK	----		
1.702	10192	0.016	----	7.322		----		
1.963	81	0.011	----	7.891		----		
2.079	351	0.041	----	8.145		----		
2.367	468	0.047	----	8.773		----		
2.464	447	0.038	----	8.984		----		
2.563	345	0.034	----	9.201		----		
2.867	806	0.058	----	9.864		----		
3.855	103	0.012	----	11.343		----		
4.223	329	0.028	----	11.776		----		
4.574	569	0.066	----	12.150		----		
4.791	1241	0.069	----	12.345		----		
5.656	180	0.023	----	13.123		----		
6.114	197	0.022	----	13.476		----		
6.299	2541	0.029	0.995	13.619	14:0 ISO	1.90	ECL deviates 0.000	Reference -0.005
6.793	291	0.023	0.987	14.000	14:0	0.21	ECL deviates 0.000	Reference -0.005
7.424	126	0.014	----	14.426		----	< min ar/ht	
7.713	1375	0.032	0.974	14.622	15:0 ISO	1.00	ECL deviates -0.001	Reference -0.007
7.849	5554	0.039	0.973	14.714	15:0 ANTEISO	4.05	ECL deviates 0.001	Reference -0.005
8.002	4736	0.122	----	14.817		----	> max ar/ht	
8.187	275	0.023	----	14.942		----		
8.431	1025	0.067	----	15.098		----		
9.289	50037	0.038	0.958	15.629	16:0 ISO	35.94	ECL deviates 0.002	Reference -0.004
9.590	1804	0.033	0.956	15.815	Sum In Feature 3	1.29	ECL deviates -0.007	16:1 w7c/15 iso 2OH
9.889	1723	0.032	0.954	16.000	16:0	1.23	ECL deviates 0.000	Reference -0.005
10.957	4550	0.039	0.947	16.631	17:0 ISO	3.23	ECL deviates 0.001	Reference -0.004
11.114	15164	0.040	0.946	16.723	17:0 ANTEISO	10.75	ECL deviates 0.000	Reference -0.004
11.229	5570	0.041	0.946	16.791	17:1 w8c	3.95	ECL deviates -0.001	
11.583	1015	0.025	0.944	17.000	17:0	0.72	ECL deviates 0.000	Reference -0.004
11.961	4278	0.162	----	17.218		----	> max ar/ht	
12.207	1238	0.063	----	17.359		----		
12.286	6588	0.055	0.941	17.405	17:0 10 methyl	4.65	ECL deviates -0.004	
12.453	2062	0.085	----	17.500		----		
12.680	9388	0.043	0.939	17.631	18:0 ISO	6.61	ECL deviates -0.001	Reference -0.004
12.913	18528	0.042	0.939	17.765	18:1 w9c	13.03	ECL deviates -0.004	
13.317	5817	0.045	0.937	17.997	18:0	4.09	ECL deviates -0.003	Reference -0.005
13.996	6085	0.041	0.935	18.388	TBSA 10Me18:0	4.27	ECL deviates -0.004	
14.284	1725	0.053	----	18.554		----		
14.422	1113	0.060	0.934	18.633	19:0 ISO	0.78	ECL deviates -0.001	Reference -0.001
14.598	2175	0.090	0.934	18.734	19:0 ANTEISO	1.52	ECL deviates 0.003	
14.912	468	0.042	0.933	18.915	19:0 CYCLO w8c	0.33	ECL deviates 0.013	Reference 0.014
15.043	639	0.034	0.933	18.990	19:0	0.45	ECL deviates -0.010	Reference -0.009
15.164	448	0.039	----	19.061		----		
15.988	748	0.062	----	19.539		----		
16.434	718	0.066	----	19.798		----		
16.470	181	0.021	----	19.819		----		
16.985	554	0.056	----	20.118		----		

Chapter

3

IMMUNOMODULATORY PROPERTY OF *NOCARDIOPSIS* MCCB110 IN *PENAEUS MONODON*

Contents

3.1 Introduction

Commercial shrimp farming began in the 1970s and the world production of shrimp farming has been increasing steeply during the last 20 years. During the past two decades, worldwide shrimp aquaculture has been greatly puzzled by diseases. Most disease occurs as a result of environmental deterioration and stress associated with intensification of shrimp farming (Tseng and Chen, 2004). Many shrimp farms have been affected by epidemics caused by virus, bacteria, protozoan and fungi. Several disease outbreaks of vibriosis caused by *Vibrio harveyi*, *Vibrio damsel* and *Vibrio alginolyticus* have been reported (Song *et al*., 1993 & Lee and Wickins, 1992).

Prevention and control of diseases are now the priority for the sustenance of aquaculture industry. The emergence of drug resistance in pathogens, problems associated with drug residues in cultured animals and awareness towards environmental problems associated with the use of

chemotherapeutics have led to greater focus on alternate methods like probiotics and immunostimulants.

3.1.1 Non-specific immune response of *Penaeus monodon*

Immunostimulants comprise compounds that enhance the non-specific defence mechanisms and provide resistance against the invading pathogenic microorganisms. Perusal of the literature indicated that immunostimulants are proven very successfully in treating or preventing microbial diseases in cultured shell fishes. A variety of cell wall components of fungal and bacterial origin can trigger non-specific responses (Soderhall and Smith, 1986). Live bacteria, killed bacteria, cell wall components like peptidoglycans, β-glucans and lipopolysaccharides (LPS) have been successfully used to initiate a series of nonspecific defense activities (Soderhall and Smith, 1986 and Persson *et al*., 1987). Immunostimulants that are reported to be effective in fish and shell fish includes synthetic chemicals like levamisole, Vitamins C and E, interferon and prolactin. Vitamin C plays a role as immunostimulant, as evidenced by the ability of *P. monodon* post larvae and juveniles to control Baculovirus and to resist *V. harveyi* and saline shock (Catacutan and Lavilla- Pitogo.,1994 and Merchie *et al*., 1998). Dietary administration of sodium alginate could enhance ability of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus* (Cheng et al., 2005). *Sargassum fusiforme* polysaccharide extracts enhance resistance to vibriosis and immune activity of *Fenneropenaeus chinensis* (Huang *et al*., 2006). Gram-negative and Gram- positive bacteria have also been used for activating some factors of immune system in crustaceans (Soderhall and Duvic, 1990; Johansson and Soderhall, 1989). Administration of β-1,3 and 1, 6-glucan extracted from the yeast, *Saccharomyces cerevisiae*, by immersion has been reported to increase the phenoloxidase activity of tiger

shrimp, *P.monodon*, and its resistance to *Vibrio vulnificus*. Oral administration of schizophyllan, a β-1,3-glucan extracted from the fungus, *Schizophyllum commune*, has been reported to enhance the immunity indices such as total hemocyte count, phenol oxidase, superoxide anion production and superoxide dismutase activity (Chang *et al*., 2003). *Litopenaeus vannamei* fed with a diet containing sodium alginate at 2.0g kg^{-1} increased its immune ability by increasing its phenol oxidase activity, respiratory burst, SOD activity and clearance efficiency against *V. alginolyticus* (Cheng *et al*., 2005).

3.1.2 Immune-related genes' expression of *Penaeus monodon*

The knowledge of immune gene expression in response to pathogens is of prime importance to understand the immune capability of shrimps and also for the establishment of a health monitoring system in shrimp culture. Genomic approaches have been used to characterize immune genes in different shrimp species. Expression profiles of selected genes have been analyzed by real time PCR. Several immune genes, encoding proteins involved in the general biodefence have been discovered and differential gene expression analyzed in penaeid shrimps, *Penaeus monodon* (de la Vega *et al*., 2006; 2007; Jiravanichpaisal *et al*., 2007), *Litopenaeus vannamei* (Wang *et al*., 2007, 2008), *Litopenaeus setiferus* (Gross *et al*., 2001) and *Penaeus japonicas* (He *et al*., 2004). The gene expression profile of shrimp in response to white spot syndrome virus, yellow head virus, *Vibrio* spp., peptidoglycan, oxytetracycline, oxolinic acid, salinity and temperature using the high throughput microarray analysis has been reviewed by Aoki *et al*, (2010). The immune related genes identified in *Penaeus monodon* include clottable protein (Yeh *et al*., 1998; Yeh *et al*., 1999); transglutaminase (Huang *et al*., 2004; Yeh *et al*., 2006); proPO (Sritunyalucksana *et al*., 1999); β-1,3- glucan binding

protein (Sritunyalucksana *et al*., 2002); *Penaeus monodon* LPS binding lectin-PmLec (Luo *et al*., 2006); penaedin-5 (Hu *et al*., 2006); clip domain serine protease homolog, c-SPH (Lin *et al*., 2006); heat shock cognate 70, hsc70 (Chuang *et al*., 2007); heat shock protein 86, hsp86 (Cimino *et al*,2002); hsp90 (Jiang *et al*., 2009); haemocyanin (Colangelo *et al*., 2004; Zhang *et al*., 2004); alpha2- macroglobulin (Lin *et al*., 2007); crustin (Chen *et al*., 2004); kazal-type serine proteinase inhibitor (Somprasong *et al*., 2006); astakine (Soderhall *et al*., 2005); peroxinectin (Sritunyalucksana *et al*., 2001); haemocyanin (Lenhert *et al*.,2002); antilipopolysachharide factors, ALF (Supungul *et al*., 2002; Tharntada *et al*., 2008); inhibitor of apoptosis protein, IAP (Leu *et al*., 2008); Translationally controlled tumor protein, TCTP (Bangrak *et al*., 2004); ribophorin I (Chotwiwatthanakun *et al*., 2008); Cathepsin C (Qui *et al*., 2008); Dicer (Su *et al*., 2008); *Penaeus monodon's* Argonaute, Pem-Ago (Dechklar *et al*., 2008); *Penaeus monodon* Toll, PmToll (Arts *et al*., 2007); Crustin-like antimicrobial peptide (Amparyup *et al*., 2008); lysosome (Xing *et al*., 2009); Ribophorin 1 (Chotwiwatthanakun *et al*., 2008; Molthathong *et al*., 2008a); Defender against apoptotic death- DAD1 (Molthathong *et al*., 2008b); and cyclophilin A (Qiu *et al*., 2009).

Accordingly, various non-specific humoral immune mechanisms present in the haemolymph of *Penaeus monodon* were analyzed by employing most widely used non-specific immune response assays. Subsequently, a study on semi quantitative RT-PCR analysis of the expression of some of the genes related to different immune-related mechanisms such as proPO system (proPO gene), cell adhesion (Peroxinectin

gene), Clotting/Coagulation (Transglutaminase gene), Proteinase inhibition (Alpha 2-macroglobulin gene), Cytokine in haematopoeisis (Astakine gene) and Antimicrobial peptides (Crustin gene, Penaeidin-3 gene) were identified along with a house keeping gene (β actin) in shrimp.

3.2 Materials and method

3.2.1 Experimental animals and rearing conditions

Adult *Penaeus monodon* (average body weight, 15-20 gm) obtained from a commercial farm located at Narayanmangalam, Trichur, Kerala were used as experimental animals. They were transported to the laboratory immediately after capture. Shrimps were reared in fiber glass tanks containing 15ppt salinity seawater and allowed to acclimate for a week. Continuous aeration was provided and shrimps were fed on a commercial shrimp diet. Physico –chemical parameters of the rearing water were monitored regularly and salinity, NH_3 and pH were estimated as per APHA (1995) and maintained at optimum level (Table 1). The faecal matter and left over feed were removed daily by siphoning. Water was exchanged to about 30-40% on alternate days. Stringed bed suspended bioreactor (Kumar *et al*., 2008) was set up in all the rearing tanks for the management of ammonia nitrogen. After acclimation for 7 days, the immunological profile was recorded from a group of shrimps (n=6) as the baseline data. The samples (n=6) were also taken after 10 days of feeding from normal and *Nocardiopsis* incorporated feed administered animals, and subsequently 48h after challenge with *V. harveyi*.

Table.1. Rearing conditions and water quality parameters

Tank capacity	150 l
Stocking density	9 nos.
Feeding level	10–15% of body weight
Feeding frequency	Twice daily
Water temperature	24–27 °C
pH	7.5–8
Salinity	15ppt
Ammonia	0.01–0.02 mg l−1
Nitrite	0.00–0.01 mg l−1
Nitrate	Below detectable level
Dissolved oxygen	6–7 mg l−1

3.2.2 Feeding Experiment

Two groups of animals were maintained: Group 1- Normal (fed with normal feed) and Group II- fed with *Nocardiopsis* biomass incorporated feed. *Nocardiopsis* (7 d old) was incorporated to the pelleted feed at a ratio of 50 ml culture broth (1x 10^6 cfu/ml) to 100 g feed and dried at 50 ^{0} C and stored at 4 ^{0} C. The shrimps were fed twice daily, at morning 9 A.M and evening 7 P.M, at a rate of 10 % of their body weight. The shrimp was fed for10 days. Physico-chemical parameters of the rearing water were monitored regularly (Table 1). After 10 days of feeding, the animals (n=6/group) were sampled (just before challenge) from each of the two experimental groups. Simultaneously, after 10 days, the animals were challenged with *V harveyi* (10^5cells/animal of 15 to 20 g size) by injection and sampled (n=6) from each of the two experimental groups. Post challenge survival was recorded for a period of seven days.

3.2.3 Sample collection for non-specific immune assays

Haemolymph sample was collected using capillary tube rinsed with the anticoagulant (0.01M Tris-HCl, 0.25 M Sucrose, 0.1M Tri Sodium Citrate, pH-7) from 6 animals belonging to each of the two groups for testing the non-specific immune parameters such as total haemocyte count, total protein, phenol oxidase activity, reactive oxygen intermediate production/ respiratory burst, alkaline phosphatase and acid phosphatase activities.

3.2.4 Non-Specific Immune assays

3.2.4.1 Total Haemocyte Count (THC)

Total haemocyte count (THC) was determined by using a Neubaeur's haemocytometer. A drop of haemolymph was placed on the haemocytometer and haemocytes were counted by observing under a bright field microscope and expressed as cells ml^{-1} haemolymph.

3.2.4.2 Phenoloxidase (PO) activity

Phenol oxidase activity was determined by incubating 100µl of haemolymph with 100µl of 10% SDS for 30 min. at 25^0C and by the addition of 2.0ml of substrate (0.19% L-DOPA in Tris-HCl buffer). The dopachrome formed was measured in a UV-Visible spectrophotometer at 490 nm, at every 30s for 3 minutes and the activity expressed as increase in absorbance $minute^{-1}$ $100µl^{-1}$ haemolymph (Soderhall *et al*., 1981).

3.2.4.3 Respiratory burst activity

Intracellular superoxide anion (O_2^-) or the reactive oxygen intermediates (ROI) or the respiratory burst activity was measured by the reduction of Nitro blue tetrazolium (NBT) (Song and Hsieh, 1994) as described by Chang

et al, (2000). A sample of 100µl haemolymph was incubated with 100µl 0.2 % NBT for 30min at 10^0C. The cells were separated by centrifugation (1200rpm, 4^0C, 10 min) and fixed in 100% methanol. It was then incubated for 10 min at room temperature (RT, 28^0C) and subjected to centrifugation (300rpm, 4^0C, 10 min). The supernatant was removed after centrifugation and the cells were dried and then rinsed in 50% methanol and solubilised in 140µl DMSO and 120µl 2M KOH. The absorbance at 620nm was recorded and the activity expressed as O.D. 100 μl^{-1}haemolymph.

3.2.4.4 Acid phosphatase (ACP)

Acid phosphatase activity (ACP) of the haemolymph was determined as described by Bisswanger (2004) with o- carboxyphenyl phosphate, under acid conditions. Briefly, a 20 µl haemolymph sample was mixed with 140 µl 0.15M acetate (pH-5) and 40 µl 3.65 mM o- carboxyphenyl phosphate in a UV microplate and incubated at 25^0C for 3min. The absorbance was taken at 300 nm at 1 min interval for 6 min and expressed as change in absorbance/minute 100 μl^{-1} haemolymph.

3.2.4.5 Alkaline phosphatase (ALP)

Alkaline phosphatase activity (ALP) was determined by the hydrolysis of the chromogenic substrate p-nitrophenyl phosphate to p-nitrophenol by the enzyme as described by Bisswanger (2004). Briefly, 170 µl 0.1M Glycine-KOH (pH -10.5) was mixed with 10 µl 0.5M p-nitrophenyl phosphate and 2 µl haemolymph in a microplate and the absorbance taken at 405 nm at 1 min interval for 6 min and expressed as change in absorbance/min 100 μl^{-1} haemolymph.

3.2.4.6 Total protein

Total haemolymph protein was estimated by the Bradford's assay (1976) and Bovine serum albumin was used as the standard. Briefly, 5 µl haemolymph was mixed with 995 µl 0.1N NaOH and 5ml Bradford's reagent and incubated at room temperature for 10 min. The absorbance was taken at 595 nm and expressed as mg/ml.

3.2.4.7 Statistical Analysis

The data collected were analyzed statistically to determine significant difference in immunological parameters between different groups by independent student t test and two way ANOVA by using SPSS 15.0. The level of significance was set at 1% and 5%.

3.2.5 Immune-related genes' expression of *Penaeus monodon* to *Nocardiopsis* MCCB 110

3.2.5.1 Sample collection for the semi quantitative RT-PCR analysis of biodefence genes

Haemolymph was collected using RNase free (diethyl pyrocarbonate (DEPC) treated) capillary needles from 3 animals belonging to each of the two groups before challenge and after challenge with minimum stress. The samples (~250 µl of haemolymph) were stored per ml TRI Reagent (Sigma) and transferred immediately to – 80 ^{0}C, and RNA extraction was carried out within a fortnight.

3.2.5.2 RNA extraction, cDNA synthesis and semi quantitative RT-PCR of bio-defence genes

Total RNA was isolated using TRI® Reagent (Sigma) following the manufacturer's protocol with slight modifications. Briefly, the samples were

macerated and placed at room temperature for 5 min to ensure complete dissociation of nucleoprotein complexes. Chloroform (200 µl) was added per ml of TRI® Reagent and shaken vigorously for 15sec and allowed to stand for 15 min at RT and centrifuged (12,000g, 4^0C, 15 min.). Of the three layers observed, the colourless top aqueous phase was carefully separated into a fresh MCT. Aliquot of 500 µl isopropanol was added and incubated for 10 min at RT and centrifuged (12,000g, 4^0C, 15 min.). The supernatant was removed and the RNA precipitated at the bottom and sides of the MCT was washed (12,000g, 4^0C, 15 min.-twice) with ethanol. After removing the supernatant, the pellet was air dried and dissolved in 20 µl DEPC treated water and incubated at 55^0C for 10 min. DNase treatment of the RNA samples were done with 0.2 U of the enzyme, RNase free DNase 1(New England Biolabs), for 1µg of RNA by incubating at 37 ^{0}C for 10 min. The enzyme inactivation was done by incubating at 75^0C for 10 min. RNA quality and concentration were determined at 260/280 nm using a UV- Visible spectrophotometer (Shimadzu).

cDNA synthesized using a sample of 5µg of the extracted RNA, was added to a 20 µl reaction mixture with RNase Inhibitor (8U), Oligo $(dT)_{12}$ primer (40pmol), dNTP mix (1mM), RTase Buffer (1X), $MgCl_2$ (2mM) and Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase enzyme (200U). The reverse transcription was carried out at 42^0C for 1 hour on a Thermal cycler (Master Cycler personal, Eppendorf).

Bio-defence genes were amplified on a Master Cycler personal (Eppendorf). The primers selected for these genes included those from literature as well as those designed from sequences deposited in GenBank (Table 2).The PCR was carried out by using 2 µl of the cDNA with specific primer sets as given in the Table. Shrimp β actin was amplified as a reference

gene. The 25 µl PCR mixture subjected for amplification contained dNTP mix (200 µM), 1X PCR buffer (Thermopol), forward and reverse primers (10pmol), cDNA (2 µl) and Taq DNA Polymerase (0.5U). The hot start PCR programme used for the immune related genes was 94^0C for 2min, followed by 35 cycles of 94^0C for 2 min, annealing for 1 min, 72^0C for 1 min and a final extension at 72^0C for 10 min. The annealing temperature, which was used for the amplification of immune-related genes, is shown along with the primer sequence in the Table 2. The PCR products were analyzed by horizontal gel electrophoresis of 10 µl of the PCR products. The gels were stained with ethidium bromide and documented under UV light with Molecular Imager® Gel Doc ™ XR+ Imaging System (Bio-Rad). Band intensity was calculated using Quantity One ® software (Bio-Rad) and the significant differences between different groups were identified from the graphical analysis of the band intensity.

Table.2. Primer sequences of the bio-defence genes selected for the study

Sl. No.	Gene	Primer sequences(5'-3') and Annealing Temperatures(^{0}C)	PCR amplicon (~bp)	Reference
1	Prophenoloxidase (proPO)	F-TGGCACTGGCACTTGATCTA R- GCGAAAGAACACAGGGTCTCT (56)	590	Jiravanichpaisal et al,2007
2	Astakine	F- GTCGCGCATTTAACAAGGAG R- CCCTGTGGATTGAGCTCACT (56)	455	
3	Peroxinectin	F- CGAAGCTTCTTGCAACTACCA R- GCAGGCTGATTAAACTGGCTT (56)	547	
4	Transglutaminase	F- TGGGYCTTCGGGCAGTT R- CGAAGGGCACGTCGTAC (56)	627	
5	Penaedin-3	F-AGGATATCATCCAGTTCCTG R-ACCTACATCCTTTCCACAAG (55)	240	
6	Crustin	F- GCACAGCCGAGAGAACACTATCAAAGAT R-GGCCTATCCCTCAGAACCCAGCACG (55)	430	
7	Alpha 2 macroglobulin	F-F-ATGGCCAATCCCGAGAGGTACCTACTG R-TGTTGCTGCAGAAGTTTGTTATCCTCAT (65)	345	Lin et al,2007a
8	B Actin	F- CTTGTGGTTGACAATGGCTCCG R- TGGTGAAGGAGTAGCCACGCTC (55)	520	Zhang et al,2007

3.3 Results

3.3.1 Non-specific immune response of *Penaeus monodon*

3.3.1.1. Total Haemocyte Count (THC)

The shrimps fed with *Nocardiopsis* incorporated feeds showed a higher number of total haemocytes both before ($42.1 \times 10^6 \pm 1.83 \times 10^5$) and after challenge ($36.8 \times 10^6 \pm 2.51 \times 10^5$) compared to normal feed administered shrimps. However, compared with the unchallenged group the haemocyte count in the challenged group was significantly lower ($p < 0.01$), but was higher than that of both the controls (Table 3; Fig. 1). Whereas in normal feed administered shrimps the THC was significantly ($p < 0.01$) below the baseline level after the challenge.

Table.3. Total haemocyte count of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

		Normal feed (Control)	***Nocardiopsis* incorporated feed (Test)**
Total Haemocyte Count	**Baseline**	$25.8 \times 10^6 \pm 35.3 \times 10^5$	
	Before challenge	$25.4 \times 10^6 \pm 34.2 \times 10^5$	$42.1 \times 10^6 \pm 18.3 \times 10^5$
	After challenge	$23.5 \times 10^6 \pm 21.7 \times 10^5$	$36.8 \times 10^6 \pm 25.1 \times 10^5$

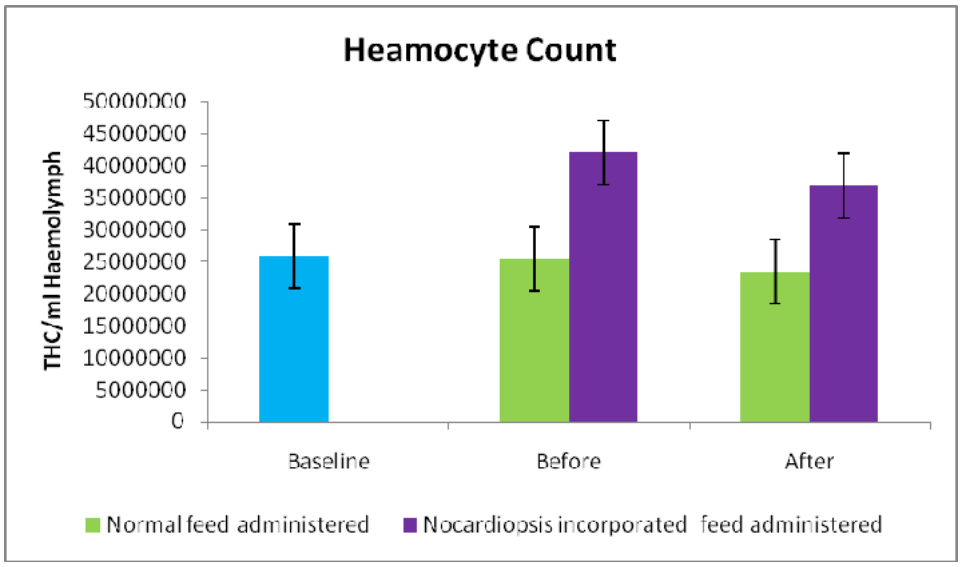


Fig.1. Total haemocyte count of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

3.3.1.2 Phenoloxidase (PO) activity

Phenoloxidase activity was found to be significantly higher ($p < 0.01$) in the haemolymph of shrimps fed with *Nocardiopsis* incorporated feed (0.577 ± 0.263) compared to normal feed administered ones (0.372 ± 0.091) prior to challenge. On challenge the phenoloxidase activity was also found to be significantly higher ($p < 0.01$) in *Nocardiopsis* fed shrimps (0.671 ± 0.203) than in normal feed administered animals (0.398 ± 0.094) (Table 4; Fig. 2).

Table.4. Phenol oxidase of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

		Normal feed (Control)	***Nocardiopsis* incorporated feed (Test)**
Phenol Oxidase	**Baseline**	0.357 ± 0.052	
	Before Challenge	0.372 ± 0.091	0.577 ± 0.263
	After challenge	0.398 ± 0.094	0.671 ± 0.203

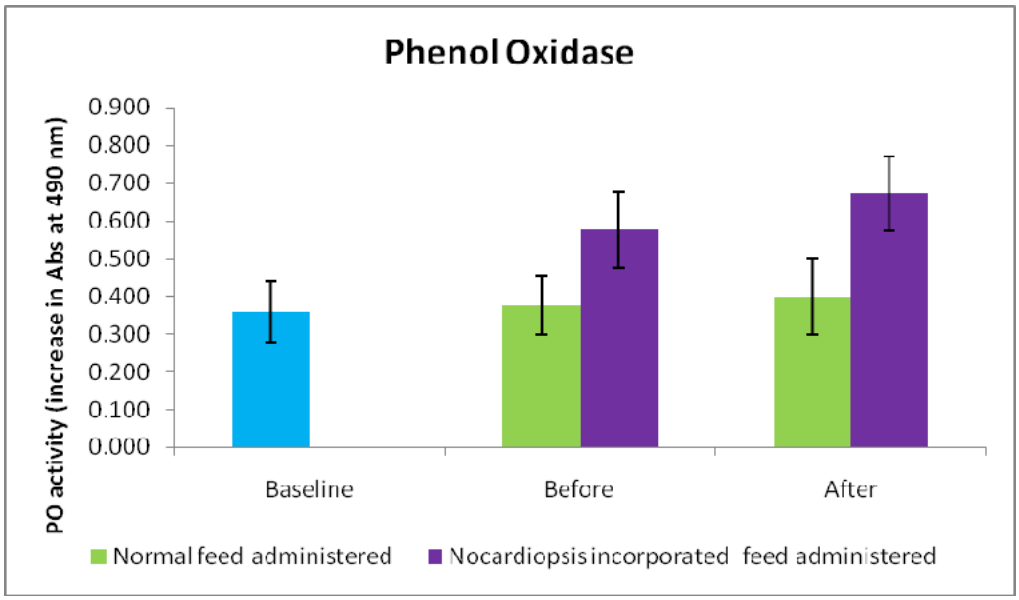


Fig.2. Phenol oxidase of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

3.3.1.3. Respiratory burst activity

Respiratory burst activity was significantly higher in the haemolymph of shrimps fed with *Nocardiopsis* incorporated feed before (0.347 ± 0.173) ($p < 0.05$) and after challenge (0.413 ± 0.054) ($p < 0.01$) compared to normal feed administered group. Meanwhile the reactive oxygen intermediates value was significantly lower in normal feed administered shrimps after challenge (Table 5; Fig. 3).

Table.5. Reactive Oxygen of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

		Normal feed (Control)	*Nocardiopsis* incorporated feed (Test)
Reactive Oxygen	**Baseline**	0.089 ± 0.039	
	Before challenge	0.148 ± 0.055	0.347 ± 0.173
	After challenge	0.102 ± 0.046	0.413 ± 0.054

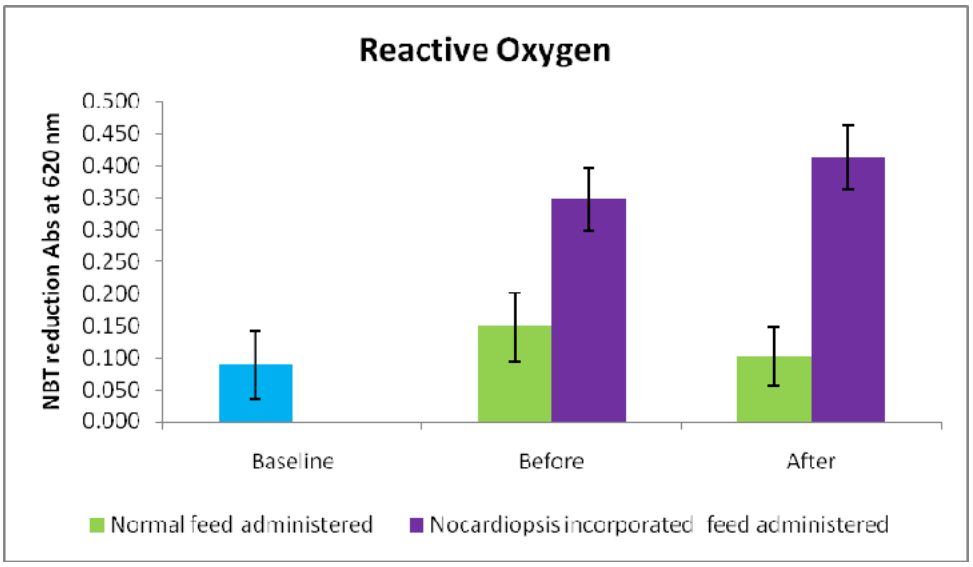


Fig.3. Reactive Oxygen of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

3.3.1.4 Acid phosphatase

The acid phosphatases activity was found to be higher in the haemolymph of shrimps fed with *Nocardiopsis* incorporated feeds before challenge (1.146 ± 0.220) ($p < 0.05$) and after challenge (1.388 ± 0.224) ($p < 0.01$) compared to that in normal feed administered group. The acid phosphatases activity showed a significant ($p < 0.01$) lowering in normal feed administered shrimps after challenge (Table 6; Fig 4).

Table.6. Acid phosphatase of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

		Normal feed (Control)	***Nocardiopsis* incorporated feed (Test)**
Acid Phosphatase	**Baseline**	0.670 ± 0.125	
	Before challenge	0.991 ± 0.192	1.146 ± 0.220
	After challenge	0.666 ± 0.204	1.388 ± 0.224

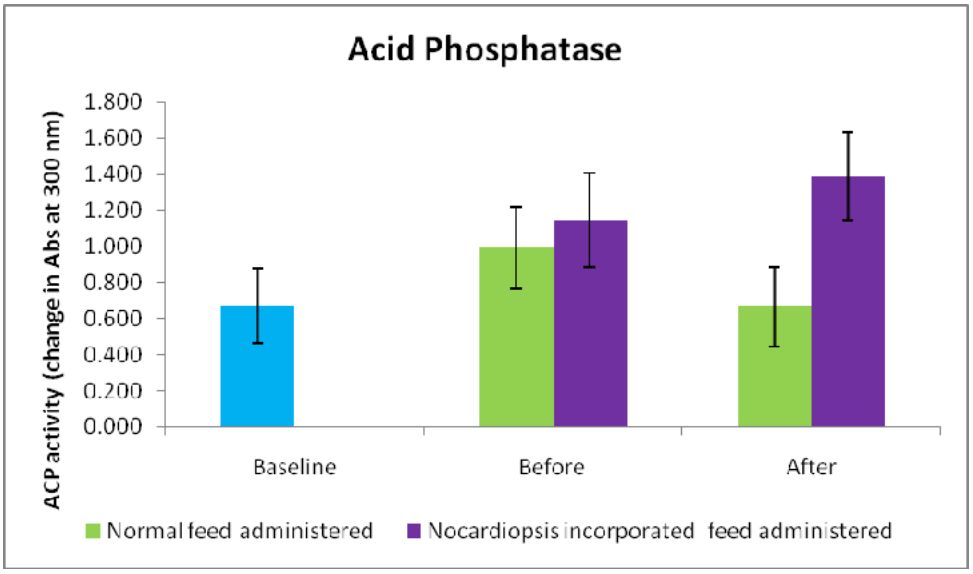


Fig.4. Acid phosphatase of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

3.3.1.5 Alkaline phosphatase

The alkaline phosphatases activity was found to be significantly ($p < 0.01$) higher in the haemolymph of shrimps fed with *Nocardiopsis* incorporated feeds before (2.168 ± 0.432) and after challenge (2.191 ± 0.530) compared to that in normal feed administered group ($p < 0.01$). The alkaline phosphatases activity did not show significant variation in normal feed administered shrimps after challenge from the base line values (Table 7; Fig.5).

Table.7 Alkaline phosphatase of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

		Normal feed (Control)	***Nocardiopsis* incorporated feed (Test)**
Alkaline Phosphatase	**Baseline**	1.494 ± 0.325	
	Before challenge	1.335 ± 0.408	2.168 ± 0.432
	After challenge	1.360 ± 0.440	2.191 ± 0.530

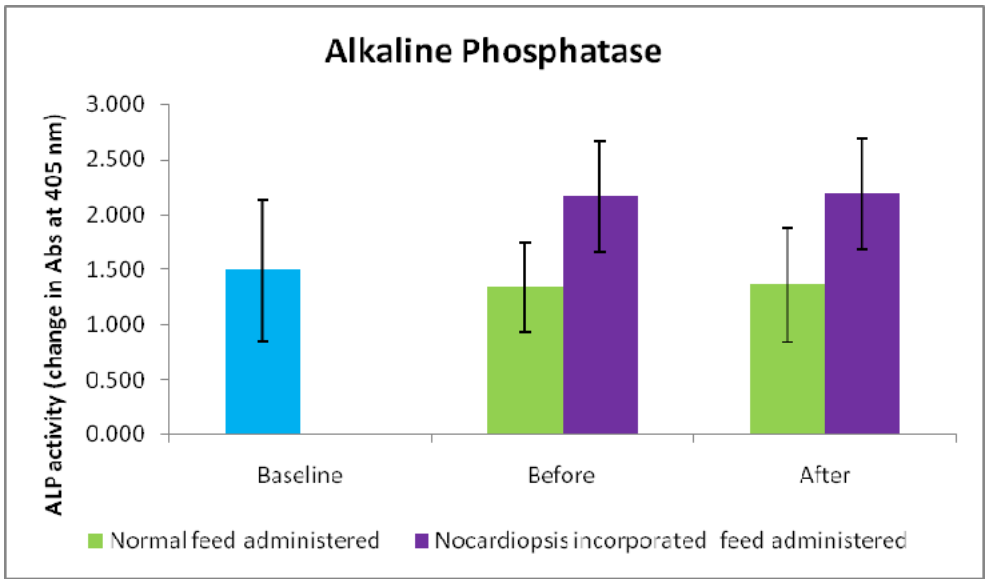


Fig.5. Alkaline phosphatase of P.monodon fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

3.3.1.6. Total protein

Haemolymph of shrimps fed with *Nocardiopsis* incorporated feed showed significantly higher total protein value before challenge (42.48 ± 16.65) and after challenge (57.76 ± 6.414) ($p < 0.05$) compared to that of the normal feed administered group. Meanwhile in normal feed administered shrimps haemolymph protein was lower before challenge followed by a significant ($p < 0.05$) increase after challenge period. (Table 8; Fig. 6).

Table.8. Total protein of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

		Normal feed (Control)	***Nocardiopsis* incorporated feed (Test)**
Total Protein	**Baseline**	16.89 ± 10.81	
	Before challenge	16.37 ± 10.48	42.48 ± 16.65
	After challenge	34.43 ± 13.07	57.76 ± 6.414

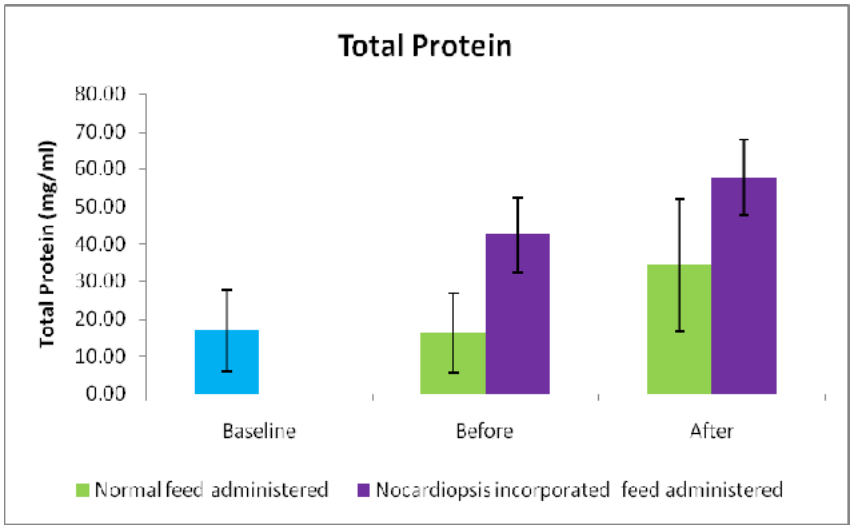


Fig.6. Total protein of *P.monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

3.3.1.7 Survival

The survival of shrimp among *Nocardiopsis* administered ones was 83% while in the normal feed administered ones the survival was only 50%, subsequent to challenge.

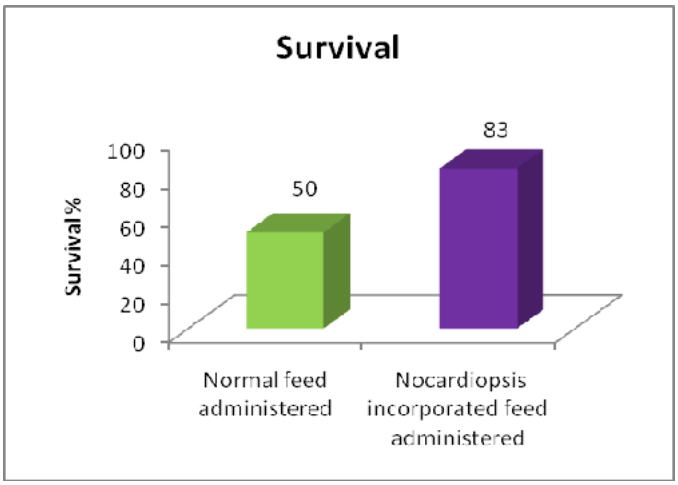


Fig.7. Percentage of survival of *P.monodon* fed on *Nocardiopsis* incorporated diet and normal feed.

Level of significance in the variation of non-specific parameters of *Penaeus monodon* between normal and *Nocardiopsis* incorporated feed administered animals, before and after challenge was obtained by t test (Table 9). The level of significance in the variation between the groups such as the one designated as base line, before and after challenge with respect to normal and *Nocardiopsis* incorporated feed administered ones was obtained by two way ANOVA as tabulated in Table 10.

3.3.1.8 Independent Sample t – test

Table.9. Level of significance of non-specific immune parameters of *Penaeus monodon* within the groups determined by t test

Serial No.	Non –specific immune parameters	Variation in immune parameters between the animals administered with normal and *Nocardiopsis* administered feed before challenge (t-values)	Variation in immune parameters between the animals administered with normal and *Nocardiopsis* administered feed after challenge (t-values)
1	Acid Phosphatase	-2.258*	-14.301**
2	Alkaline Phosphatase	-7.311**	-5.102**
3	Reactive Oxygen	-2.685*	-10.401**
4	Heamocyte Count	-11.906**	-11.601**
5	Total Protein	-2.888*	-2.800*
6	Phenol Oxidase	-6.294**	-8.253**

** ➔ Difference is significant ($p < 0.01$).
* ➔ Difference is significant ($p < 0.05$).

3.3.1.9 Two – way Analysis of Variance

Table.10. Level of significance of non-specific immune parameters of *Penaeus monodon* between the groups determined by two-way ANOVA

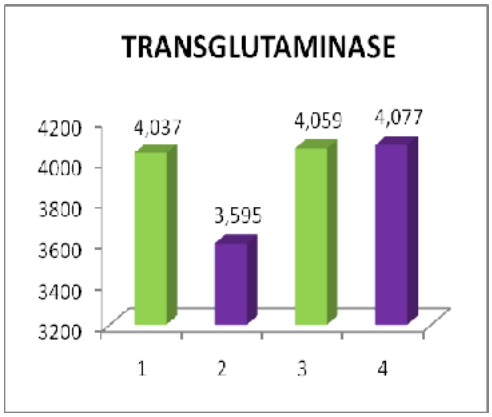
Serial No.	Non-specific immune parameters	Variations between baseline parameters with those of the normal feed administered and *Nocardiopsis* feed administered groups before & after challenge (F-values)	Variations in the immune parameters between the groups of animals fed on normal diet and *Nocardiopsis* incorporated diet before and after challenge (F-values)
1	Acid Phosphatase	24.771**	93.528**
2	Alkaline Phosphatase	10.303**	57.748**
3	Reactive Oxygen	3.502*	21.947**
4	Heamocyte Count	9.691**	55.293**
5	Total Protein	10.137**	8.550**
6	Phenol Oxidase	14.841**	58.964**

** ➔ Difference is significant ($p < 0.01$).
* ➔ Difference is significant ($p < 0.05$).

3.3.2 Immune-related genes' expression of *Penaeus monodon*

Semi quantitative RT-PCR analysis of the 8 selected genes in haemolymph showed difference in expression between normal feed and *Nocardiopsis* incorporated feed administered groups of animals before and after challenge. The selected genes were amplified with the same PCR cycling condition with variation in the annealing temperatures only (Table 2). The house keeping gene beta actin showed amplification in all the experiments. Generally an up-regulation of immune genes could be observed on administration of the *Nocardiopsis* incorporated diet except for astakine.

3.3.2.1 Transglutaminase



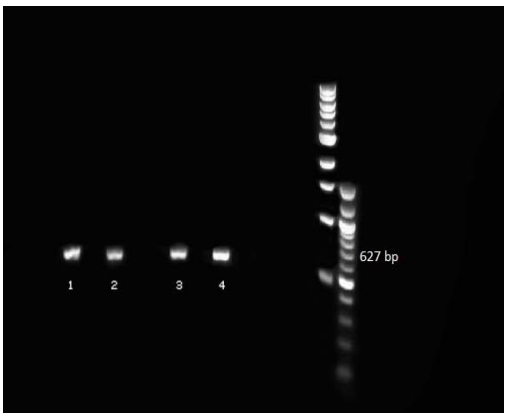


Fig.8. Transglutaminase gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1: Normal feed administered before challenge

2. *Nocardiopsis* incorporated feed administered before challenge

3. Normal feed administered after challenge

4. *Nocardiopsis* incorporated feed administered after challenge

The transglutaminase gene expression in normal feed administered group did not vary much between the challenged and unchallenged groups. However, in the group of animals fed on the *Nocardiopsis* incorporated diet the gene expression was considerably low before challenge which showed heightened expression after challenge suggesting up regulation of the transglutaminase gene in the *Nocardiopsis* administered group. But, this up regulation was not beyond the level of expression of the gene in the groups of animals fed on the normal diet suggesting a down regulatory effect of *Nocardiopsis* which was further up regulated on challenge.

3.3.2.2 Phenoloxidase

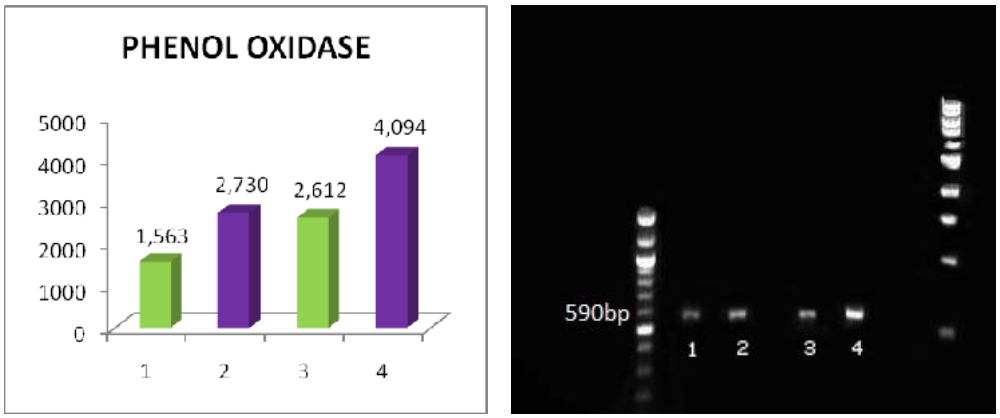


Fig.9. Prophenol oxidase gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1: Normal feed administered before challenge

2. *Nocardiopsis* incorporated feed administered before challenge

3. Normal feed administered after challenge

4. *Nocardiopsis* incorporated feed administered after challenge

The phenol oxidase gene showed up regulation in the group of animals fed on *Nocardiopsis* incorporated diet compared to normal feed administered group even before challenge. The gene was further up regulated in the group administered with *Nocardiopsis* incorporated diet. The up regulation was seen in the group of animals fed on normal diet after challenge but much lesser than the one observed in the *Nocardiopsis* administered group subsequent to challenge. Data clearly indicate that the administration of *Nocardiopsis* along with diet has stimulatory effect on phenoloxidase gene especially on challenge.

3.3.2.3 Peroxinectin

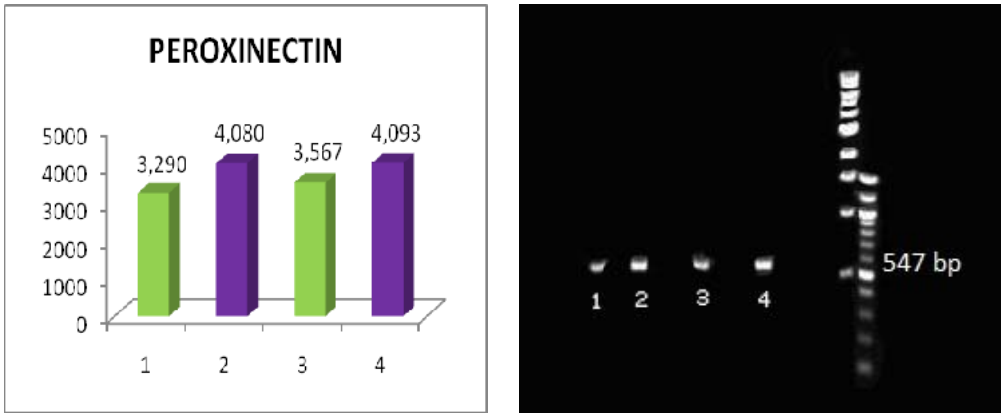


Fig.10. Peroxinectin gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1: Normal feed administered before challenge

2. *Nocardiopsis* incorporated feed administered before challenge

3. Normal feed administered after challenge

4. *Nocardiopsis* incorporated feed administered after challenge

Administration of *Nocardiopsis* incorporated diet showed up regulation of Peroxinectin gene which was further marginally up regulated on challenge. The same trend was shown in the group of animals administered with normal diet after challenge. Nevertheless, data is indicative of positive effect of *Nocardiopsis* in peroxinectin gene.

3.3.2.4 Alpha 2 Macroglobulin

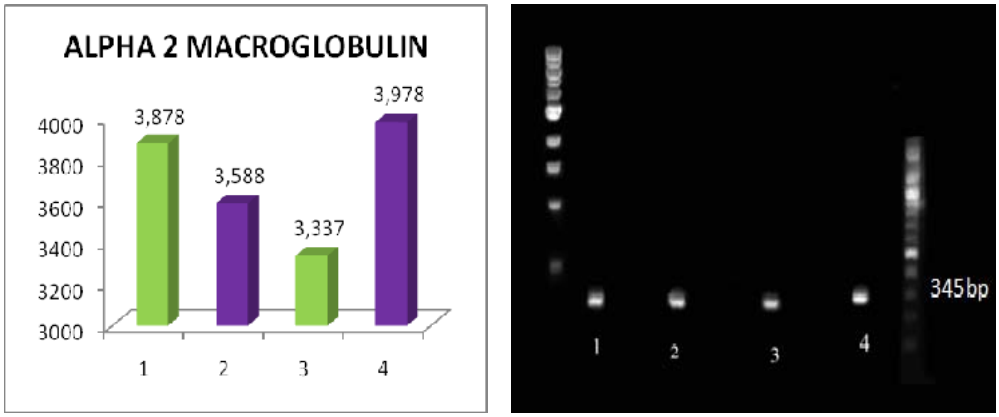


Fig.11. Alpha 2 Macroglobulin gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1: Normal feed administered before challenge

2. *Nocardiopsis* incorporated feed administered before challenge

3. Normal feed administered after challenge

4. *Nocardiopsis* incorporated feed administered after challenge

Alpha 2 macroglobulin gene in the animals administered with normal diet exhibited down regulation on challenge. On the other hand the animals administered with *Nocardiopsis* expressed up regulation on challenge suggesting a positive role played by *Nocardiopsis* in the up regulation of the bio-defense gene Alpha 2 macroglobulin. It has to be pointed out that the gene was down regulated in the animals administered with normal diet on challenge.

3.3.2.5 Astakine

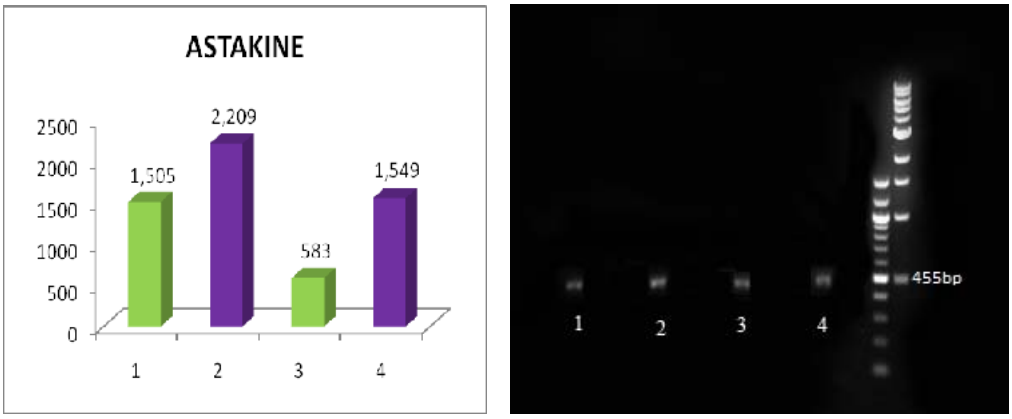


Fig.12. Astakine gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1. Normal feed administered before challenge
2. *Nocardiopsis* incorporated feed administered before challenge
3. Normal feed administered after challenge
4. *Nocardiopsis* incorporated feed administered after challenge

Compared to the group of animals administered with normal diet the ones with the *Nocardiopsis* incorporated diet expressed upregulation of astakine gene. However, it was down regulated on challenge. But, the expression level was much better than the one administered with normal diet. Overall, the data suggest that *Nocardiopsis* has positive regulatory effect on astakine gene.

3.3.2.6 Penaeidin

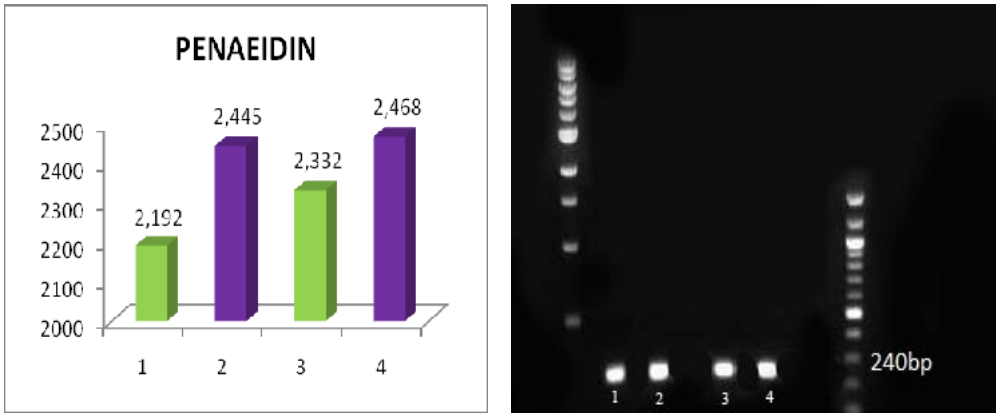


Fig.13. Penaeidin gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1: Normal feed administered before challenge

2. *Nocardiopsis* incorporated feed administered before challenge

3. Normal feed administered after challenge

4. *Nocardiopsis* incorporated feed administered after challenge

Prior to challenge even, Penaeidin gene exhibited up regulation in the group of animals administered with *Nocardiopsis* incorporated diet compared to the one with normal diet. However, on challenge the animals administered with *Nocardiopsis* incorporated diet did not show substantial up regulation. Meanwhile, in the case of the group of animals administered with normal diet challenge brought in notable up regulation of the gene.

3.3.2.7 Crustin

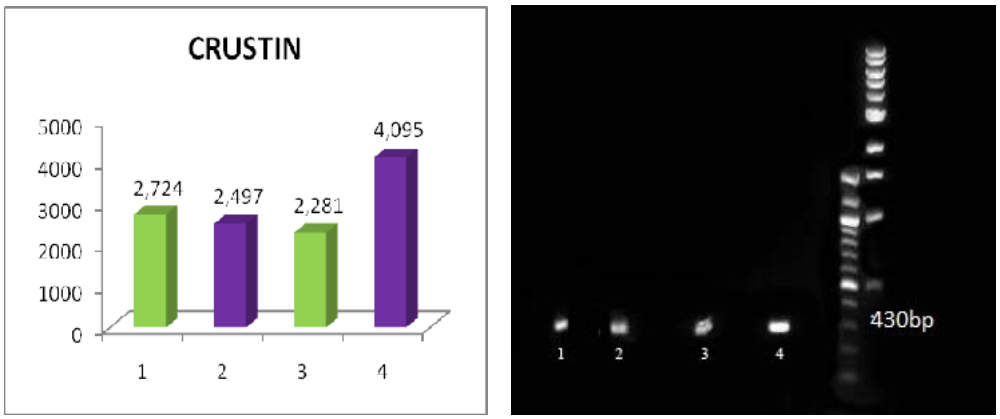


Fig.14. Crustin gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1: Normal feed administered before challenge

2. *Nocardiopsis* incorporated feed administered before challenge

3. Normal feed administered after challenge

4. *Nocardiopsis* incorporated feed administered after challenge

Substantial up regulation was exihibited by the group of animals administered with *Nocardiopsis* incorporated diet on challenge. However, prior to challenge the gene expression was almost similar to that of the group administered with the normal diet. In the animals administered with normal diet a marginal down regulation of the gene could be observed after challenge. The data clearly indicate protective effect of dietary incorporation of *Nocardiopsis* to *Penaeus monodon.*

3.3.2.8 B Actin

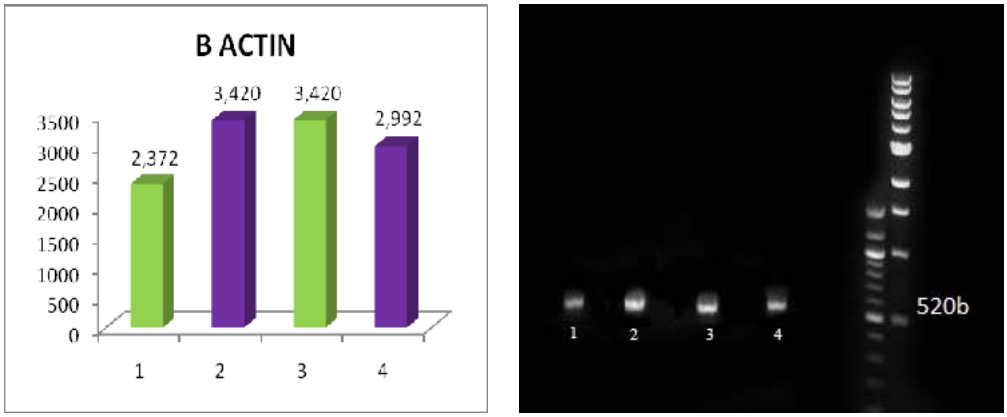


Fig.15. B actin gene expression in *P monodon* fed on *Nocardiopsis* incorporated feed and normal feed before and after challenge.

1: Normal feed administered before challenge

2. *Nocardiopsis* incorporated feed administered before challenge

3. Normal feed administered after challenge

4. *Nocardiopsis* incorporated feed administered after challenge

There was marginal difference in the expression of beta actin gene in the samples described.

3.4 Discussion

Shrimp farming constitutes an important source of revenue and employment in many developing countries. However, the shrimp industry has always been affected by infectious diseases, mainly of bacterial and viral etiology (Lightner *et al*., 1983; Kroll *et al*., 1991; Mohney *et al*., 1994; Hasson *et al*., 1995; Flegel, 1997) causing great loss of production. The sustainability of the shrimp industry depends largely on disease control and the health status of shrimp. For this reason, disease prevention is a priority and shrimp

immunology has become a prime area of research. From this point of view, the immune system is a tool to assess shrimp health (Bachere *et al*., 1995) and workers have suggested furthermore the value of immune parameters as biomarkers in ecotoxicology. In such a perspective, studies into the value of cellular and humoral parameters as indicators of shrimp condition are being carried out, with the intention of developing criteria for sanitary surveys, immunomodulation studies and selection programs for shrimp with high resistance to pathogens.

Haemocytes play a central role in crustacean immune defense. Firstly, they remove foreign particles in the hemocoel by phagocytosis, encapsulation and nodular formation (Soderhall and Cerenius, 1992). Secondly, haemocytes take part in wound healing by cellular clumping and initiation of coagulation processes through the release of factors required for plasma gelation (Johansson and Soderhall, 1989; Omori *et al*., 1989; Vargas-Albores *et al*., 1998), and carriage and release of the prophenoloxidase (proPO) system (Johansson and Soderhall, 1989; Hernandez-Lopez *et al*., 1996). They are also involved in the synthesis and discharge in the haemolymph of important molecules, such as α 2-macroglobulin (α2M) (Rodriguez *et al*., 1995; Armstrong *et al*., 1990) agglutinins (Rodriguez *et al*., 1995) and antibacterial peptides (Destoumieux *et al*., 1997; Schnapp *et al*., 1996; Lester *et al*., 1997).

For crustaceans, some information exists on the importance of THC in pathogen resistance. Persson *et al*. (1987) reported in *Pacifastacus leniusculus* a relationship between haemocyte number and its resistance to the parasitic fungus *Aphanomyces astaci*. They demonstrated that a decrease in the haemocyte number of crayfish harbouring *A. astaci* as a latent infection resulted in an acute infection with incomplete melanisation of fungus hyphae,

leading to the death of the crayfish. Le Moullac *et al*. (1998) observed that *Penaeus stylirostris* with a low THC due to a hypoxia situation became more sensitive to infections with highly virulent *Vibrio alginolyticus*.

In this study, the result of total hemocyte values showed that there was significant increase in the THC values of *Nocardiopsis* administered shrimp compared to the ones fed on normal diet. Even though there was a decline in THC values in both the groups of animals after challenge, the THC values in *Nocardiopsis* fed shrimp did not decline below the base line whereas in the group with normal diet the THC values went below. Haemocyte lysis (Omori *et al*., 1989), cell recruitment towards infected tissues, nodule formation (Martin *et al*., 1998) or interference with haematopoeisis could contribute to lower THC. The results of this study is in tune with findings of Hennig *et al*.1998, where THC was found increased in peptidoglycan- fed kuruma shrimp, resulting in enhanced resistance against WSSV infection.

Phagocytosis is the most common reaction of cellular defense. During phagocytosis, particles or microorganisms are internalized into the cell which later forms a digestive vacuole called the phagosome. The elimination of phagocyted particles involves the release of degradative enzymes into the phagosome and the generation of ROIs. This last process is known as the respiratory burst. The first ROI generated during this process is the superoxide anion (O_2^-). Subsequent reactions will produce other ROIs, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$). The first evidence that crustacean haemocytes produce ROIs was given by Bell and Smith (1993) in the shore crab *Carcinus maenas*. The respiratory burst activity was found to be higher in the haemolymph of shrimps fed with *Nocardiopsis* incorporated feeds both before challenge and after challenge groups when compared to normal feed administered

shrimps. On the contrary the reactive oxygen value was found declined in normal feed administered shrimps after challenge. Song and Hsieh (1994) demonstrated *in vitro* the phenomenon known as respiratory burst in *P.monodon* haemocytes, thought to be related to phagocytosis, when shrimp responded to immunostimulants such as zymosan and β- glucan. An increase in ROI has been observed in *P.monodon* administered with specific amount of feed incorporated with β 1, 3 glucan, certain Indian herbs, Vitamin C with its derivatives, copper and Vitamin E (Chang *et al*., 2000, 2003; Citarasu *et al*., 2006; Lee and Shiau, 2002a, 2002b, 2003, 2004). The observations and results of this study are in parallel to the above studies.

The PO is responsible for the melanisation process in arthropods. The PO enzyme results from the activation of the proPO system. The proPO activating system has been very well studied in crustaceans, especially in crayfish, for which a few reviews have been written (Soderhall and Cerenius, 1998; Soderhall *et al*., 1996). Melanin and its reactive intermediates have shown to be fungistatic (Soderhall and Ajaxon, 1982; Persson *et al*., 1987). The process of activation of the proPO system has been studied in several penaeid shrimp (Hernandez-Lopez *et al*., 1996; Vargas-Albores *et al*., 1997; Perazzolo and Barraco, 1997; Sung *et al*., 1998). The proPO has been cloned and sequenced in crayfish (Aspan *et al*., 1995) and *P. monodon* (Sritunyalucksana *et al*., 1999). Using these different approaches, the function of the proPO system can be better understood in relation to the health status of shrimp. A few studies have shown that proPO could be used as health and environmental markers because changes are correlated with infectious state and environmental variations (Le Moullac and Haffner, 2000) which have recently been confirmed also at the gene expression level. Increase in PO

activity has been reported in *P.monodon* (after challenge) after the administration of beta glucan (Chang *et al*., 2003) immunostimulant herbs (Citrasu *et al*., 2006) and 'DNA vaccination' (Rajeshkumar *et al*., 2008).The analysis of PO activity in the present study also fall in tune with the above observations where the phenol oxidase activity increased significantly in the haemolymph of shrimp fed with *Nocardiospis* before and after challenge.

Alkaline and acid phosphatases are important defense enzymes in crustaceans. In the present study both the alkaline and acid phosphatases activity were found to be higher in shrimps fed with *Nocardiopsis* incorporated diet. The concurrent over production of phosphatases shows that these enzymes are being activated by *Nocardiopsis* diet. Acid phosphates (ACP) and alkaline phosphatase (ALP) were composed of many kinds of phosphomonoesterases, which were very important to the crustacean immune system. Liu et al. (1999) reported that ACP was a sign of lysosome activity to digest the invading organisms in shrimps. ACP had been released during phagocytosis, nodule and capsule formation to hydrolyze phosphate groups of the invaders. ALP had taken part in the transfer of phosphate groups and metabolism of Ca and P as the key regulatory enzymes. It has been shown that two phosphates played important role in the immune system as a key compound of lysosomal enzymes (Wang *et al*., 2005). In the present study, the acid phosphatases and alkaline phosphatases activities were found to be higher in the haemolymph of shrimps fed with *Nocardiopsis* incorporated feeds both before and after challenge compared to normal feed administered shrimps. The acid phosphatases activity showed a considerable decline in normal feed administered shrimps after challenge compared to *Nocardiopsis* fed shrimps whereas there was not much variation in alkaline phosphatases activity.

Evidence has been given regarding the physiological importance of the plasma protein concentration and its susceptibility to environmental or physiological changes in the animal. High concentration of haemolymph proteins has been reported for crustaceans after WSSV challenge by Lo *et al*. (1997). In the present study, shrimps fed with *Nocardiopsis* incorporated diet showed an increase in total protein. This may be due to the over expression of defence protein to counteract the effects of *V. harveyi*.

Analysis of the results of immune gene expression in *P. monodon* showed that *Nocardiopsis* administration through diet up-regulated the expression levels of six out of seven immune genes of shrimps selected under this study. Alpha 2 macroglobulin (α-2 macroglobulin) has been defined as a ubiquitous high molecular proteinase inhibitor. It is a plasma glycoprotein that arrest proteinases avoiding the degradation of the host protein removing them from circulation. It has been observed that α-2 macroglobulin expression in haemocytes was significantly induced by administration of peptidoglycan (immunostimulant) in kuruma shrimp and *P. mono*don (Gollas- Galvan *et al*., 2003 & Lin *et al*., 2007). The RT-PCR m-RNA transcript analysis of α-2 macroglobulin in *P. mono*don showed up regulation, in the *Nocardiopsis* fed shrimps in the present study whereas in normal feed administered shrimp there was down regulation after challenge and is in parallel to the above reported study.

Astakine is a cytokine which is reported to be involved in cell (haemocyte) proliferation and / or cell differentiation (Jiravanichpaisal *et al*., 2007). In the present study, there showed a decrease in the level of expression of astakine gene both in normal feed and *Nocardiopsis* incorporated feed administered shrimp after challenge with *Vibrio harveyi*. This is in agreement

with the previous work where a general down-regulation of the immune genes has been reported with bacterial or viral infection (Okumura, 2007).

Penaeidins are a unique family of antimicrobial peptides in crustaceans, originally isolated and characterized in the shrimp, *P.vannamei*. Isoforms of penaedins are classified according to their similarity in amino acid sequence into penaeidin 2 (PEN2), penaeidin 3 (PEN3), penaeidin 4 (PEN4) and penaeidin 5 (PEN5) (Wang *et al*., 2007 & Hu *et al*., 2006). Pen-3, the most abundant and representative member of the penaeidin family is reported to be highly expressed in haemocytes (Munoz *et al*., 2002 and Wang *et al*., 2007c). Penaeidin-3 was found to be up-regulated on administration of probiotic *Bacillus* MCCB 101(Swapna *et al*., 2011). Strong antibacterial activity especially against Gram-positive bacteria has already been reported for penaedins (Destoumieux *et al*., 2000a; 2000b). In *Nocardiopsis* administered *P.monodon*, in the present study, Pen-3 was found to be up-regulated both before and after challenge and hence can be said that an antimicrobial component has been activated.

Transglutaminase can catalyze the cross – linking reaction between soluble clotting protein molecules from the plasma for the prevention of excess blood loss from wounds and obstructing microorganisms from invading the wound in crustaceans. Transglutaminase (TG) stabilizes the clots by intermolecular covalent cross-linking. Of the two types of shrimp transglutaminases (STGI and STGII), STGII was found to be involved in coagulation and that transglutaminase expression was decreased after 24 hr post infection in *F.chinensis* (Liu *et al*., 2007). Even though in the present study there was only a slight variation in the level of up regulation between normal feed and *Nocardiopsis* incorporated feed administered shrimps after challenge, there was a considerable difference in the level of expression in

Nocardiopsis administered shrimps after challenge. The up regulation of transglutaminase of *Nocardiopsis* administered *P.monodon* indicated the activation of coagulation factors.

The prophenoloxidase (proPO) system is considered as a constituent of the immune system and forms an important part of an immune-recognition process of the defense mechanism in invertebrates (Lai *et al*., 2005). ProPO can be activated by cell wall components of microbial origins, such as β-1,3-glucan, lipopolysaccharides (LPS) and peptidoglycan. Activation of proPO can be regulated by environmental, biological and experimental factors, including calcium sodium dodecyl sulfate (SDS), trypsin, pH and temperature. ProPO is an inactive form and is converted to an active form phenoloxidase (PO) after limited proteolysis by serine proteinases. In addition, Ca^{2+} is required for the conversion of the proPO-activating enzyme (ppAE) to an active proteinase that transforms proPO to active phenoloxidase (PO). The prophenoloxidase system (proPO) is the best studied crustacean immune defence system that functions as an oxygen transferring enzyme to catalyze the dehydrogenation of catechols to orthoquinones and the orthohydroxylation of phenols to catechols (Strothkamp and Mason, 1974; Sritunyalucksana and Soderhall, 2000). Pro phenoloxidase is capable of converting phenolic substrates such as dihydroxyphenylalanine to dopaquinone, which then polymerize non-enzymatically to melanin. Intermediate compounds in the melanin pathway have bactericidal properties and are involved in immune reactions (Johansson and Soderhall, 1989). ProPO is also involved in cell adhesion, encapsulation and phagocytosis (Gillespie *et al*., 1997). In the present study, the level of proPO gene expression was found to be highest in *Nocardiopsis* fed shrimps

after challenge. The up-regulation of proPO gene of *P.monodon* administered with *Nocardiopsis* has shown the activation of proPO system.

Peroxinectin is involved in cell adhesion activity. There was only a slight variation in the level of expression of peroxinectin gene after challenge in *Nocardiopsis* fed shrimps.

Crustins are antibacterial proteins of 7-14 kDa with a characteristic four disulphide core –containing whey acidic protein (WAP) domain, expressed by the circulating haemocytes of crustaceans. In case of crustins also, up-regulation of the gene could be noticed in *Nocardiopsis* fed shrimps. This is in agreement with the previous works where crustins have been found to be up-regulated with bacterial invasion in the shrimps (Jiravanichpaisal *et al*., 2007; Amparyup *et al*., 2008; Brockton and Smith, 2008; Rattanachai *et al*., 2004b, Swapna *et al*., 2011). There are also contrasting results where a down-regulation of the crustin gene was observed on administration of LPS or Gram-negative bacteria in *Litopenaeus vannamei* (Okumura, 2007; Jimenez-Vega *et al*., 2004; Vargas-Albores *et al*., 2004; Supungul *et al*., 2008).

Biodefence mechanisms like protease inhibition involving alpha 2 macroglobulin, antimicrobial activity involving penaeidin-3, coagulation involving transglutaminase, phenol oxidase system involving proPO, antibacterial activity involving crustin and cell adhesion involving peroxinectin were found to be active as the genes involved in these mechanisms were up- regulated in *Nocardiopsis* administered shrimp. However, reduction in gene expression could be noticed in the expression profile of Astakine. This higher number of up-regulated genes in *Nocardiopsis* administered group showed that bio-defence has been elicited after administration of *Nocardiopsis* coated diet and

it clearly demonstrated that *Nocardiopsis* could enhance the immune status in shrimps.

Immunostimulants are substances that stimulate immune responses and increase the ability of the immune system to fight against infections and diseases. The immunostimulatory effect of various substances like glucans, peptidoglycans, lipopolysaccharides (LPS), sodium alginate, levamisole, polyherbal formulations, and other polysaccharides has been widely studied in crustaceans (Kumari *et al*., 2004; Sritunyalucksana *et al*., 1999; Chang *et al*., 2003; Baruah and Prasad, 2001; Smith *et al*., 2003; Chang *et al*., 2011).

There is a growing need to control, prevent or minimise the devastating effects of disease in crustacean culture, without recourse to toxic chemicals or antibiotics. In keeping with approaches to disease control in fish and higher mammals, interest is getting developed in compounds that confer protection and/or enhance immune reactivity to likely pathogens in shellfish. The findings of the this chapter shows that *Nocardiopsis* MCCB 110 is having immunostimulatory property and points to the conclusion that *Nocardiopsis* MCCB 110 can be successfully used to enhance resistance of *P.monodon* against *V harveyi* infection. It has to be pointed out that on challenge studies *Nocardiopsis* administered shrimp demonstrated 83% survival against 50% in the control.

In conclusion, shrimps fed on *Nocardiopsis* incorporated diet have shown better survival with concurrent increment in hematological parameters. Therefore, *Nocardiopsis* MCCB110 can be utilized for shrimp aquaculture systems as an effective immunostimulant against *V. harveyi*.

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Chapter

4

ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF THE BIOACTIVE COMPOUND PRODUCED BY *NOCARDIOPSIS* MCCB 110

Contents

4.1 Introduction

Members of the Actinobacteria are prolific sources of secondary metabolites. Secondary metabolites are produced for a variety of reasons, including defense and communication. Marine-derived actinomycetes have been studied for isolation of several novel secondary metabolites (Fenical and Jensen, 2006; Das *et al*., 2006a). However, to date there have only been a few studies (Das *et al*., 2006b; Kumar *et al*., 2006; Lakshmi, 2008) that have considered Actinobacteria for their application as probiotics in aquaculture. Most probiotics proposed as biological control agents in aquaculture are lactic acid bacteria (*Lactobacillus, Carnobacterium* etc.), *Bacillus* spp, and *Pseudomonas* spp (Verschuere *et al*., 2000).

You *et al*. (2005) described the potential of actinomycetes against shrimp pathogenic *Vibrio* spp. and recommended marine actinomycetes as

potential probiotics due to their ability to degrade macromolecules, such as starch and protein, in culture ponds, the production of antimicrobials, and the formation of heat- and desiccation-resistant spores. More recently, there were a few studies on the possible use of marine actinomycetes in disease prevention against aquatic pathogens. Das *et al*. (2006b) reported a preliminary study on the use of *Streptomyces* on the growth of black tiger shrimp. Kumar *et al*. (2006) extracted an antibiotic from marine actinomycetes and incorporated it into feed to observe the *in vivo* effect on white spot syndrome virus in black tiger shrimp. You *et al*. (2007) reported the activity of marine actinomycete as potential organisms against biofilms produced by *Vibrio* spp. and recommended the use of actinomycetes to prevent vibriosis. All these investigations indicate the paramount importance of marine actinobacteria in aquaculture. Despite being the source of several novel antibiotics, marine actinobacteria have not been given adequate importance as probiotic in aquaculture.

Species of *Nocardiopsis* are distributed ubiquitously in the environment (Kroppenstedt & Evtushenko, 2002). Like other actinomycetes, *Nocardiopsis* spp. are capable of producing metabolites with biological activities.

As investigations on the nature of the inhibitory compounds and their role in eliminating pathogens were found inevitable for their application in aquaculture, this chapter looks into the isolation, purification and partial characterization of the bioactive compound produced by the organism. The antagonistic potential against *V.harveyi* and a few bacteria used as probiotics and cytotoxic effect on a mammalian cell lines apart their luminescence inhibitory property against *V harveyi* have all been dealt with in this chapter.

4.2 Materials and method

4.2.1 Antagonistic property of *Nocardiopsis* MCCB110 to beneficial bacteria

In addition to *Vibrio harveyi* MCCB111, the antagonistic property of *Nocardiopsis* MCCB 110 to a few probiotics organism such as *Micrococcus* sp (MCCB104), *Pseudomonas aeruginosa* (MTCC741), and *Bacillus* sp (MCCB101) were also examined to know whether these beneficial forms are being spared by the antagonistic organism.

4.2.2 Fermentation and antimicrobial assay

4.2.2.1 Fermentation media

4.2.2.2 Seed Medium

An aliquot of 10 ml seed medium (starch 1%, yeast extract 0.4%, peptone 0.2%, seawater 30 g/L salinity, pH 7) was prepared in test tubes and autoclaved at 15lbs for 15min.

4.2.2.3 Production Medium

An aliquot of 100 ml of production medium (glycerol 7%, glucose 3%, beef extract 3%, peptone 0.8%, $NaNO_3$ 0.2%, $MgSO_4.7H_2O$ 0.01%, seawater 30 ppt 100ml, pH 7) was prepared in 250ml Erlenmeyer flask, autoclaved at 15 lbs for 15 min and used as fermentation medium for secondary metabolite production.

4.2.2.4 Assay for antibacterial activity of the culture

4.2.2.4.1. Inoculation and incubation

A loopful of the slant culture of *Nocardiopsis* MCCB110 was inoculated into 10 ml seed medium and the tubes were incubated at 28^0C for 2 days. After 48 h of incubation, the growth was confirmed and the seed culture was

transferred to 100ml production medium. The flasks were incubated at room temperatures (28+-2^{0}C) on a rotary shaker at 120 rpm for seven days.

4.2.2.4.2 Antibacterial assay

Antibacterial activity of the *Nocardiopsis* MCCB110 was determined following agar diffusion method (Kirby-Bauer method, 1959). Lawn cultures of the test organisms, *Vibrio harveyi* MCCB111, *Micrococcus MCCB104, Pseudomonas aeruginosa* MTCC741, and *Bacillus MCCB101* were made on nutrient agar plates (peptone 0.5g, beef extract 0.3 g, agar 2g, seawater 50 ml & distilled water 50 ml). After swab inoculation, sterile filter paper discs (Whatman 6 nos.) were placed on the swabbed agar surface aseptically. The discs were impregnated with 30µl *Nocardiopsis* MCCB110 culture broth using micropipette. The plates were incubated at room temperature ((28±2^{0}C) for 24 hrs and examined for evidence of antibacterial activities, represented by a zone of inhibition of bacterial growth around the paper disc.

4.2.3 Centrifugation and filtration of the culture supernatant

Nocardiopsis MCCB110 culture after seven days of incubation was centrifuged at 10000g at 4^{0}C for 15 min and the supernatant and pellet were separated. They were independently tested for their antagonistic property following agar diffusion method. After confirming the antibacterial activity of the culture supernatant it was filter sterilized initially using 0.45µM and subsequently by 0.2 µM cellulose acetate filters, for further testing the antibacterial property.

4.2.4 Extraction of the bioactive molecule

4.2.4.1 Selection of solvent system

A series of solvents (Table 1) were tested for examining the efficiency for extracting the bioactive molecule from the culture supernatant. An efficient solvent is the one in which the antagonistic compound dissolves well and the activity is preserved. Solvents were chosen on the basis of differing polarities.

Table.1. Selected solvents for extraction along with polarity index and boiling point

Solvent	Polarity index	Boiling point
Acetone	5.1	56
Acetonitrile	5.8	82
Benzene	2.7	80
n-butanol	3.9	118
Chloroform	4.1	61
Cyclohexane	0.2	81
1,2 Dichloroethane	3.5	84
Ethylacetate	4.4	77
Diethyl ether	2.8	35
n-hexane	0.0	69
Methanol	5.1	65
n-propanol	4.0	92

4.2.4.2 Extraction and concentration

An aliquot of 15 ml filter sterilized supernatant taken in a boiling tube (50 ml) was mixed with 5 ml of solvent, sealed using parafilm and thoroughly mixed using a Cyclomixer. The agitated mixture was transferred into a separating funnel and allowed to separate under gravity. The solvent phase and

aqueous phase were separated and the activities of both the phases were tested subsequently following the disc diffusion method. The extract after separation from the aqueous phase was concentrated by evaporation to dryness in a water bath at 80^0C.

4.2.4 Heat stability of the solvent extract

An aliquot of 1ml of the extract taken in Eppendorf tubes was kept in a water bath at various temperatures (40^0C, 60^0C, 80^0C, 100^0C) for 30 min. The extract was also subjected to autoclaving at 10 lbs for 10 min and 15 lbs for 15 min. The heat treated extract was subjected to test its antagonistic property following the method described earlier.

4.2.5 Thin layer chromatographic separation of the bioactive compound

TLC plates having 0.2 mm thickness were prepared following standard procedures using 30 gm silica (silica gel G) in 60 ml distilled water. Concentrated solvent extract was spotted near the bottom of the plate and ran in different solvent systems to compare the Rf values and to select the best solvent system. Rf values of different spots were measured for each solvent system by placing the TLC plates in iodine vapour. Various solvent systems used for selection were: Ethylacetate: n-hexane (50:50), Ethylacetate: n-hexane (60:40), Ethylacetate: methanol (95:5), Chloroform: acetone (50:50), Butanol: acetic acid: water (60:20:20) and Acetic acid: water (50:50).

For testing the antibacterial property, different spots from the TLC plates were scraped off and dissolved in distilled water and centrifuged at10000g for 15 min at 4^0C. Antibacterial activity of each fraction was tested by disc diffusion method. The scrapped off bands after dissolving in distilled water and centrifugation were lyophilised and used for further studies.

4.2.6 HPLC analysis of the bioactive compound

The purified active fraction was analyzed by HPLC to check its purity. Analysis was carried out on a Perkin-Elmer Series 200 instrument using C-18 column and with a UV detector tuned at 237 nm. A gradient method was used for eluting samples, employing solvent system A and B: solvent A was water and solvent B was acetonitrile. The sample was eluted at a rate of 1ml/min

4.2.7 UV-Vis and FT-IR spectra of the bioactive compound

The bioactive compound extracted and purified by the method described earlier was subjected to spectrophotometric analysis. The absorption spectrum of the active molecule was determined with a Shimadzu UV-1601 spectrometer. The infrared spectrum was determined with a ThermoNicolet AVATAR 370DTGS FT-IR spectrometer.

4.2.8 MS-MS analysis of the bioactive compound

MS-MS analysis of the active compound was carried out on an Applied Biosystem API 2000 instrument. The scan range was from 100 to 1000m/z.

4.2.9 ^{1}HNMR spectral analysis

Proton magnetic resonance (^{1}H NMR) of the active compound in D_2O was recorded on a Bruker AMX400MHz High Resolution multinuclear FT-NMR spectrometer.

4.2.10 Cytotoxicity studies of the bioactive compound

Cytotoxicity of the active compound was evaluated against African green monkey kidney cells (VERO cells) and shrimp haemocyte by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide) assay

(Mosmann, 1983). Briefly, 200µl of a 4x 10^5cells/ml suspension was pipetted into each well of a 96- well microplate. This plate was incubated in CO_2 incubator at 37^0C for VERO cell line and 25^0C for shrimp haemocytes for 12 h in order to allow cell attachment. Different concentrations of the active compound ranging from 0 – 1000 ppm were added to the wells in quadruple and incubated in CO_2 incubator at the respective temperatures for 14 hrs. After removing the culture supernatant, 50µl of MTT solution (Sigma) (5mg/ml) was added to each well and incubated for 5hrs.The media was carefully removed and MTT-formazan crystals were dissolved in 200µl dimethyl sulfoxide and the absorbance was measured at 570 nm in a micro plate reader (TECAN Infinite Tm, Austria). Cells cultured with medium in the absence of the bioactive compound were used as control.

4.2.11. Luminescence inhibition studies of the culture supernatant

Overnight cultures of *V harveyi* adjusted to an optical density of 0.1 at Ab600 were inoculated to get 50 ml nutrient broth cultures taken in conical flasks. Aliquots of centrifuged and filter sterilized cell free culture supernatants were added to these flasks with 10, 20, 30, 40 and 50 ml respectively. Required volumes of saline were added to get a final volume of 100 ml in each conical flask. Aliquots of 50 ml *V harveyi* culture in nutrient broth along with 50 ml saline served as control. Cultures were incubated with shaking at 28^0C. Aliquots (1ml) were removed in 3 hrs interval during first 24 hrs and once in 6 hrs in the next 24 hrs for the determination of luminescence. Luminescence was quantified using a Modulus luminometer (Turner Biosystem) and reported as relative light unit (RLUs). Absorbance of the preparations was measured at 600 nm every 6 hr intervals for the first 24 hrs and once in 12 hrs in the next 24 hrs using a UV-Vis spectrophotometer

(Shimadzu UV-1601). Growth inhibitory effect of the culture supernatant was worked out at the lowest concentration tested at the 18th hour of incubation, using the formula, Inhibitory effect (Ie) = (1-Ab_{600} of test sample / Ab_{600} of control) X 100 % (Anderson *et al*., 2001)

4.3 Results

4.3.1 Assay for antibacterial activity of the culture

Results of the antimicrobial assays showed that the isolate *Nocardiopsis* MCCB110 exhibited clear antibacterial activity towards, *Vibrio harveyi* MCCB111, *Micrococcus* sp MCCB104 and *Bacillus* sp. MCCB101 (halo zone diameter 22mm). The activity was weak to moderate against *Pseudomonas aeruginosa* MTCC741.

4.3.2 Assay for antibacterial activity of cell free culture supernatant.

After centrifugation of the culture, the activity of the cell free culture supernatant and pellet were tested independently and found that the cell free culture supernatant had the activity and not the pellet. After confirming the antibacterial activity of the cell free culture supernatant, it was subjected to filtration and the antibacterial activity of the filtrate was also confirmed.

4.3.3 Selection of solvent system and extraction of the bioactive compound

The culture supernatant was fully soluble in acetone, acetonitrile, methanol and propanol and found not useful in this study. Benzene, n-butanol, chloroform, cyclohexane, 1, 2 dichloroethane, ethylacetate, diethyl ether and n- hexane were not miscible with the culture supernatant. After thorough agitation with immiscible solvents, the following observations were made (Table 2).

Table.2. General observation of the solvent and aqueous phases

Solvent	Solvent phase	Aqueous phase
Butanol	Turbid and formed the upper phase	Similar to filter sterilized supernatant
Ethylacetate	Turbid and formed the upper phase	Similar to filter sterilized supernatant
Dichloroethane	Crystal like, solvent phase formed below the aqueous phase.	Similar to filter sterilized supernatant
Cyclohexane	Clear solvent phase formed above the aqueous phase	Similar to filter sterilized supernatant
Chloroform	White turbid precipitate formed below the aqueous phase	Similar to filter sterilized supernatant
Diethyl ether	No change in solvent phase, formed above the aqueous phase.	Similar to filter sterilized supernatant
Benzene	Crystal like solvent phase formed above the aqueous phase.	Similar to filter sterilized supernatant
n-hexane	No change, no mixing	Similar to filter sterilized supernatant

4.3.4 Antagonistic activity of different solvent extracts

Out of eight solvent extracts tested for antibacterial activity, the extracts of butanol, benzene, dichloroethane, chloroform and ethylacetate have shown activity. Among them the ethylacetate extract has shown comparatively better halo zone (diameter 18-20 mm). (Table 3)

Table.3. Antagonistic activity of different solvent extracts

Solvent fraction	Inhibition zone diameter(mm)
n-butanol	10-12
Benzene	10-12
Dichloroethane	10
Chloroform	15-18
Ethylacetate	18-20

4.3.5 Heat stability of the solvent extracted fraction

The heat stability of the ethylacetate fraction (which was chosen for further study) confirmed that the bioactive compound was heat stable; it could withstand 120^0C without any change of activity.

4.3.6 Thin layer chromatographic separation of the bioactive compound

The comparison of Rf values of the fractions in different solvent systems was made to find out the best system for further use (Table 4). The results showed that ethyl acetate: n-hexane 60:40 was the one with clear separation and movements of the bands (Table 4). Preparative TLC of the extract led to four fractions with Rf values within the following ranges: 0.05-0.25 (Band I); 0.25-0.5(Band II); 0.5 – 0.75 (Band III) and >0.75 (Band IV). The biological assays of the four fractions showed that only fraction Band II (Rf value 0.25-0.5) possessed antibacterial activity against the target organisms.

Table 4. Solvent systems and Rf values of Thin layer chromatograph

SL No:	Solvent System	RF values						
1	EtAc :n-hexane 50:50	0.08	0.18	0.34	0.44	0.63		
2	EtAc :n-hexane 60:40	0.08		0.32		0.57	0.78	
3	EtAc :methanol 95:5		0.13	0.35		0.67		
4	$CHCl_3$:Acetone 50:50				0.49		0.81	
5	Butanol: Acetic acid: Water 60:20:20	0.075			0.54		0.8	
6	Acetic acid : Water 50:50					0.71	0.85	0.97

4.3.7 HPLC analysis of the bioactive compound

The retention time (Rt value) of the active fraction was 33.71 minutes, with a peak area of 95.25%, showing that the bioactive fraction was sufficiently pure (Fig 1).

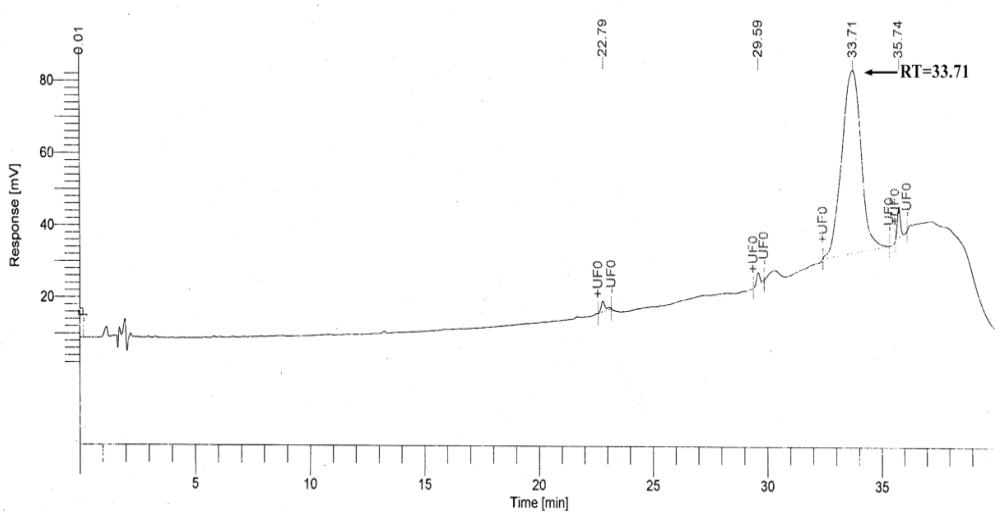

Fig.1. HPLC analysis of the purified fraction

4.3.8 UV-Vis and FTIR spectra of the bioactive compound

The UV-visible spectrum (H_2O) exhibited a peak at 237.5 nm with a shoulder at around 270nm.

The IR spectrum in potassium bromide pellet exhibited prominent signals at 3407.17λ, 1622 -1562 λ, 1418 λ and 1142 λ (Fig 2).

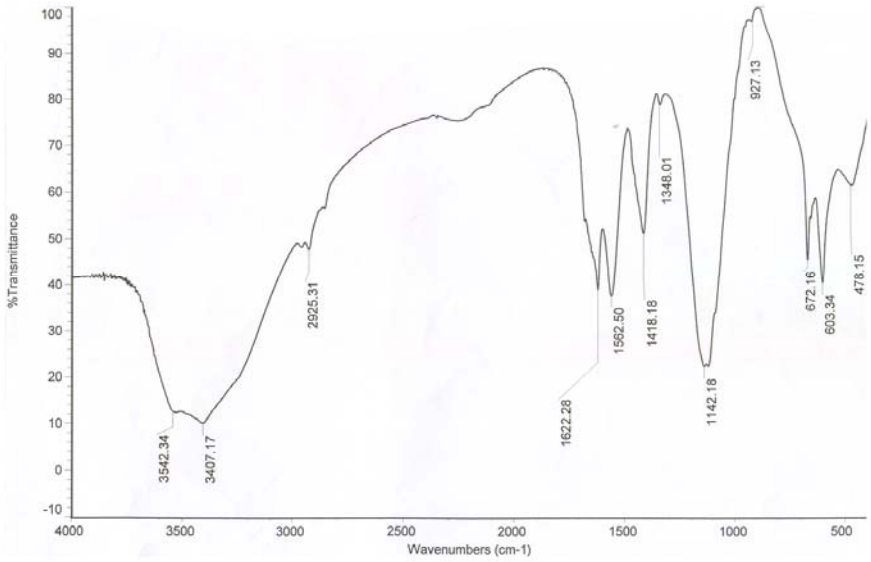

Fig.2. FT-IR spectrum of the purified compound

4.3.9 MS-MS analysis of the anti-vibrio compound

The MS-MS analysis of the active fraction demonstrated a protonated molecular ion at m/z 437.5 (Fig 3).

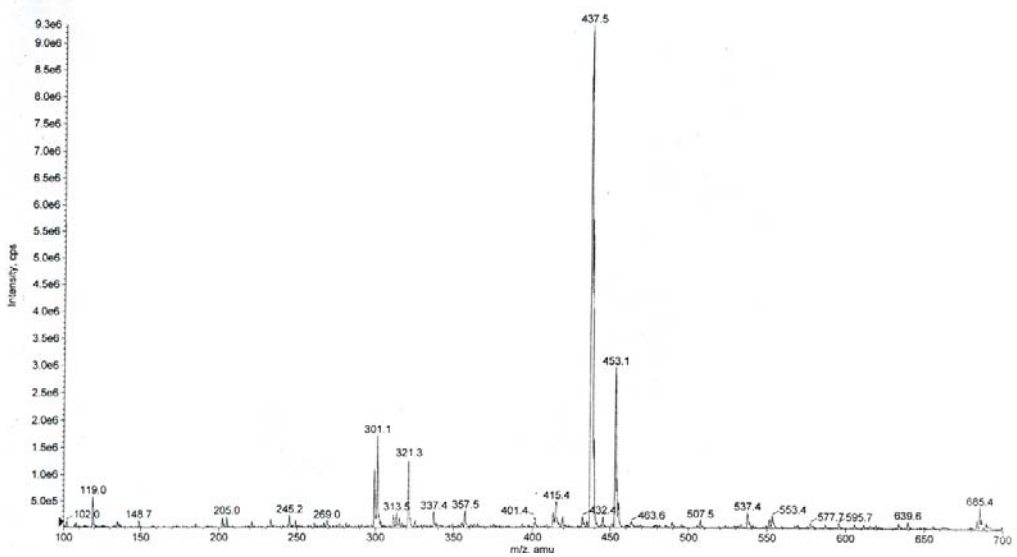

Fig.3. MS-MS spectrum of the purified fraction

4.3.10 ^{1}HNMR spectral analysis

In the proton magnetic resonance (^{1}H NMR) spectrum (recorded in D_2O) of the active molecule (Fig.4), signals were observed at δ 3.63 (m), 3.49 (dd), 3.39 (dd), 2.07 (s) and 1.76(s) ppm in a 1:2:2:4:8 ratio. Since the spectrum was recorded in D_2O, signals due to NH and OH protons were not observed.

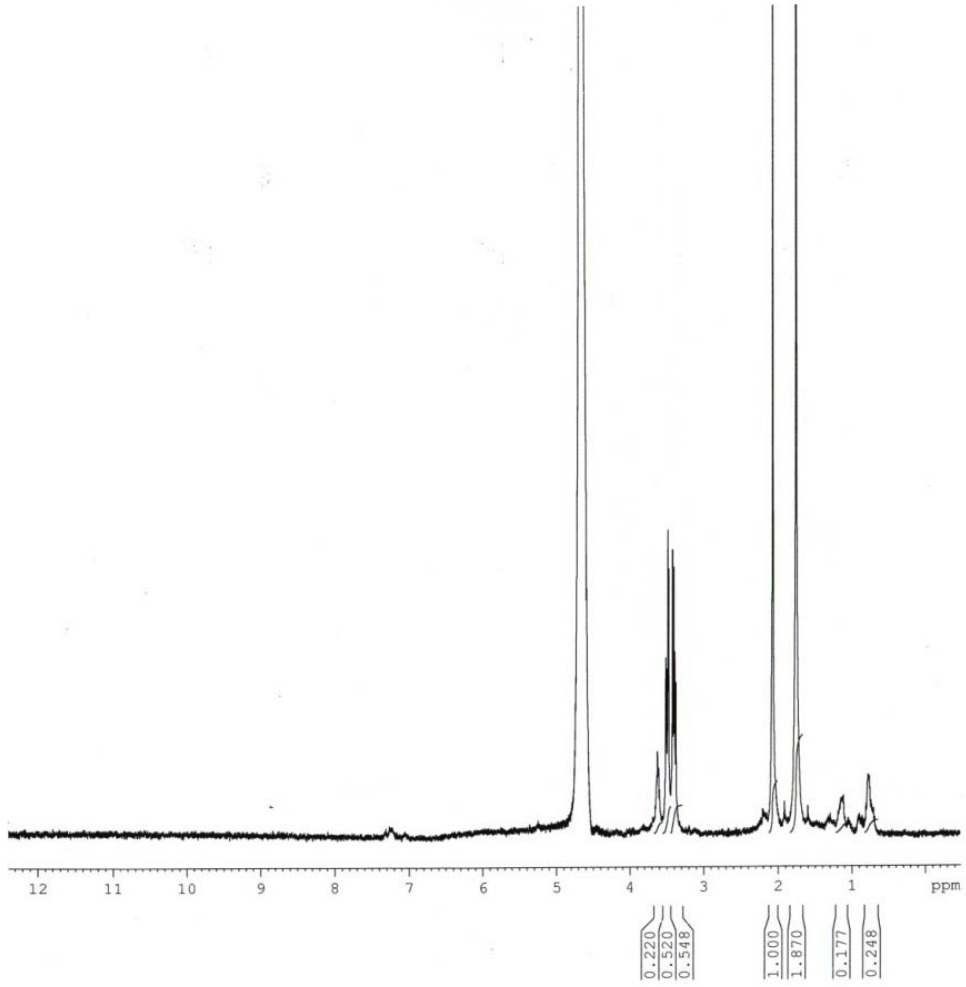

Fig.4. ^{1}HNMR spectrum of the purified fraction

4.3.11 Cytotoxicity Test

Cytotoxicity of the active compound was evaluated with a mammalian cell line and shrimp cell culture by the MTT assay. The active compound was not toxic to VERO and shrimp haemocyte cells (Fig5 & Fig 6) up to 1000 ppm.

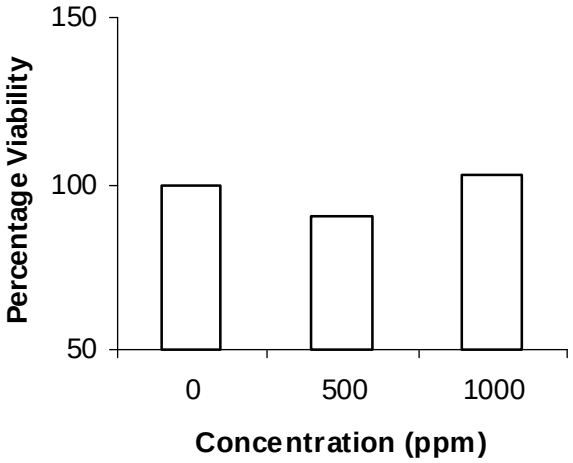

Fig.5. Percentage viability of VERO cells exposed to antivibrio fraction

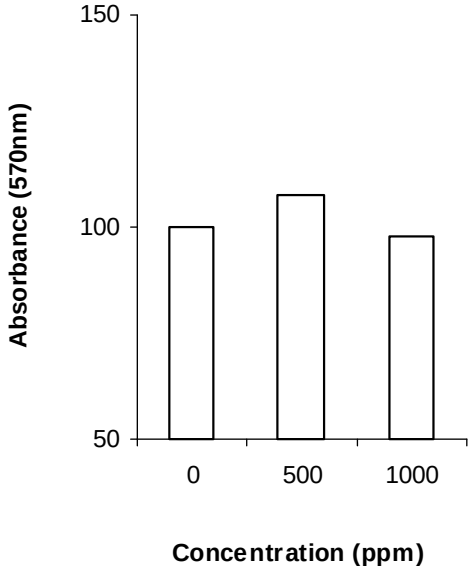

Fig.6. Percentage viability of shrimp haemocyte cells exposed to antivibrio fraction

4.3.12 Bioluminescence assay and growth

The culture supernatant could bring forth inhibition of luminescence (Fig. 7), most apparent at the 3rd hr of incubation. At the concentrations tested, there was inhibition of growth of V *harveyi* as measured by optical density (Fig 8).

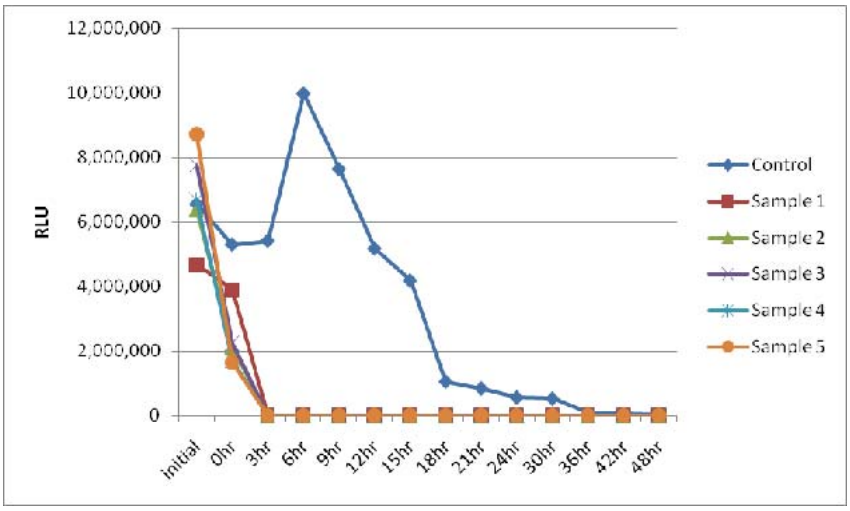

Fig.7. Inhibition of luminescence of *V harveyi* exposed to the culture supernatant at different time intervals

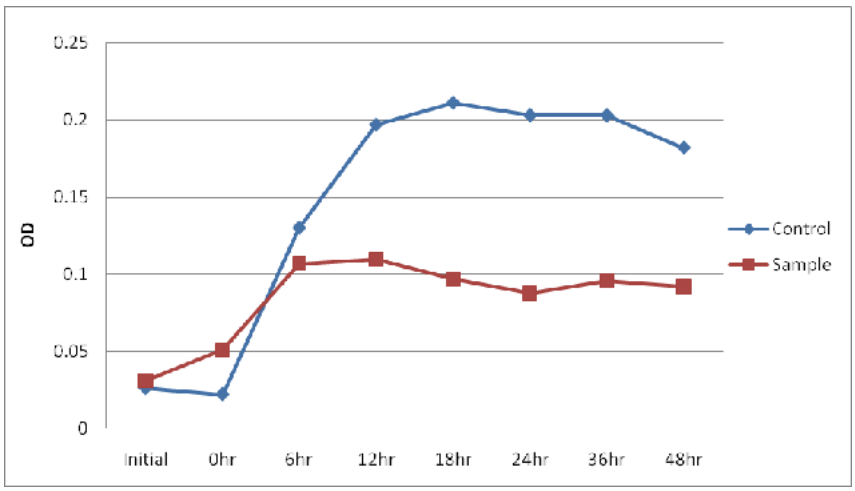

Fig.8. Growth curve of *V harveyi* exposed to the antagonistic culture supernatant

4.4 Discussion

As a result of increasing prevalence of antibiotic-resistant pathogens and the pharmacological limitations of antibiotics, there is an exigency for new antibacterial substances. Filamentous actinomycetes are widely recognized as industrially important microorganisms because of their ability to produce many kinds of natural products including antibiotics (Jensen *et al*.,2005), and marine actinomycetes have been proved to be rich source of such compounds useful for the development of new antimicrobials (Blunt *et al*., 2004; Faulkner, 2000; Haefner, 2003). In recent decades, many studies on the antimicrobial activity of the actinomycetes isolated from marine environment have been done, but the results are different. Zheng *et al*. (2000) found that 43.6% of actinomycetes isolated from the marine environment showed antimicrobial activities, but only 12.8% of the actinomycetes produced antimicrobial metabolites against *Vibrio* spp.

Through separation techniques the biological activity was found in fraction B_2 (Rf value 0.25-0.5) in TLC which was further confirmed of purity by HPLC to have a single well defined peak of Rt 33.71 minutes with a peak area of 95.75% registering sufficient purity for structural elucidation.

Efforts were taken to determine the structure of the active compound on the basis of standard spectroscopic analysis data. The solution-phase spectral analysis was carried out in H_2O/D_2O due to the poor solubility of the compound in common organic solvents. The UV-visible spectrum (H_2O) exhibited a peak at 237.5 nm with a shoulder at around 270nm. These features strongly suggested the absence of aromatic and other highly conjugated residues. But presence of a keto group was a distinct possibility (^{1}H NMR and

IR spectral data). Therefore, it was inferred that aromatic and highly conjugated structural fragments were not present in this compound. In the IR spectrum (KBr) OH and NH stretching frequencies appeared as broad peaks at 3542 and 3407 cm^{-1} respectively. NH bending frequencies were observed at 1622 and 1562 cm^{-1} and OH bending frequency could be observed at 1418 $cm^{-1.}$ C-O stretching frequency was observed at 1142 cm^{-1}. Other minor peaks ascribable to C-H (aliphatic) stretching and bending and out of plane hydrogen bonded OH bending frequencies could also be observed at appropriate positions. The signal appearing as a shoulder around 1680 cm^{-1} suggested the presence of a carbonyl group in the molecule (Fig.2).

In the last two decades several new species of *Nocardiopsis* have been described and many interesting substances, such as enzymes and antibiotics have been discovered (Takahashi *et al.,*1986; Brazhnikova *et al*., 1977; Cho *et al*., 2007; Dolak *et al*., 1980; He *et al*., 2007; Kase *et al*., 1986; Tsujibo *et al*., 1988; Kim *et al*., 1997; Shin *et al*., 2003; Newton *et al*., 1996; Nishiwaki *et al*., 1991; Schumacher *et al*., 2001; Tsujibo *et al.,* 1990; Wu *et al*., 2005, Kusakabe *et al.,* 1987).

The aim was to compare the spectral data generated here with those reported for various biologically active compounds isolated from *Nocardiopsis*. Based on IR and UV-Vis spectral data the new compound was demonstrated not as Kalafungin (Tsujibo *et al*., 1990). Absence of peaks in the aromatic region (6-9 ppm) ruled out K252 (Kase *et al*., 1986), indole nucleoside (Schumacher *et al*., 2001), Griseusins (He *et al*., 2007), Pendolmycin (Nishiwaki *et al*., 1991) and phenazine antibiotics (Tsujibo *et al*., 1988). Absence of vinylic proton signals in the δ 4.8-δ 5.8 regions ruled out the structures such as Apoptilidin (Kim *et al*.,

1997), Nocamycin (Brazhnikova *et al*., 1977) and others as having 3-methyl-4-ethylideneproline - containing peptide units such as Lucentamycins (Cho *et al*., 2007). Some of the methyl signals in Portmicin (a polyether antibiotic) (Kusakabe *et al*. 1987) should appear as either doublets or triplets in δ 0.8- δ 2.1 region which was absent in the NMR spectrum. Absence of anomeric proton signals around δ5.33 regions ruled out polyamine type Dopisamine (Takahashi *et al*., 1986), disaccharide antibiotic 3-trehalosamine (Dolak *et al*., 1980), thiols such as MSH (Newton *et al*., 1996) and tetrodotoxin like structures (Tsujibo *et al*., 1990). Since all the methyl signals appear as singlets, cyclic tetra peptide structures like MKN-349A (Shin *et al*., 2003) could be ruled out. The above comparative study strongly suggested that the active molecule was different from all other known compounds reportedly produced by different species of *Nocardiopsis*. The MS-MS analysis showed that the molecule possessed a molecular weight of 453 (Fig.3) indicating the presence of odd number of nitrogen atoms in the molecule.

One of the striking features of the bioactive compound is the lack of cytotoxicity as proved in VERO and shrimp haemocyte culture even up to 1000 ppm.

Vibrio harveyi is a serious pathogen of marine fish and invertebrates, particularly penaeid shrimp (Austin and Zhang, 2006). However, the pathogenicity mechanism remains elusive. Several studies have suggested that biofilms may be important for survival, virulence, and stress resistance of *Vibrio* spp. (Wai *et al*., 1998; Watnick and Kolter, 1999; Watnick *et al*., 2001; Wang *et al*., 2003; Zhu and MeKalanos, 2003; Faruque *et al*., 2006). The persistence and survival of *V. harveyi* in shrimp hatcheries has been attributed

to its ability to form biofilms with resistance to disinfectants and antibiotics (Karunasagar *et al*., 1994). Quorum sensing (QS) plays an important role in biofilm formation (Schembri *et al*., 2002). *Vibrio* spp. have been reported to utilize N-acylated homoserine lactones (AHLs) to coordinate expression of virulence in response to the density of the surrounding bacterial population (Zhu and MeKalanos 2003; Hammer and Bassler, 2003). The AHL molecules are produced by LuxI homologues and constitute, in complex with LuxR homologues, transcriptional regulators (Waters and Bassler, 2006). Fighting *Vibrio* infection by interfering with their command language and thereby disrupting virulence expression, instead of inhibiting growth, could serve as an alternative to conventional procedures (Thomas *et al*., 2005). Another strategy would be based on microorganisms or small molecules with variations in their chemical composition that would allow them to block the AHL receptor site of the LuxR homologues, such as TraR, or, alternatively, to block the formation of active dimmers that are required for binding to and expression of target genes (Thomas *et al*., 2005). For example, furanones from alga *Delisea pulchra* was found to inhibit AI-2 signalling as well as homoserine lactone signalling (Ren *et al*., 2001), and furanone could protect the host from the pathogenic *V. harveyi* by disruption of AI- 2 QS (Defoirdt *et al*., 2006; Tinh *et al*., 2006). The luminescence quenching property detected in the culture supernatant of *Nocardiopsis* MCCB 110 might be having similar mechanisms interfering with the expression of virulence. The compound could shut down bioluminescence of *V harveyi* within 3 hours exposure to a crude preparation at the lowest concentration tested. This suggests that the AHL-dependent luminescence phenomenon of *V harveyi* is susceptible to regulation by the bioactive compound produced by *Nocardiopsis.* At the lowest concentration tested it also exhibited a growth inhibitory effect of 52.4% (Fig. 8).

This suggests that the antibacterial compound produced by *Nocardiopsis* may find application in luminescence quenching and there by inhibiting the virulence in *V. harveyi* and considering its non toxicity it has greater prospects in using in aquaculture system especially in the management of *V. harveyi*.

To make the shrimp aquaculture industry more sustainable, alternative strategies to control infections are urgently needed. One of the most successful alternative methods has been reported to be the use of probiotics as biological control agents in shrimp aquaculture. As potential probiotics in shrimp culture, actinomycetes have many following advantages: (1) production of antimicrobial and antiviral agents (Austin, 1989; Oskay *et al*., 2004); (2) degradation of complex biological polymers, such as starch and protein (Barcina *et al*., 1987), lignocellulose, hemicellulose, pectin, keratin, and chitin (Williams *et al*., 1984) which shows the potential to involve in mineralization and nutrient cycles in the culture ponds and in feed utilization and digestion once getting colonized into the host intestine; (3) competition for nutrients, particularly iron in marine microbes (Kesarcodi *et al*., 2008); (4) mostly non–pathogenic to the target animals in aquaculture (Yang *et al*., 2007); and (5) formation heat– and desiccation– resistant spores and the retention of viability during preparation and storage.

The above described features of actinomycetes along with the major findings of this chapter such as the antivibrio property, luminescence quenching property and novelty with regard to *Nocardiopsis* MCCB110 may facilitate its potential application as a probiotic in shrimp culture in particular and for improving the shrimp aquaculture system in general.

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Chapter

5

CONCLUSION AND SCOPE FOR FUTURE RESEARCH

Shrimp farming constitutes an important source of revenue and employment in many developing countries. However, the shrimp industry has always been affected by infectious diseases, mainly of bacterial and viral etiology causing great loss of production. The sustainability of the shrimp industry depends largely on disease control and the health status of shrimp. For this reason, disease prevention is a priority and shrimp immunology has become a prime area of research.

As a result of increasing prevalence of antibiotic-resistant pathogens and the pharmacological limitations of antibiotics, there is an exigency for new antibacterial substances. Filamentous actinomycetes are widely recognized as industrially important microorganisms because of their ability to produce many kinds of natural products including antibiotics and marine actinomycetes have been proved to be rich source of such compounds useful for the development of new antimicrobials. Considering this situation the present work was undertaken with the following objectives:

1) Selection of the organism, its characterization and identification.

2) Immunomodulatory property of *Nocardiopsis* MCCB110 in *Penaeus monodon*.

3) Isolation, purification and partial characterization of the bioactive compound produced by *Nocardiopsis* MCCB 110.

Overall achievements in this work are summarized below:

- Six isolates of actinomycetes were screened for their potential to generate antibacterial compounds against *Vibrio harveyi*. In this process the most potent isolate, MCCB110 was segregated based on its inhibitory property.
- A cell wall fatty acid pattern, including a major percentage of branched-chain fatty acids with high percentages of C17:0, anteiso-C18:0, and C18:0 10-methyl fatty acids, was reported as characteristic of *Nocardiopsis* genus.
- 16S rRNA gene sequencing allowed the definitive identification of the isolate as *Nocardiopsis* sp.
- Alignment of the total nucleotide sequence of 1495 bp (accession number: EU008081) of the 16S rRNA gene of MCCB110 followed by matching with reported 16S rRNA gene sequences in the GenBank showed a high similarity (99%) to *Nocardiopsis* sp.
- Phylogenetic analyses based on a dataset consisting of 1495 bp showed that the novel isolate fell into a distinct clade with another *Nocardiopsis* species, *Nocardiopsis* sp (AN10)
- Results of the antimicrobial assays showed that the isolate *Nocardiopsis* MCCB110 exhibited clear antibacterial activity towards, *Vibrio harveyi* MCCB111, *Micrococcus* sp MCCB104

and *Bacillus* sp. MCCB101. The activity was weak to moderate against *Pseudomonas aeruginosa* MTCC741.

- It was found that the cell free culture supernatant had the activity and not the pellet.
- In the present study, the total hemocyte count, phenol oxidase activity, respiratory burst activity, total protein values, acid phosphatases and alkaline phosphatases activities were found to be higher in the haemolymph of shrimps fed with *Nocardiopsis* MCCB 110 incorporated feeds both before and after challenge when compared to normal feed administered shrimps.
- The survival of shrimp among *Nocardiopsis* administered ones was 83% while in the normal feed administered ones the survival was only 50%, subsequent to challenge.
- Analysis of the results of immune gene expression in *P. monodon* showed that *Nocardiopsis* MCCB 110 up-regulated the expression levels of six out of seven immune genes of shrimps selected under this study.
- Biodefence mechanisms like protease inhibition involving alpha 2 macroglobulin, antimicrobial activity involving penaeidin -3, coagulation involving transglutaminase, phenol oxidase system involving proPO, antibacterial activity involving crustin and cell adhesion involving peroxinectin were found to be active as the genes involved in these mechanisms were up- regulated in animals administered with *Nocardiopsis* MCCB 110 incorporated feed.

- This higher number of up-regulated genes and concurrent increase in non specific immune parameters along with the higher rate of survival in *Nocardiopsis* administered group showed that bio-defence had been elicited after administration of *Nocardiopsis* coated diet and clearly showed that *Nocardiopsis* MCCB 110 enhanced the immune status in shrimp.
- Out of eight solvent extracts tested for antibacterial activity, the extracts of butanol, benzene, dichloroethane, chloroform and ethylacetate have shown activity. Among them the ethylacetate extract has shown comparatively better halo zone.
- The heat stability of the ethylacetate fraction confirmed that the bioactive compound was heat stable; it could withstand 120^0C without any change of activity.
- Comparison of various solvent systems showed that ethyl acetate: n-hexane (60:40) was the one with clear separation and movements of the bands in thin layer chromatography.
- The biological assays of the four fractions showed that only fraction Band II (Rf value 0.25-0.5) possessed antibacterial activity against the target organisms.
- HPLC analysis of the active fraction showed a retention time (Rt value) of 33.71 minutes, with a peak area of 95.25%, registering sufficient purity for structural elucidation.
- The IR spectrum in potassium bromide pellet exhibited prominent signals at 3407.17λ, 1622 -1562 λ, 1418 λ and 1142 λ

- The UV-visible spectrum (H_2O) exhibited a peak at 237.5 nm with a shoulder at around 270nm. These features strongly suggested the absence of aromatic and other highly conjugated residues. Therefore, it was inferred that aromatic and highly conjugated structural fragments were not present in this compound.
- In the IR spectrum (KBr) OH and NH stretching frequencies appeared as broad peaks at 3542 and 3407 cm^{-1} respectively.
- NH bending frequencies were observed at 1622 and 1562 cm^{-1} and OH bending frequency could be observed at 1418 $cm^{-1.}$ C-O stretching frequency was observed at 1142 cm^{-1}.Other minor peaks ascribable to C-H (aliphatic) stretching and bending and out of plane hydrogen bonded OH bending frequencies could also be observed at appropriate positions. The signal appearing as a shoulder around 1680 cm^{-1} suggested the presence of a carbonyl group in the molecule.
- In the proton magnetic resonance (^1H NMR) spectrum (recorded in D_2O) of the active molecule, signals were observed at δ 3.63 (m), 3.49 (dd), 3.39 (dd), 2.07 (s) and 1.76(s) ppm in a 1: 2:2:4:8 ratio.
- Based on the absence of signals above δ 4, the active compound was identified as saturated aliphatic. The α- substituted methyl group at δ 2.07 and the β-substituted methyl group at δ 1.76 were both appearing as singlets indicating a unique substitution pattern. The signal appearing at δ 2.07 was attributable to an acetyl (CH_3CO) group.

- Since only very few signals were observed, the molecule was concluded to be highly symmetric.
- The MS-MS analysis showed that the molecule possessed a molecular weight of 453 indicating the presence of odd number of nitrogen atoms in the molecule.
- The comparative study of spectral data with those reported for various biologically active compounds isolated from *Nocardiopsis*, strongly suggested that the active molecule was different from all other known compounds reportedly produced by different species of *Nocardiopsis.*
- One of the striking features of the bioactive compound was the lack of cytotoxicity as proved in VERO and shrimp haemocyte culture even up to 1000 ppm tested.
- The culture supernatant containing the compound could shut down bioluminescence of *V harveyi* within 3 hours exposure at the lowest concentration tested. This suggested that the AHL-dependent luminescence phenomenon of *V harveyi* is susceptible to regulation by the bioactive compound produced by *Nocardiopsis.* At the lowest concentration tested it also exhibited a growth inhibitory effect of 52.4%.
- The luminescence inhibition property of *Nocardiopsis* suggested that the antibacterial compound in the culture supernatant of *Nocardiopsis* may find application in luminescence quenching and there by inhibiting the virulence in *V. harveyi,* and considering its non toxicity it has greater prospects in using in aquaculture systems especially in the management of *V. harveyi.*

- The above described features of *Nocardiopsi*s MCCB110 along with the major findings of this chapter such as the antivibrio property and luminescence quenching property may facilitate the potential application of *Nocardiopsis*MCCB110 as a probiotic in shrimp culture in particular and for improving the shrimp aquaculture system in general.

Scope for future Research

- Complete structural elucidation of the active molecule by the application of newer spectroscopic methods.
- Species level identification of *Nocardiopsi*s MCCB110 using advanced molecular techniques.
- Development of a cost effective downstream process for the large scale commercial production of the active molecule.
- Determination of the mechanism of action of the probiotic in the immune system of shrimp.
- Determination of the mode of action of the active molecule in the luminescence quenching property against *V. harveyi*.

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Appendix

Publications arising out of this thesis

1) **Purification and partial characterization of an antibacterial compound from a marine *Nocardiopsis* MCCB110 with luminescence quenching on *Vibrio harveyi* (To be communicated)**
K.S.Sunish, S.Prathapan, Mathew Biji, Philip Rosamma, S.Vrinda, A. Mohandas, I. S. Bright Singh

2) **Immunomodulatory property of a Marine Actinomycete, *Nocardiopsis* MCCB 110 on the giant tiger shrimp, *Penaeus monodon* (To be communicated)**
K.S.Sunish, Mathew Biji, Philip Rosamma, S.Sreedharan, N.S.Sudheer., A. Mohandas, I. S. Bright Singh

Genbank Accession

***Nocardiopsis sp*. MCCB 110 16S ribosomal RNA gene, partial sequence. ACCESSION NO: EU008081**

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