Marine Actinomycetes as Source of Antiviral Agents and as Probionts for *Penaeus monodon* Culture Systems

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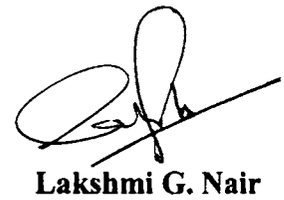
DECLARATION

I hereby do declare that the thesis entitled **"Marine Actinomycetes as Source of Antiviral Agents and as Probionts for *Penaeus monodon* Culture Systems"** is an authentic record of research work carried out by me under the supervision and guidance of Dr. Rosamma Philip, Senior Lecturer, Department of Marine Biology, Microbiology & Biochemistry, Cochin University of Science & Technology for the degree of Doctor of Philosophy in Microbiology and that no part thereof has been presented before for the award of any other degree in any university.



Lakshmi G. Nair

Cochin

May 7, 2008

CERTIFICATE

This is to certify that the thesis entitled **"Marine Actinomycetes as Source of Antiviral Agents and as Probionts for *Penaeus monodon* Culture Systems"** is an authentic record of research work carried out by Mrs. Lakshmi G. Nair under my supervision and guidance in the Department of Marine Biology, Microbiology & Biochemistry, Cochin University of Science & Technology in partial fulfuillment of the requirements for the degree of Doctor of Philosophy in Microbiology and that no part thereof has been presented before for the award of any other degree, diploma or associateship in any university or institution.

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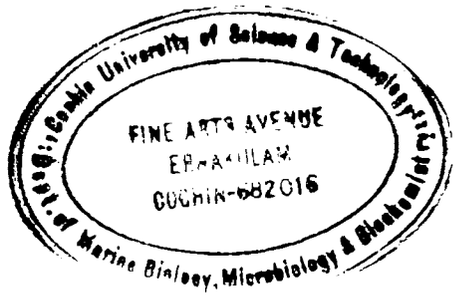

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CHAPTER 1

General Introduction

Aquaculture has developed rapidly over the last three decades to become an important activity worldwide. The Food and Agricultural Organization (FAO) of the UN acknowledge that global fishery output must be increased by atleast 50 % to offset projected shortfalls in dietary protein by 2030. At present , production by traditional fisheries and aquaculture is approximately 150 million tonnes. Out of this, commercial and artisanal fisheries account for around 94 million tonnes of seafood. Since most of the worlds fisheries have already exceeded maximum sustainable yields or are being fished at maximum permissible levels, aquaculture therefore, presents the only method of offsetting predicted fishery shortfalls.

Crustacean aquaculture is considered as a high value activity world wide and tend 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. Recent estimates suggest that the level of farmed marine shrimp is around 40 % of the total (*Rosenberry*,

2001). The bulk of farming activity is in Asia, particularly Thailand followed by China, Indonesia and India. To a lesser extent, shrimps are produced in Latin America, with Ecuador as the leading country. The major species cultured are *Marsupenaeus japonicus , Penaeus monodon, Penaeus chinensis, Penaeus merguiensis, Fenneropenaeus indicus* and *Litopenaeus vannamei.* Much of the world shrimp production still comes from extensive culture.

During the past two decades, the worldwide shrimp aquaculture has been greatly puzzled by diseases. Most diseases occur as a result of environmental deterioration and stress associated with intensification of shrimp farming. Many shrimp farms have been particularly affected by epidemics of viruses like white spot syndrome virus (WSSV), Monodon baculovirus (MBV) , Yellow head virus (YHV) , and Taura syndrome virus (TSV) (*Wang et al.* (2000), *Lo et al.* (1996), *Lightner et al.* (1987) & *Smith et al.* (2003)). Several disease outbreaks have also been associated with vibriosis-causing bacteria like *Vibrio harveyi, Vibrio damsela, Vibrio alginolyticus* (*Song et al.* (1993) & *Lee et al.* (1996)). A few diseases had fungal and protozoan aetiologies (*Destomieux et al.*, 2000). These pathologies particularly hamper larval production and lead to profitability problems due to stock mortalities. They also lead to the overfishing of shrimp larvae and an overexploitation of broodstock.

In India, commercial shrimp farming started gaining roots only during the mid-eighties, *Penaeus monodon* being the predominant species cultured . Cultured shrimps contribute a major portion of national income through high export earning. 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 people (Aquaculture Authority News, 2002). The boom period of

commercial scale shrimp culture in India started in 1990 and the bust came in 1995-96, with the outbreak of viral disease. The White spot disease (WSD) has played havoc and its repeated occurrence has demoralized shrimp farmers all over the world. 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). However, control over spreading of pathogen or introduction of new pathogens across borders should be dealt with quarantine protocols including pathogen free certification of stock. Diagnostic methods for rapid detection of aquatic diseases have been improved to a greater extent with the aid of recent biotechnological tools, but at the same time treatment of the infected stock is still lagging behind, especially for viral diseases.

The potential manipulation of shrimp defense responses in order to increase protection and resistance to infections could be fundamental for shrimp health management . The popular quote prevention is better than cure sheds light to the importance of proactive disease management measures to be taken to reduce the risk factors in aquaculture. A proactive disease management strategy, at least in shrimp aquaculture, is a multidisciplinary subject where ecology, environment, nutrition, physiology and genetics of the organism should be taken care of. (*Bachere*, 2000). In this context, a better understanding of the crustacean immune system is crucial in order to establish the basis of susceptibility and resistance of shrimps to different pathogens.

1.1 Strategies for Controlling Diseases in Shrimp Aquaculture

There is an extensive literature, dating back some thirty years, on the problem of disease in cultured shellfish (*Anderson and Conroy*, 1968), (*Fisher et al.*, 1976). Both partial and full culture carry the risk of financial losses due to disease either through mortality or reduced meat quality, resulting in reduced profit margins. It is notoriously difficult to estimate effects of shrimp disease since a common strategy is to organize emergency harvests at the first sign of disease but reportedly in Central and South America, shrimp production fell by ca.17 % during the period 1998-1999, mainly through virus infections (*Rosenberry*, 2001). For Asia, a survey of the shrimp aquaculture in Thailand found that approximately 66 % of farmers had experienced at least one disease outbreak per year producing a financial loss of over US $6000 per hectare per year (*Hambrey and Lin*). It is clear that this problem is very severe, a fact which has been acknowledged by the World Bank who recommended that an investment of US $275 million should be made in shrimp disease research during the period 1996-2010 .

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 often kept under stressful conditions of overcrowding , high food values, 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 become prevalent. This can compound the problem associated with more pathogenic organisms and is further worsened by repeated restocking of ponds, leading to accumulation of pathogens and

opportunistic bacteria in the water and sediment. The potential for a disease outbreak poses a continual threat to the existence of any farm and once an infection occurs it can prove devastating to the entire stock. 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* (1998) & *Grant and Briggs* (1998)), as well as causing mortality or impaired growth in juvenile stock (*Stuck et al.* (1992) & *Swastika et al.* (1992)). The repeated application of antibiotics, in the long term ,is also encouraging the spread of drug resistant pathogens (*Brown* (1989), *Juwans* (1990), *Aoki* (1992) & (*Karunasagar et al.*, 1994)) and this practice atleast in Europe is phased out. Moreover, chemical disinfection maybe incompatible with geographic location of the farm and the physical requirements of the stock. As Bachere (*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 programmes and the use of genetically modified strains are still a long way from 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 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.

1.2 The Crustacean Defence System

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. However, the great success of those primitive organisms , particularly arthropods , is definitely attested by their enormous numbers on earth and the diversity of environments they can colonise, which often abound in opportunistic microbes. The immune system of crustaceans is primarily related to their blood or haemolymph and to its circulating cells or haemocytes.

Shrimps possess both humoral and cellular immune responses, although they are less specialized than vertebrate immune responses The cellular components is related to haemocytes, that are involved in immediate defensive reactions such as nodulation, encapsulation and phagocytosis. The humoral component is characterised by a temporarily enhanced antimicrobial activity in the cell-free haemolymph (*Niere et al.*, 1999).

Based on the cytochemistry, function and morphology, crustacean haemocytes have been classified into three; *viz.* hyaline cells, and two kinds of granular cells - semigranular and granular cells (*Hose et al.*, 1990). It is well established that in arthropods, the defence of the host against invasive or opportunistic microorganisms is effected principally by the phagocytic, encapsulating and agglutinating activity of the circulating haemocytes (*Ratcliffe et al.*, 1985).

1.2.1 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 microbicidal proteins (*Smith and Chilsolm* (1992) & *Smith and Chilsolm* (2001)). They are involved in cellular responses, including clotting, non-self recognition, phagocytosis, melanisation, encapsulation, cyto-toxicity and cell-to-cell communication. There appears to be partitioning of these functions between the different cell types, although there maybe species difference in the way this occurs. In crabs, phagocytosis and production of reactive oxygen are mainly executed by hyaline cells, although in other species, semigranular cells may also be phagocytic. *Marsupenaeus japonicus* is unusual in that the hyaline cells appear not to be phagocytic ; this function being provided by the granular cells. (*Itami et al.*, 1998).By contrast, all three cell types display atleast some phagocytic activity in freshwater prawn, *Macrobrachium rosenbergii*.

Of the three types of haemocytes, hyaline cells in most decapod crustaceans are characterized by the absence of granules, although some cytoplasmic inclusion bodies have been reported by electron microscopic observations (*Martin and Garves.*, 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). Semigranu-lar 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 not respond to the microbial polysaccharides directly unless they are pre-treated with some haemolymph proteins called pattern recognising 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.

In decapods, haemocytes are produced within specialised haematopoietic tissue (HPT), the location and architecture vary greatly, even within close taxonomic groups. In lobsters, crabs and crayfish, haematopoietic cells of different morphology are organized and densely packed in small lobules and located over the cardiac stomach or the heart (*Martin et al.*, 1993). However, the arrangement is different in penaeid shrimps, where haematopoiesis is believed to occur in paired epigastric nodules, which consists of an extensive network of vessels derived from ophthalmic artery. Morphology of the cells in the haematopoietic tissue of penaeid shrimps was studied by van de *Braak et al.* (2002) at light and electron microscopic level.

The regulation of haematopoiesis in decapod crustaceans is poorly understood, but

is probably influenced by physiological processes such as moulting, reproduction and health status, as well as by environmental conditions like temperature and water quality (*Johnson* (1980), *Bauchau* (1981) & *Hose et al.* (1992)). Production of haemocytes occurs almost exclusively within the HPT, since mitotic haemocytes are rarely observed in the peripheral circulation. Cells released from the lobules appear identical to circulating cells. However, large granular haemocytes are not common in the HPT, suggesting that they can also develop from circulating small granular haemocytes (*Martin et al.*, 1993). The mechanism by which maturing haemocytes are released into circulation is not clear. In shrimps, haemocytes migrate into the lumen of the haematopoietic tubule, which is continuous with the ophthalmic artery (*Martin et al.*, 1987).

1.2.2 Cellular Immune Responses

1.2.2.1 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 reaction, 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). When haemocyte monolayers were treated with β-1,3-glucan, a trigger of proPO system

and a five to seven times higher degree of phagocytosis were observed than untreated control monolayers . But the factors, which act as, an opsonin in crustacean haemolymph is yet to be isolated.

1.2.2.2 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 heavily. 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) & *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.

1.2.2.3 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 in-

vading pathogens. Some opsonin factors present in the haemolymph can also mediate the encapsulation process.

1.2.3 Humoral Immune Responses

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.

1.2.3.1 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 that 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; EC1.14.18.1) catalyses the oxidation of phenols to quinones followed by several intermediate steps that lead 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 enzyme (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 these 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 recognized 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 opsonin (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 peroxidases.

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 were been identified 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). The activity of phenol oxidase has been reported for many crustaceans including Brown shrimp, *Farfantepenaeus californiensis*, Tiger shrimp, *Penaeus monodon*, White shrimp, *Litopenaeus vannamei*, Sao Paulo shrimp, *Farfantepenaeus paulensis* and Blue shrimp *Litopenaeus stylirostris*.

1.2.3.2 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 recognize 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.* (1988) 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 species (*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. (*Vargas-Albores et al.*, 1993) reported the ability of purified lectin to react with different marine species of *Vibrio*.

1.2.3.3 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 wide spread 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 characterized 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), *Destomieux et al.* (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 characterized 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.

1.2.3.4 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 (O_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 & 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). A schematic overview of the important factors in the crustacean defence system is given in *Fig.(1.1)* (*Smith et al.*, 2003).

1.3 Vaccination in Shrimps - A Possibility

Vaccination, is a strategy developed for generating long lasting protection through immunological memory. Adaptive secondary memory immune response of vertebrates depends on immunoglobulins (Igs), T Cell receptors (TCRS), Major Histocompatibility Complex (MHC) and memory T cells (*Klein*, 1989). It is the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously. It is very evident that there exists an anticipatory (memory) and non-anticipatory immune response in vertebrates, whereas only non-anticipatory immune responses were observed in invertebrates (*Klein*, 1997).

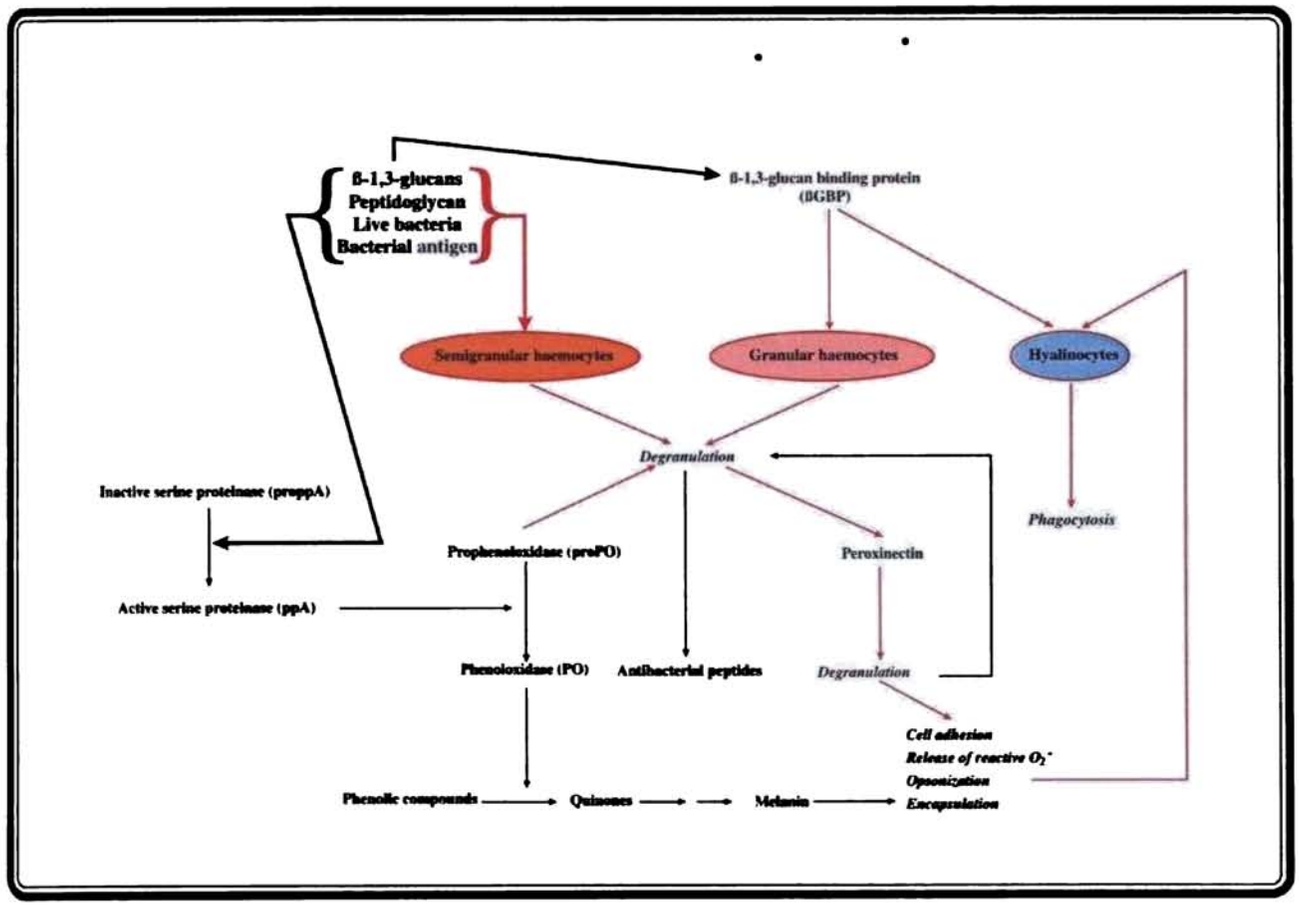


Figure 1.1: Flow diagram showing Crustacean defence system

There is much confusion in the literature as to the terms vaccination and immunostimulation with respect to disease prevention / control in shellfish. Sometimes the terms are used interchangeably . Vaccination is a term that should strictly be applied only when the purpose is to confer long lasting protection through immunological memory. It requires primary challenge with antigen and is dependent upon clonally derived lymphocyte sub-sets to be implemented. In vaccination, a harmless inactivated form of a pathogen is used to stimulate the primary antibody response, so that when the real pathogen is met, there is pre-existing immunity and the secondary response can be evoked to boost the level of immunity very quickly. It often include the use of adjuvants (killed mycobacterial cells, aluminium salts, or mineral oil) to provide a depot effect and enhance the antibody response. These usually act on one or more non-specific innate components of the immune system, such as cytokines and antigen presentation. In essence they are acting as immunostimulants to maximize a particular specific response. However, immunostimulation can be achieved in a more general sense by, for instance, targeting complement activation, phagocytosis, and cytokine secretion, without necessarily or purposefully requiring a specific response to a defined antigen. Examples include zymosan, glucans, peptidoglycans and lipopolysaccharides and these are best described as true immunostimulants . Thus immunostimulants, in shellfish aquaculture refers to any substance that is used with an intent to boost immune reactivity and improve resistance to, or survival following infection by harmful micro-organisms.

However, as in other arthropods, crustaceans have a non-adaptive (innate) immune system, which means that there is little logic in trying to immunize these animals whereas it is possible to enhance the immune capacity for a limited period of time by vaccination / immunostimulation. Attempts have been made to vaccinate shrimps and lobsters. *Adams* (1991) reported vaccination of shrimp by exposure to heat killed preparations of pathogens.

It has been reported that treatment of *P. monodon* with β-1,3-glucan (*Kenkyu*, 1994), killed vibrios (*Timmons et al.*, 2001) significantly enhanced resistance to infection by vibrios. It shows that treatment with dead vibrios and β-1,3-glucan is more effective in the protection against vibriosis than treatment with dead Vibrios alone. *Keith et al.* reported that vaccination against gaffkemia infection in lobsters was effective with inactivated bacteria. But all these responses are short-lived and usually last for a few hours or a day or so. It was observed that treatment with β-1,3-glucan induced a higher percentage of haemocytes with superoxide anions than with other immunostimulants in *P. monodon* (*Song and Hsieh*, 1994). *Chaves and Sequeira* (2000) observed a secondary immune response in *P.japonicus* which can be fit into the designation of immune memory stated by *Hildemann.* (1984). It remains unclear whether such results are due to the existence of an adaptive immune response in invertebrates homologous to that observed in vertebrates or to a distinct type of immunoprotective pathway.

1.4 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 fro the production of seedstock, 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 are 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 of a sustainable shrimp culture sector. Disease control depends on a complex of three factors; diagnosis, treatment

and preventive measures (*Sindermann and Lightner*, 1988). Treament 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 defense 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 defense 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 probiotic bacteria, a 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 microbiota", which includes photosynthetic bacteria, *Lactobacillus*, *Nitrobacteria*, Denitrifying bacteria, *Bifidobacteruim*, Yeast etc.

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. No reports could be obtained on the use of actinomycetes as immunostimulants and probiotics in shrimp aquaculture.

1.5 Marine Microbes as Source of Bioactive Compounds

Although the diversity of life in the terrestrial environment is extraordinary the greatest biodiversity is in the oceans (*Donia and Hamann*, 2003). More than 70 % of our planets surface is covered by oceans and it harbours more than 80 percent of all life on earth. The ocean cover contain over 2,00,000 invertebrate and algal species. These organisms live in complex communities and in close association with other organisms both macro (algae, sponges) and micro (non filamentous bacteria , fungi and actinomycetes) organisms. Marine organisms are capable of surviving and growing in habitats of extremes. In addition to high salinity , many of them have to face high hydrostatic pressure and low or high temperature. In order to survive and grow in a highly competitive habitat , many organisms must compete for the limited resources. A variety of offensive and defensive mechanisms have evolved to allow organisms to gain selective advantage and to cope with competitors. The physiological manifestation of these defence abilities of marine organisms is in the form of bioactive metabolites. The biological diversity of marine environment thus offers

enormous scope for the discovery of novel natural products several of which are potential targets for biomedical development.

Marine micro-organisms has been recognized as a new source for the production of bioactive secondary metabolites. The large number and diversity of marine bacteria suggest that this resource will be of significant importance in the discovery of new drugs. Of all the marine forms, the actinomycetes merit special consideration in view of the proven biosynthetic capabilities of numerous isolates from soil. They are considered to be the most economically and biotechnologically valuable prokaryotes.

Actinomycetes are a group of gram-positive bacteria that tend to form branching filaments, which in some families develop a mycelium. They produce aerial mycelia or substrate mycelia or both. But these structures may break into rods or cocci, giving bacterial appearance. The diameter of the filaments vary from 0.5-2μm. Filaments are not always observed because some families as stated above, tend to fragment and leads to the formation of coccoid, elongate or diphtheroid elements. In some families true spores are formed on aerial or substrate hyphae. The spores vary greatly in shape and are produced at the tip of filaments. Spore formation occurs in response to nutrient depletion. Actinomycetes are widely distributed in nature and found in a variety of habitats. They are temperature tolerant and can withstand desiccation.

Actinomycetes are responsible for the production of about half of the discovered bioactive secondary metabolites (*Berdy*, 2005), notably antibiotics (*Strohl*, 2004), antitumor agents (*Cragg et al.*), immunosuppressive agents (*Mann*, 2001) and enzymes (*Oldfield et al.* (1998) & ?). About 75 % of worlds antibiotics are derived from actinomycetes. Because of the excellent track record of actinomycetes in this regard, a significant amount of

effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds 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 habitats be pursued as sources of novel bioactive secondary metabolites. As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characterstics from those of terrestrial counterparts. It is likely that this is reflected in the genetic and metabolic diversity of marine actinomycetes, which remains largely unknown. Indeed, the marine environment is a virtually untapped source of actinomycete diversity and therefore, of new metabolites (*Lam*, 2006).

The present work is aimed at the utilization of marine actinomycetes as a source of antiviral compounds and as probiotics for application in penaeid prawn growout systems. A detailed study of the immunological and antioxidant responses of *Penaeus monodon* administered with actinomycte diets is also dealt with. The present study was undertaken with the following objectives.

1 Isolation of marine actinomycetes and screening for antiviral activity against white spot virus in *Penaeus monodon*.

2 Screening of marine actinomycetes for hydrolytic and antagonistic property and segregation of potent strains as putative probionts for prawn culture systems.

3 Testing selected probionts for immunostimulant property in *Penaeus monodon* culture systems.

4 Testing selected probionts for antioxidant property in *Penaeus monodon*.

The thesis comprises of 6 Chapters. In Chapter I, a general introduction on the various aspects of penaeid shrimp aquaculture, its present status and various constraints confronted by the industry is dealt with. A focus on the defence mechanisms of crustaceans, and the probable measures undertaken to combat diseases and the relevance of marine actinomycetes as source of bioactive compounds is also discussed. In Chapter 2, a study on the efficacy of marine actinomycetes as source of antiviral compounds against WSSV in *P.monodon* is done . Chapter 3 deals with testing the efficacy of marine actinomycetes as probiotics in *P.monodon* . Chapter 4, gives a detailed account on the immunological responses of marine actinomycete fed *P.monodon* to white spot viral disease. In Chapter 5, a detailed account on the antioxidant responses of marine actinomycete fed *P.monodon* to white spot viral disease is carried out, followed by summary and conclusion in Chapter 6.

CHAPTER 2

Screening of Marine Actinomycetes for Antiviral Activity Against White Spot Viral Diseases in *Penaeus monodon*

2.1 Introduction

Shrimp aquaculture has grown into a major commercial venture in the tropics , particularly in the Asian subcontinent. The intensification of shrimp farming over the last few decades has been accompanied by development of infectious diseases of viral, bacterial and in some cases, fungal origin (*Destomieux et al.*, 2000). Approximately about 20 viruses has been reported in shrimp culture. Among the various viruses of the penaeid shrimp, the white spot syndrome virus (WSSV) is responsible for a major proportion of the diseases plaguing commercial shrimp ponds, and has resulted in high mortality and economic losses (*Lightner* (1996), *Flegel* (1997)). In cultured shrimp, WSSV infection can cause a

cumulative mortality upto 100 % within 3 to 10 days (*Lightner*, 1996). The first reported epidemic due to this virus is from Taiwan in 1992 (*Chen*, 1995) , followed by outbreaks in Japan and Korea in the same year, Thailand , India and Malaysia in 1994 and by 1996 it had severely affected East Asia and South Asia (*Cai et al.* (1995), *Flegel* (1997)). WSSV was reported from the United States in 1995 (*Kosower and Kosower*, 1978), and from Central and South America since early 1999 (*Rosenberry*, 2001). In India, WSSV infection was first reported from the Kandaleeru creek-fed shrimp farms in Andhra Pradesh during 1994 and was subsequently reported from ponds located all along the Indian coasts.

White spot syndrome virus (WSSV) is an extremely virulent pathogen of penaeid shrimp and is a large dsDNA virus belonging to the virus family Nimaviridae, genus Whispovirus (*Mayo.*, 2002). *Wongteerasupaya et al.* (1995) , first described the TEM morphology of WSSV by negative staining to reveal tail like appendages. WSSV virions are enveloped , have a bacilliform to ovoid shape, are about 275 nm in length by 120 nm in width and have a tail-like appendage at one end (*Rajan et al.*, 2000). The nucle-ocapsid have striations perpendicular to the rod shaped long axis of about 300 x 70 nm (*Wongteerasupaya et al.*, 1995). It has a broad host range including marine and freshwater crustaceans such as crabs, crayfish, insects and lobsters (*Lo et al.*, 1996) and are reported to get infected with variable severities depending on the life stage of the host and presence of external stressors (temperature, salinity, bacterial diseases, pollutants etc). Clinical signs of WSSV include a sudden reduction in food consumption, lethargy, loose cuticle and often reddish discolouration and the presence of white spots of 0.5 to 2.0 nm in diameter on the inside of the carapace , appendages and cuticle over the abdominal segments. Chemical composition of the spots is similar to the carapace , calcium forming 80-90% of the total material and is suggested to have derived from abnormalities of the cuticular epidermis. (*Wang et al.*, 1997). The complete DNA sequence of WSSV genome has been assembled

into circular sequence of 292,967 base pairs (*Hulten et al.*, 2001).

Transmission of the virus is mainly through oral ingestion and water borne routes in farms (horizontal transmission) and from infected mother prawns (vertical transmission) in case of shrimp hatcheries. Rapid and specific diagnosis of the virus is presently carried out using two step-nested polymerase chain reaction (*Kim et al.*, 1989) and in situ hybridisation assay (*Wongteerasupaya et al.* (1996), *Chang et al.* (1998)). Histopathological changes include prominent intracellular eosinophilic to basophilic inclusions in the infected cells and cellular degeneration with hypertrophied nuclei and chromatin margination in the cuticular epidermis, gill epithelium, antennal gland, haematopoetic tissue, nervous tissue and connective tissue and cellular necrosis and detachment of intestinal epithelial tissue (*Wongteerasupaya et al.*, 1995).

Numerous works are being undertaken all over the world to control the spread of the disease. Strategies for prophylaxis and control of WSSV, theoretically include improvement of environmental conditions, stocking of specific pathogen free shrimp post-larvae and enhancement of disease resistance by using immunostimulants (*Citarasu et al.*, 2006).

2.2 Measures Undertaken to Enhance Disease Resistance Against WSSV Infection in Penaeid Shrimps

The use of aquatic plants and animals for biomedical research and the potential of microorganisms as sources of pharmaceuticals have opened new vistas to the whole scenario of aquaculture activities. Recent studies revealed that the extracts (containing polysaccharide fraction) from several kinds of seaweeds had an impressive ability to improve the immune

status or disease resistance of cultured animals. Fucoidan from *Cladosiphon okamranus* inhibited white spot virus (WSSV) in the shrimp *M.japonicus* (Takahashi et al., 1998). Oral administration of crude fuciodan extracted from *Sargassum polycystum* reduced the impact of WSSV infection in *P.monodon* (*Chotigeat et al.*, 2004).

The mechanism of action of the seaweed polysaccharides to inhibit pathogens or improve immunity is unclear. However, Witvrouw and De Clercq proposes that they may act by preventing viral attachment on host cell. Oral administration of *Sargassum fusiforme* polysaccharide extracts had enhanced disesase resistance and immune status in *Fenneropenaeus chinensis* (*Huang et al.*, 2006). Methanolic herbal extracts from *Cyanodon dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa* and *Eclipta alba* have proved to be successful against WSSV in *P.monodon* (*Citarasu et al.*, 2006).Plant extract from *Lantana camera, Cyanodon dactylon, Aegle marmelos, Ocimum sanctum, Mimosa pudica, Circuma longa* and *Allium sativum* has proved to have prophylactic and therapeutic properties against WSSV in penaeid shrimps (*Flegel*, 1997) . The disease enhancing property of various microbial cell wall components like glucans, peptidoglycans, lipopolysaccharides and other polysaccharides have been widely studied in fish and crustaceans. Oral administration of peptidoglycan derived from *Bifidobacterium thermophilum* could enhance disease resistance against WSSV in *P.japonicus.* (*Itami et al.*, 1998). Oral administration of β-1,3 glucan from *Schizophyllum commune*, effectively improved the survival of WSSV-infected *P.monodon.* (*Chang et al.*, 2003).

The potential to vaccinate *P. monodon* shrimp against WSSV using WSSV envelope proteins VP19 and VP28 has been evaluated and the results show that some sort of protection can be imparted despite the absence of a true adaptive immune system in shrimps (*Witteveldt et al.*, 2003). It has been demonstrated that crustaceans produce virus-inhibiting

proteins and that some genes are upregulated upon viral infection (*Pan et al.* (2000), *Rojtinnakorn et al.* (2002)). Recently, RNA interference (RNAi), a sequence-specific down regulation of RNA has been used as an alternative to vaccination. RNA interference has been described in a wide range of eukaryotic organisms including invertebrates ((*Fire et al.*, 1998) & (*Valdes et al.*, 2003)). RNAi is triggered by dsRNAs, which are processed into shorter 21-25 bp small interfering RNAs (siRNAs) . The siRNAs are incorporated into the RNA-induced silencing complex which facilitates the binding of the siRNAs to the homologous mRNAs upon which the mRNA will be degraded. RNAi has been shown to be effective against several virus infection (*Wang et al.* (2003), *Tan and Yin* (2004)). Injection of the vp28 and vp15 siRNAs into *P. monodon* gave a significant reduction in mortality upon WSSV infection (*Westenberg et al.*) . However, for practical applications , more advanced delivery systems need to be developed such as the edible dsRNA producing bacteria used for RNAi applications in *C. elegans* (*Timmons et al.*, 2001). A DNA vaccination strategy using four recombinant constructs encoding WSSV structural proteins had proved to impart protection upon WSSV challenge. These reports suggest that contrary to the previous belief that invertebrates rely entirely on innate immune system, some aspects of specific immunity like, inducibility, appear to be present in some cases. However, the protection conferred by these recombinant protein vaccines has been found to be short-lived. Cidofovir, an antiviral drug (acyclic nucleoside phosponate) has been effective against human DNA viruses. In shrimp, *Litopenaeus vannamei*, cidofovir induced a significant delay in mortality in WSSV infected shrimp. This study opens perspectives for antiviral drugs to treat shrimp infected with WSSV (*Rahman et al.*, 2006).

Against this background, the efficacy of marine actinomycetes in enhancing disease resistance against white spot viral disease in *P.monodon* was tested. Actinomycetes are extensively distributed in soil and provide many important bioactive compounds of high

commercial value many of which are pharmaceutically important (*Takizawa et al.*, 1993). They can also be isolated from marine sediments, sea water, marine plants and animals. About half of the discovered bioactive secondary metabolites (*Berdy*, 2005), notably antibiotics (*Berdy* (2005) & *Strohl* (2004)) , antitumor agents (*Itami et al.*, 1989),immuno-suppressive agents and enzymes (*Oldfield et al.* (1998) & ?) have been derived from actinomycetes. This fact is not surprising when taking into consideration the ubiquitous nature of this bacteria and the prolific activity of its species in the production of secondary metabolites.

Pentalactones isolated from the fermentation broth of *Streptomyces* sp. M-2718 has been reported to be active against several DNA viruses (*Nakagawa et al.*, 1985). The antiviral activities of pentalactones and pyrrole-2-carboxylic acid against herpes simplex virus had already been assayed and described (*Laren et al.*, 1983). *Yokomizo et al.* (1999) have reported that Fattiviracin, an antiviral agent from *Streptomyces microflavus*, could inhibit herpes simplex type I and human immunodeficiency virus type I. Researchers have reported that guanine-7-N-oxide produced by *Streptococcus* sp. was found to inhibit *in vitro* replication of the fish herpes virus (Onchorhynchus Masou Virus), rhabdovirus (Infectious Hematopoitic virus) and a fish virus (Infectious Pancreatic necrosis Virus) (*Hasobe et al.*, 1985).

Because of the excellent track record of actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds 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 habitats be pursued as sources of novel bioactive

secondary metabolites. The exploitation of marine actinomycetes as a source for secondary metabolites is in its infancy. Even with the limited screening efforts that have been dedicated to date to marine actinomycetes, the discovery rate of novel secondary metabolites has recently surpassed that of their terrestrial counterparts, as evident by the isolation of many new chemical entities from marine actinomycetes (*Jensen et al.* (2005), *Fiedler et al.* (2005), *Blunt et al.* (2004), *Blunt et al.* (2005) & *Blunt et al.* (2006)).

2.3 Metabolites Produced by Marine Actinomycetes

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 (2.1) shows some examples of novel secondary metabolites isolated during the period 2003 to 2005 covering many different diverse structures with biological activities. In this respect , future success relies on our ability to isolate novel actinomycetes from marine environments. Recent investigations using enrichment techniques, new selective methods and media have led to the isolation of novel actinmoycetes from sediment samples. Further development work in improving isolation strategies in the recovery of marine actinomycetes is of utmost importance for ensuring success in this area. This work is targeted at harnessing the potential of marine actinomycetes as a source of bioactive compounds to be specifically employed towards prawn pathogens, particularly against white spot virus.

Compound	Source	Activity
Abyssomicins	*Verrucosispora* sp.	Antibacterial
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer
Bonactin Bonactin	*Streptomyces* sp.	Antibacterial;Antifungal
Caprolactones	*Streptomyces* sp.	Anticancer
Chandrananimycin	*Actinomadura* sp.	Antialgal;Antibacterial;Anticancer;Antifungal
Chinikomycins	*Streptomyces* sp.	Anticancer
Chloro-dihydroquinones	*Novel Actinomycete*	Antibacterial;Anticancer
Diazepinomicin (ECO-4601)	*Micromonospora* sp.	Antibacterial;Anticancer;Antiinflammatory
3,6 disubstituted indoles	*Streptomyces* sp.	Anticancer
Frigocyclinone	*Streptomyces griseus*	Antibacterial
Glaciapyrroles	*Streptomyces* sp.	Antibacterial
Gutingimycin	*Streptomyces* sp.	Antibacterial
Helquinoline	*Janibacter limosus*	Antibacterial
Himalomycins	*Streptomyces* sp.	Antibacterial
IB-00208	*Actinomadura* sp.	Anticancer
Komodoquinone A	*Streptomyces* sp.	Neuritogenic activity
Lajollamycin	*Streptomyces nodosus*	Antibacterial
Marinomycins	*Marinispora*	Anticancer;Antibacterial ;Anticancer
Mecherchamycins	*Thermoactinomyces* sp.	Anticancer
MKN-349A	*Nocardiopsis* sp.	Unknown Biological Activity
Salinosporamide A (NPI-0052)	*Salinospora tropica*	Anticancer
Sporolides	*Salinospora tropica*	Unknown Biological Activity
Trioxacarcins	*Streptomyces* sp.	Antibacterial; Anticancer,Antimalarial

Table 2.1: Novel metabolites produced by marine actinomycetes (*Lam*, 2006)

2.4 Materials and Methods

2.4.1 Isolation of Actinomycetes from Sediment Samples

Actinomycetes (77 Nos.) were isolated from sediment samples collected from the West coast of India. The sediment samples were pre-treated for selective isolation of actinomycetes. Samples were subjected to heat treatment at 50-60°C for one hour. 1g of the sediment was mixed with 0.1 g $CaCO_3$ and incubated at 26°C for a week in humidity controlled environmental chamber. The pre-treated samples were serially diluted, vortexed and plated onto Actinomycete Isolation Agar (Himedia) supplemented with anti-fungal agent Bavistin (BASF India Limited, Bombay) (13.75 mg/ 100 ml) and antibacterial compound Novobiocin (Himedia) (2.5 mg/ 100 ml). Colonies with characteristic appearance of actinomycetes were isolated into Marine Actinomycete Growth medium. Twenty-two actinomycete strains isolated from sediment samples collected from the South West coast of India and maintained in the Microbiology Laboratory of School of Marine Sciences were also used for the present study.

2.4.2 Screening for Antiviral Compounds from Marine Actinomycetes

2.4.2.1 Primary Screening

Preparation of Culture Broth

Actinomycetes isolates (99 Nos) were inoculated into Marine Actinomycetes Growth medium (Table.2.2) (100 ml) and incubated for 10 days at room temperature. The culture broth was concentrated (100 ml to 5 ml) using vacuum evaporator (Thermo Savant, USA).

Starch	-	10g
Yeast Extract	-	4g
Peptone	-	2g
Agar	-	20g
Sea Water(15 ppt)	-	1 L
pH	-	7

Table 2.2: Composition of Marine Actinomycetes Growth Medium (MAG)

Preparation of medicated diet

A commercial diet (Higashimaru) was used as the basal diet in the study. Concentrated actinomycete broth (5 ml, 99 Nos) were incorporated into the diet (20 g) separately. Incorporation was done using binder (Bindex gel) and dried at room temperature (28 ± 2°C). Thus 99 feeds were prepared along with the control diets. Two control diets included feed incorporated with the medium (CL1) and normal diet (CL2) were used for this study.

Feeding Experiments

Experimental Animals

Penaeus monodon post larvae (PL-30) of the size range 0.025- 0.03g were used for the experiment (*Fig.2.1*). The larvae were brought from Matsyafed hatchery (Ponnani, Kerala) and was acclimatized to laboratory conditions. They were PCR screened and found to be negative for WSSV. These larvae were maintained on control diets for a period of one week.

Experimental design

Twenty five animals were stocked in Fibre Reinforced Plastic (FRP) tanks of 30L capacity containing 20L seawater (*Fig.2.2*).Feeding experiments were done in triplicate for each actinomycete isolate (99 Nos) incorporated diet along with the two control diets for a period of 15 days. The prawns were fed twice daily, morning 10 A.M and evening 8 P.M, at a rate of 10-15% of their body weight. The faecal matter and left over feed were removed daily

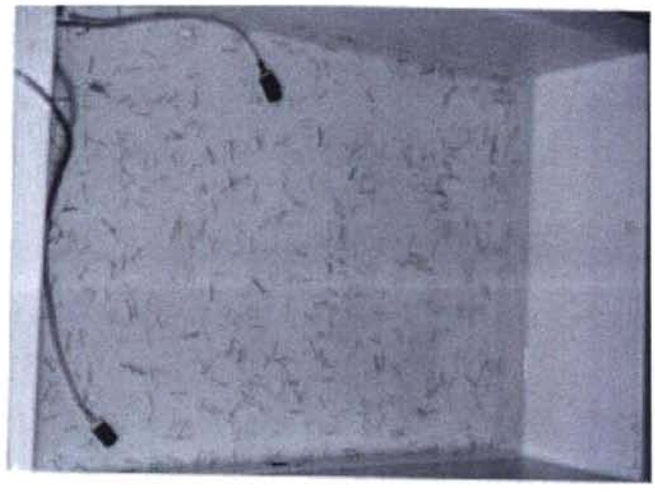


Figure 2.1: *P.monodon* post larve for testing antiviral property of marine actinomycetes against WSSV infection

by siphoning. About 30-40% water exchange was done daily morning. Physico-chemical parameters of the rearing water were monitored regularly and salinity, NH_3-N, NO_2-N, NO_3-N and dissolved oxygen were estimated as per APHA (1995) and maintained at optimal level by the water exchange (Table2.3). Continuous aeration was provided from 1 HP compressor.

Challenging with White Spot Syndrome Virus.

After 15 days of feeding experiment the animals were challenged with White Spot Syndrome virus (WSSV) via oral administration of white spot virus infected prawn flesh . The animals were starved for 12 hrs before the challenge to ensure the intake of infected prawn flesh and the animals were then maintained on test feeds. Infection with WSSV was confirmed by examining the carapace of dead larvae for white spots on it. Post challenge survival was recorded for a period of seven days.

Figure 2.2: Bioassay system used for feeding experiment with *P.monodon* post larve for testing antiviral property of marine actinomycetes

2.4.2.2 Secondary Screening

Sixteen actinomycete isolates, which supported a survival rate of 40% or above after challenge with WSSV (A2, L8, A8, L10, L27, L33, L35, L39, L45, L56, L83, L84, L85, L102, L117, B451) were selected for secondary screening. The same methodology as used in the primary screening was followed for secondary screening. Post challenge survival results were noted for a period of seven days.

2.4.2.3 Screening of Actinomycete Biomass and Culture Supernatant Separately for Antiviral Property

Microorganisms Used

Seven actinomycete isolates, which supported a survival rate of 40% or above after challenge with WSSV (L10, L27, L33, L35, L45, L56, B451) were selected for further study. In order to find out whether the bioactive principle is in the biomass or get released into the

Initial Weight	-	0.025g
Stocking Density	-	25 PL/tank
Tank Capacity	-	20 L
Feeding Level	-	10-15% body wt.
Feeding Freequency	-	Twice Daily
Feeding Period	-	15 days
Water Temperature	-	24-27°C days
Feeding Period	-	15 days
pH	-	7.5 - 8 days
Salinity	-	15 - 20 ppt
NH_3	-	0.01 - 0.02 mg/L
NO_3	-	Below Detectable Level
NO_2	-	0 - 0.01 mg/L
Dissolved Oxygen	-	6-7 mg/L

Table 2.3: Rearing Conditions and Water Quality

culture broth (supernatant) both the biomass and supernatant of the selected seven isolates were screened separately.

2.4.2.4 Identification of the selected seven actinomycetes using 16S rDNA sequencing

DNA Isolation from Actinomycetes

Actinomycete cultures (I.5 ml) at log phase grown in Lauria Bertani (LB) medium (Bacto tryptone, 1 g; Bacto yeast,0.5 g; NaCl ,1.5 g; Deionized water,100 mL) was harvested at 10000 rpm for 5 min.The cell pellet thus obtained was resuspended in 500 μl of TEN buffer (1000mM Tris HCL;10mM EDTA and 250mM NaCl) and centrifuged at 10000 rpm for 5 min. The cell suspension was again resuspended in 500 ul TE buffer (10mM Tris HCl -pH 7.5 ;1mM EDTA), added 50 μl 20% SDS and 20μl of proteinase K (20mg/ml) and incubated at 370 °C in a water bath for overnight. Then extracted with 500 μl of Tris equilibrated phenol and centrifuged at 10000 rpm for 15 min.The top layer was transferred to a

new tube avoiding interface and the process was repeated two times. It was then extracted with equal volume of chloroform isoamyl alcohol (24:1) and centrifuged at 10000 rpm for 10 min. After transferring the aqueous layer to a new vial 0.1 volume of 3M Sodium acetate (pH 5.2) was added ,mixed gently and precipitated with 0.6 volume of isopropanol at -20 °C overnight. DNA pellets were washed twice with 70 % ethanol and once with absolute ethanol ,then suspended in sterile TE and stored at -20 °C.

Determination of the quality of DNA

Quality and quantity of isolated DNA was checked by measuring optical density in a UV spectrophotometer and visualizing DNA using gel electrophoresis .The ratio of absorbance at 260 nm and 280 nm is an indication of the DNA quality. The ratio ranges from 1.6 to 1.8 for pure DNA .For quantification of DNA the OD at 260 nm was taken and the concentration of DNA was calculated as follows.

1 OD of Double stranded DNA = 50 μg/ml

DNA concentration (μg/ml) = OD x Dilution factor x 50

Gel Electrophoresis

Extracted DNA was diluted to a concentration of 100ng/μl.0.8 % agarose gel was prepared in 1 X TBE (Tris base, 10.8 g; 0.5 M EDTA, 4ml; Boric acid, 5.5g; Double Distilled water, 100ml; pH-8) Ethidium bromide (0.2mg/ml stock stored in dark) was added to the melted agarose to a final concentration of 0.2 μg/ml. After cooling to about 45°C, the agarose was poured on to gel tray and was allowed to solidify. The gel tray was transferred in to a buffer tank and was submerged in 1 X TBE buffer. Appropriate quantity of DNA in a volume of 2-3 μl was mixed with 5 μl of of 6 X loading dye (Bromophenol Blue,0.125g; Xylene Cyanol, 0.125g; Glycerol,15ml; Double Distilled water 50ml) and loaded into the well .Electrophoresis was done at a voltage of 3- 4 volt/cm till the bromophenol blue dye

front migrated to the middle of the gel.The gel was visualized on a UV transilluminator (Syngene, Imagen Technologies,USA)

PCR Amplification of 16S rDNA

After checking the quality and quantity of DNA ,appropriate dilutions were made to make up the concentration to 100ng per microlitre.The 16S rDNA of the actinomycetes was amplified individually using a set of primers complementary to the conserved regions of both 5' and 3' ends of 16S rRNA gene (1500 bp). The following forward and reverse primers were used (Table2.4) (*Reddy et al.*, 2000)

Primer F (16S1) 5'- GAGTTTGATCCTGGCTCAG-3'

Primer R (16S2) 5'- ACGGCTACCTTGTTACGACTT- 3'

Sl.No.	Item	Amount (μl)
1	10 X PCR Buffer	2.5
2	250mM $MgCl_2$	1.5
3	2.5 mM dNTP	2
4	10pmol Primer F	1
5	10pmol Primer R	1
6	Template DNA 100ng/μl	1
7	Autoclaved MilliQ Water	15
8	Taq Polymerase U/μl	1
	Total	25

Table 2.4: PCR Reaction Mixture

The amplification was done according to the following protocol. Initial denaturation was carried out at 95°C for 5 min.The samples were placed in ice and 1 μl Taq polymerase was added.This was followed by 35 cycles of denaturation at 94°C for 20 Seconds,annealing at 60°C for 30 Seconds,extension at 68°C for 2 min and then a final extension step at 68°C for 10 min.The PCR products were observed on 1 % agarose gel . The PCR products

were cleaned using Wizard SV gel PCR clean up System (Promega) and directly used for sequencing.

DNA Sequencing

The purified 1.5 kb DNA product was sequenced using primers 16S1 and 16S2. Sequencing was done by Sangar dideoxy chain teminator sequencing (automated fluorescent DNA sequencing) using ABI Prism model 3700 Big Dye Sequencer (Applied Biosystems ,USA) at Microsynth AG, Switzerland. The nucleotide sequences obtained were assembled using Autoassembler (ABI Prism,USA) software and were aligned to find regions of similarity between sequences in the GenBank database through BLAST (Basic Local Alignment Search Tool) search at National Centre for Biotechnology Information web site (NCBI) USA. (http://www.ncbi.nlm.nih.gov/). Based on the percentage similarity with the GenBank the sequences, the isolates were identified.

Preparation of Actinomycete Biomass and Supernatant

Actinomycetes (7 nos- L10, L27, L33, L35, L45, L56 and B451) (*Fig.2.4, Fig.2.6, Fig.2.5*) were used for the study. The cultures were inoculated into marine actinomycete growth medium and incubated at (28 ± 2°C for ten days. Culture broths were centrifuged at 10,000 rpm for 15 minutes at 4°C in a cooling centrifuge (Remi C-30). Biomass and the supernatants were collected separately. Biomass was kept in a deep freezer at -20°C until used. The supernatants were concentrated in a vacuum evaporator and kept at -20°C until used (*Fig.(2.3)*).

Preparation of Medicated Diet

Both the biomass and concentrated supernatants of the seven actinomycetes were separately incorporated into a commercial diet (Higashimaru). The same methodology as used for the primary screening was followed here also. Two control diets included a feed

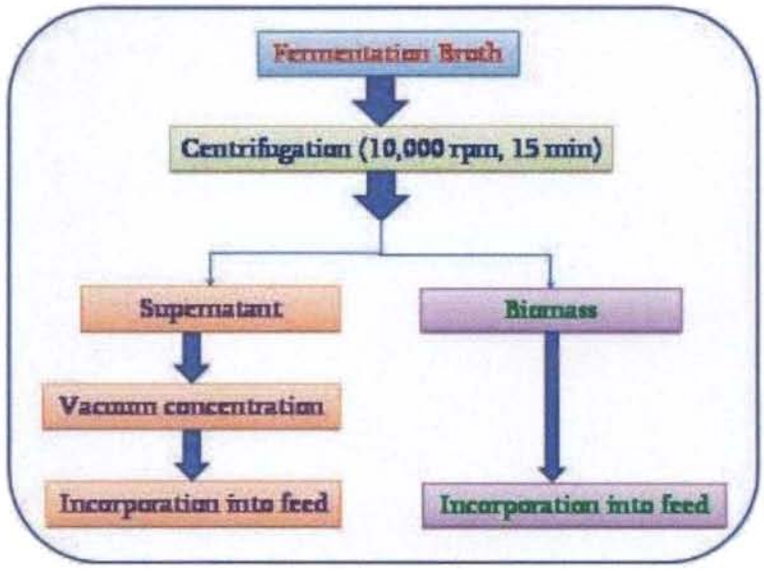


Figure 2.3: Flow diagram showing Preparation of Biomass and Supernatant

Figure 2.4: Culture broths of the selected seven actinomycete isolates

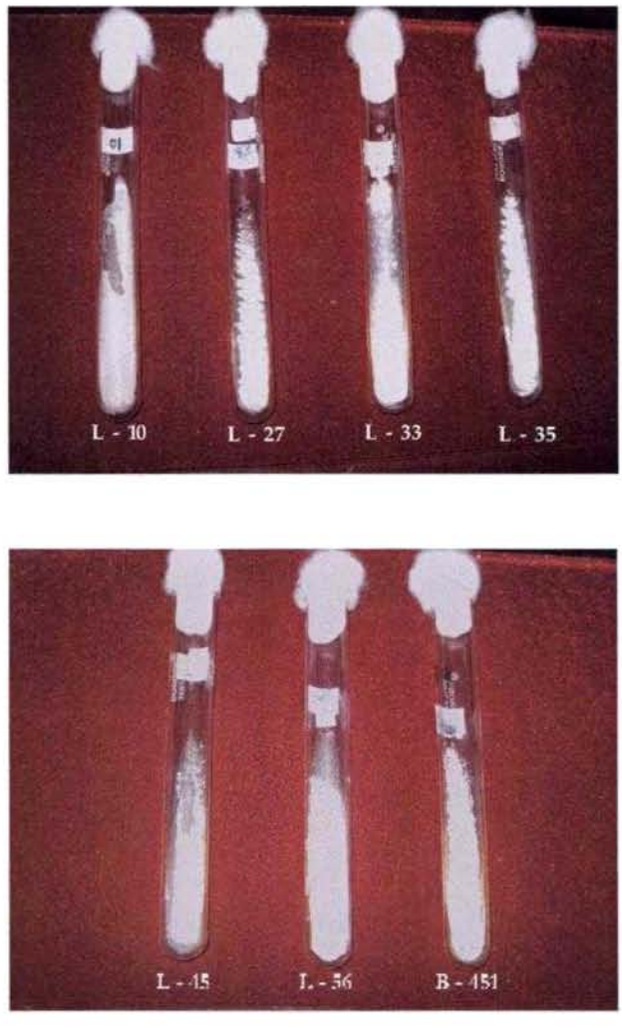


Figure 2.5: Slant culture of the selected seven strains

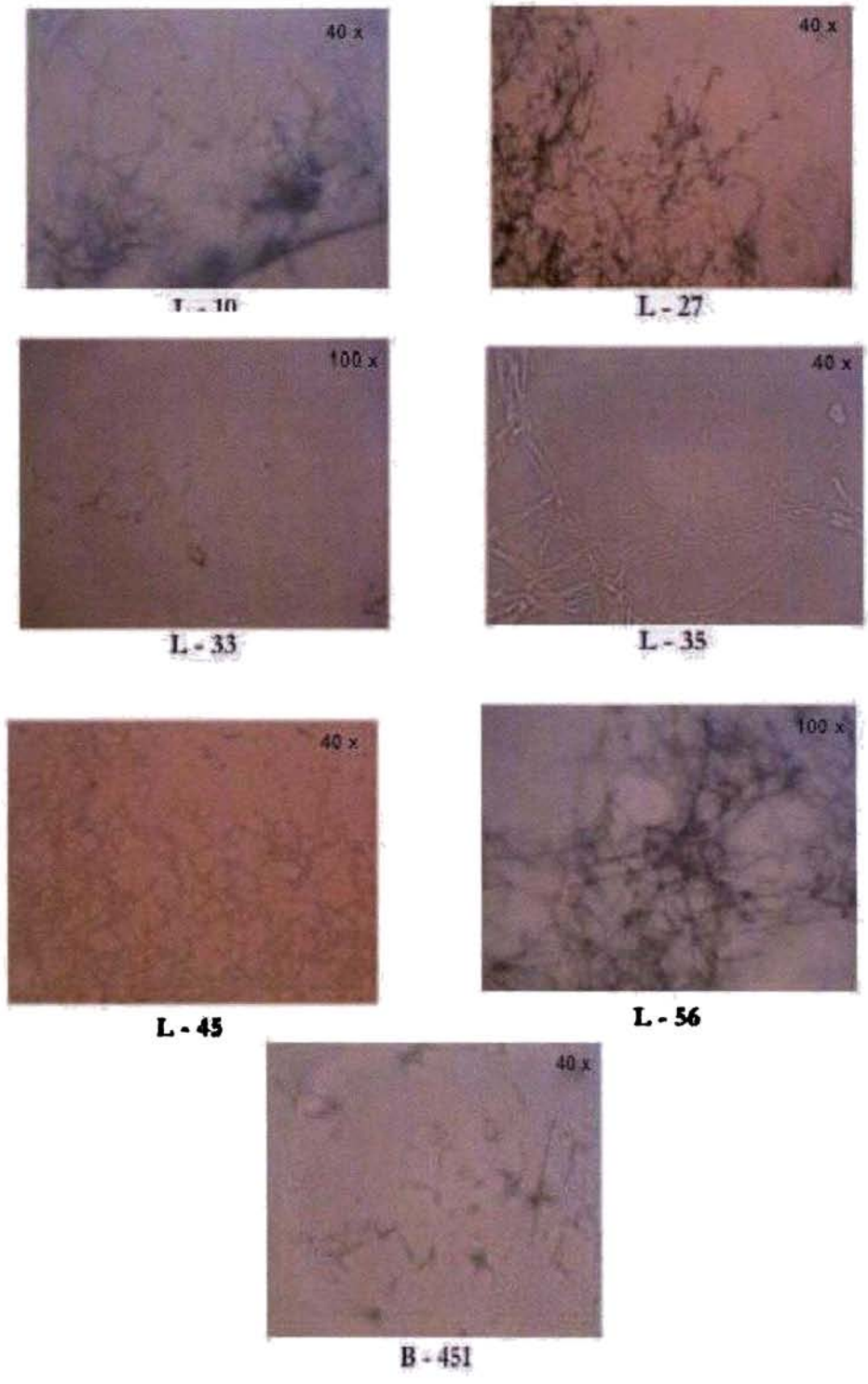


Figure 2.6: Slide culture of the selected seven strains

incorporated with the medium (CL1) and a normal feed (CL2) were used for this study . Post challenge survival results were noted for a period of seven days.

Experimental Setup

Penaeus monodon post larvae (PL 30) of the size range 30-40 mg were used for the study. The animals were brought from a hatchery at Kannamali, Cochin. They were PCR screened and found to be negative for WSSV. The experiment was performed as in the case of primary or secondary screening. After 15 days feeding the animals were challenged with WSSV and the post challenge survival was noted for a period of 7 days.

2.4.2.5 Screening of Methanol Extract of Actinomycete Culture Supernatant

1 Virus Neutralization Test

2 Administration of Medicated Diet (Methanol Extract)

Preparation of Culture Broth

14 day old culture broths (1 L) of the selected seven isolates (L10, L27, L33, L35, L45, L56 and B451) were prepared and biomass was separated by centrifuging at 10, 000 rpm for 15 min at 4°C in a cooling centrifuge (Remi C-30, Mumbai).

Preparation of Methanol Extract

A glass chromatography column of 45 × 3 cm (l × dia) size with a stopcock at the bottom was used. Amberlite (XAD -16) was mixed with water and then packed into the column upto 20 cm height. The culture supernatant (1L) was passed through the column at a rate of 5-ml/min. The bioactive principle was eluted using methanol (100 ml). This methanol extract was concentrated in a vacuum evaporator. The residue was dissolved in 5 ml distilled water.

Preparation of Viral Suspension

Gill tissue (300 mg) was taken from an infected *P. monodon* and mascerated in 10 ml cold PBS (NaCl, 8g ; KCl, 0.2g; NA_2HPO_4, 1.15g; KH_2PO_4, 0.2g; DDW, 1000 ml) with glass wool to a homogenous mass using mortar and pestle in an ice bath. The homogenate was centrifuged at 10,000 rpm in a refrigerated centrifuge for 20 minutes (REMI C 24) at 4°C and the supernatant fluid was made bacteria free by passing it through 0.22μm pore size membrane filter. The filtrate was used as the viral suspension for the experiment.

1. Virus Neutralization Test

Equal quantities of viral suspension and methanol extract concentrates from various actinomycetes (7 nos.- L10, L27, L33, L35, L45, L56 and B451) were mixed and kept for a period of 3 hrs. Virus suspension was incubated with PBS (phosphate buffered saline) for the same period as a control.

Experimental Animals

Penaeus monodon adults of the size range 13-15 g collected from a private farm at Cherthala were used for the study. They were PCR screened for WSSV. The animals were acclimatized to laboratory conditions for a week.

Experimental Setup

The animals were transferred ten each into rectangular fibre glass tanks of 30L capacity. 50 % water exchange was done daily and the water quality parameters were maintained as given in Table I. Animals were maintained on a commercial diet (Higashimaru, Kochi). After one week of maintenance the animals were challenged with the various treatments.

Toxicity Test

For testing the toxicity of the methanol extract of the actinomycetes culture supernatant,

an aliquot (20μl each) was injected into *P.monodon* adults (10 nos.) and maintained on commercial diet. The various treatments are given Table (2.5). Animals were maintained on the commercial diet and the post challenge survival was monitored for a period of seven days.

Serial No.	Code Used	Treatment Groups
1	$L10P_0$	(Methanol extract concentrate of L10 + PBS at 0 hr)
2	$L27P_0$	(Methanol extract concentrate of L27 + PBS at 0 hr)
3	$L33P_0$	(Methanol extract concentrate of L33 + PBS at 0 hr)
4	$L35P_0$	(Methanol extract concentrate of L35 + PBS at 0 hr)
5	$L45P_0$	(Methanol extract concentrate of L45 + PBS at 0 hr)
6	$L56P_0$	(Methanol extract concentrate of L56 + PBS at 0 hr)
7	$B451P_0$	(Methanol extract concentrate of B451 + PBS at 0 hr)

Table 2.5: Methanol extract preparations used for Toxicity test

Infectivity Test

Viral suspension after neutralisation with various methanol extract (seven acinomycetes) were injected (20μl each) into *P.monodon* adults (10 nos.) and maintained on commercial diet. A possitive control injected with viral suspension and PBS was also maintained. In order to test the effect of 3 hour incubation on virus survival, the viral suspension with PBS after three hour incubation was also injected into a set of animals (10 nos.). The various treatments are given in Table (2.6).The post challenge survival was monitored for a period of seven days.

2. Administration of Medicated Diet (Methanol Extract)

Experimental Animals

Penaeus monodon post larvae (PL-30) of the size range 0.015- 0.03g were used for the

Serial No.	Code Used	Treatment Groups
1	VP_0	(Viral suspension+ PBS at 0 hr)
2	$V_3 3P_3$	(Viral suspesion + PBS at 3 hr)
3	$L10V_3$	(Methanol extract concentrate of L10 + Viral suspension at 3 hr)
i 4	$L27V_3$	(Methanol extract concentrate of L27 + Viral suspension at 3 hr)
5	$L33V_3$	(Methanol extract concentrate of L33 + Viral suspension at 3 hr)
6	$L35V_3$	(Methanol extract concentrate of L35 + Viral suspension at 3 hr)
7	$L45V_3$	(Methanol extract concentrate of L45 + Viral suspension at 3 hr)
8	$L56V_3$	(Methanol extract concentrate of L56 + Viral suspension at 3 hr)
9	$B451V_3$	(Methanol extract concentrate of B451 + Viral suspension at 3 hr)

Table 2.6: Preparations used for Infectivity test

experiment. The larvae were brought from Matsyafed hatchery (Ponnani, Kerala). They were PCR screened and found to be negative for WSSV. The animals were acclimatized to laboratory conditions for a week.

Experimental Design

Fibre reinforced rectangular plastic (FRP) tanks of 30L capacity were used for the study. *Penaeus monodon* post larvae (PL 30) of the size range 15-30 mg were used for the study. The animals were acclimatized to laboratory conditions for a period of one week. These animals were transferred 15 each into rectangular fibreglass tanks. Triplicate tanks were set for each treatment, 50% water exchange was done on alternate days and the water quality parameters were maintained as given in Table 1 .Feeding experiment was done twice daily *ad libitum* and the faecal matter was removed daily in the morning. 7 experimental feeds and a control feed (Higashimaru) was used for the study. The animals were maintained on test feeds for a period of 15 days and then challenged with white spot virus orally (feeding white spot virus infected prawn meat). Post challenge survival was noted everyday for a period of seven days. Mortality by WSSV infection was confirmed by checking the

characteristic cuticular white spots on the carapace and other shell parts of the infected animal.

2.5 Statistical Analysis

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

2.6 Results

2.6.1 Primary Screening

99 actinomycetes were screened for the production of antiviral compound during primary screening. Of the 99 actinomycetes 16 isolates supported more than 40 % survival in prawns. Maximum post challenge survival was observed for prawns maintained on L45 *Streptomyces grieus* (57.4%); incorporated diet followed by L10 *Streptomyces sp.* (57.3%); L35 *Nocardia nova* (54.6%); L56 *Streptomyces sp.* (54.6%); L33 *Streptomyces flavido-fuscus* (51.86%) ; L27 *Brevibacterium linens* (50.18 %) and B451 *Streptomyces fradiae* (42.16%). The mortality rate in the controls sharply increased from day 3 and the controls CL1 and CL2 fed animals showed a survival rate of 7% and 3.6 % respectively at the end of the experiment. Based on the above results 16 actinomycetes were segregated for further screening (*Fig.2.7*).

2.6.2 Secondary Screening

During secondary screening of the actinomycetes for antiviral property it was found that maximum post challenge survival could be obtained with L45 *Streptomyces grieus* incorporated diet (60.27%) , followed by L56 (54.6%), L10 (51.1%), B451 (49%), L33 (47.2%),

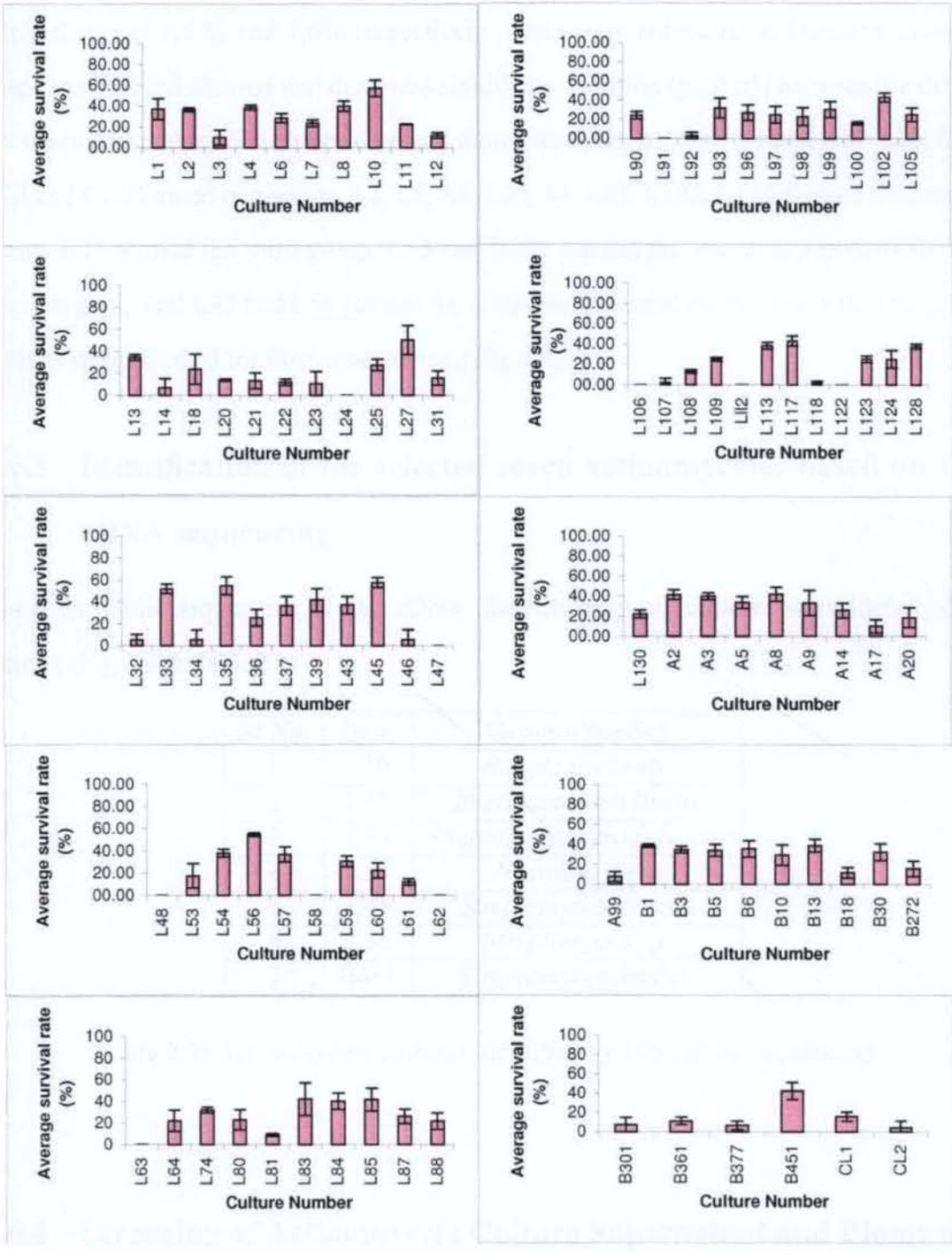


Figure 2.7: Primary screening : Post challenge survival (%) of *P.monodon* fed on different actinomycete incorporated diets and challenged with WSSV

L35 (40.2%) and L27 (40.0%). The controls CL1 And CL2 blank fed animals showed a survival rate of 1.5 % and 1.6% respectively . Data were subjected to Duncans multiple range analysis and showed that there was significant variation ($p<0.05$) between the different treatment groups . Six groups emerged during analysis, animals treated with L35, L39, CL1 and CL2 formed one group, A2, L8, A8, L83, 84, L85, L102, L117 formed the second group, L27 formed the third group, L33 and B451 formed the fourth group, L10 formed the fifth group and L45 and L56 formed the sixth group. Based on the above results, seven isolates were selected for further screening.(*Fig.2.8*).

2.6.3 Identification of the selected seven actinomycetes based on 16S rDNA sequencing

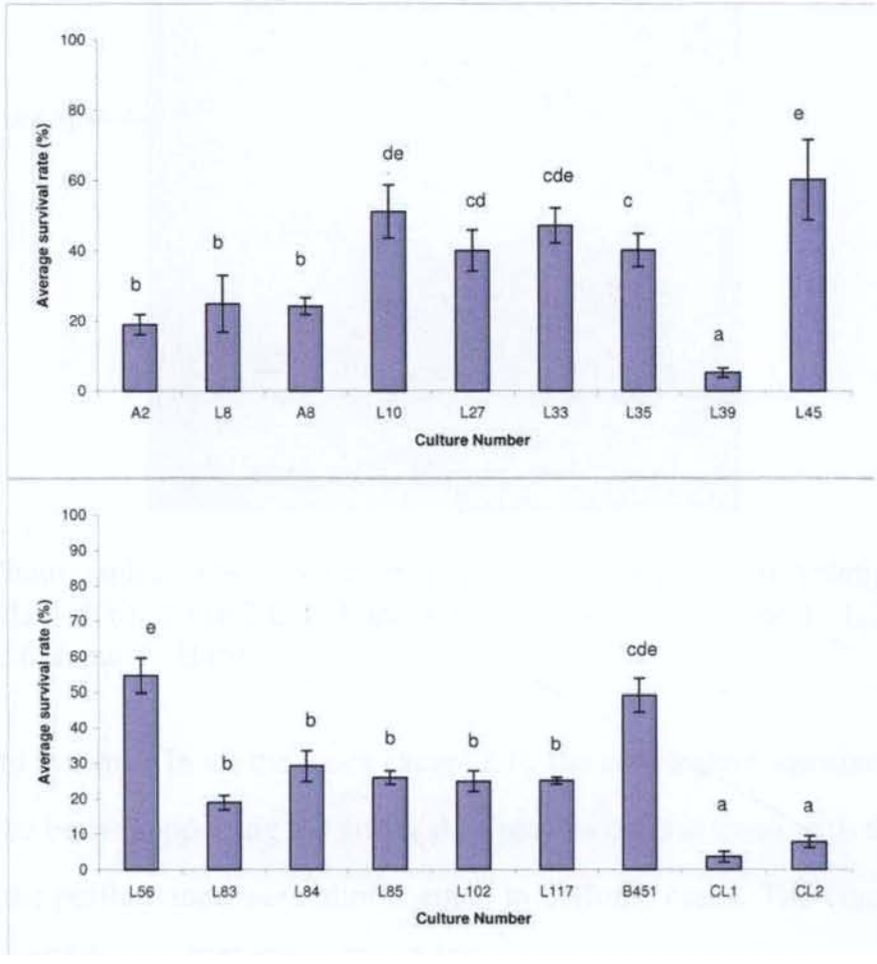
Based on partial sequencing of 16S rDNA ,the actinomycete cultures were identified as follows (Fig.2.9 & Table2.7)

Sl.No.	Item	Genera/Species
1	L10	*Streptomyces* sp.
2	L27	*Brevibacterium linens*
3	L33	*Streptomyces flavidofuscus*
4	L35	*Nocardia nova*
5	L45	*Streptomyces griseus*
6	L56	*Streptomyces* sp.
7	B451	*Streptomyces fradiae*

Table 2.7: Actinomycete cultures identified by 16S rDNA sequencing

2.6.4 Screening of Actinomycete Culture Supernatant and Biomass

In order to find out whether the antiviral principle of the selected seven isolates is in the biomass or supernatant of culture broths, these were separately incorporated with the diet



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

Culture No:	Post challenge survival rate %	Culture No:	Post challenge survival rate %
A2	18.97 ± 02.89	L56	54.60 ± 11.38
L8	24.86 ± 08.13	L83	18.90 ± 02.01
A8	24.20 ± 02.43	L84	29.20 ± 04.35
L10	51.12 ± 07.56	L85	25.90 ± 01.89
L27	40.04 ± 05.84	L102	24.86 ± 02.92
L33	47.22 ± 05.00	L117	25.07 ± 00.97
L35	40.16 ± 04.74	B451	49.02 ± 04.70
L39	05.25 ± 01.37	CL1	03.06 ± 01.50
L45	60.27 ± 11.38	CL2	07.81 ± 01.66
	(Mean ± S.D)		**(Mean ± S.D)**

Figure 2.8: Secondary screening : Post challenge survival (%) of *P.monodon* fed on different actinomycete incorporated diets and challenged with WSSV

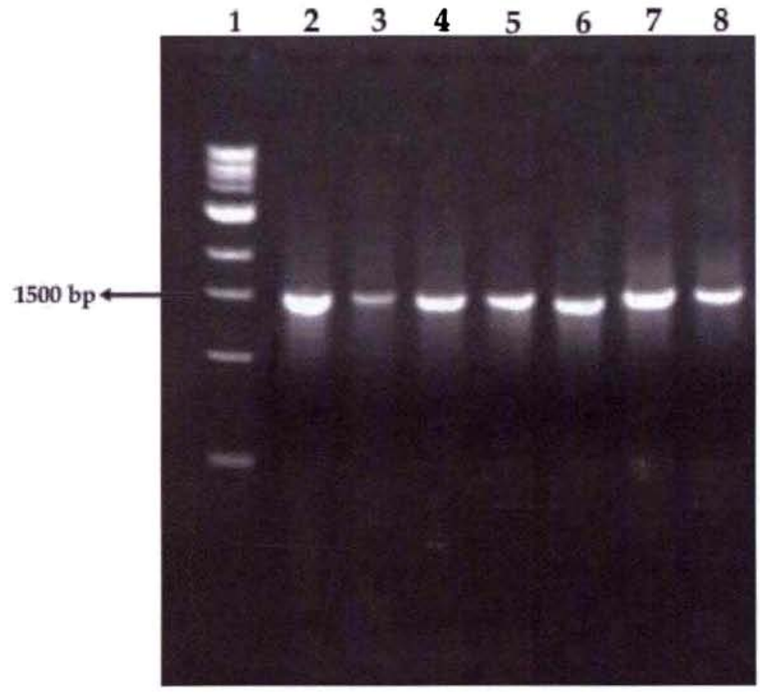
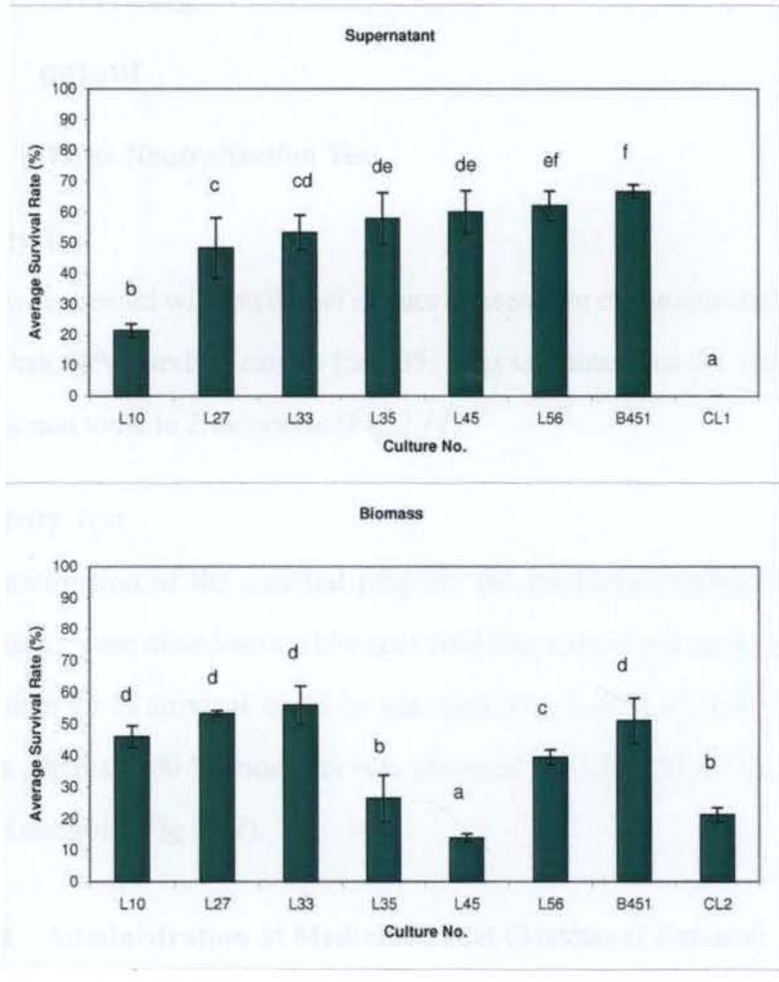


Figure 2.9: Gel Photograph of 16s rDNA amplification of selected seven Actinomycetes Lane 1- DNA Ladder (1Kb) , Lane 2 L10, Lane 3 L 27, Lane 4 L33, Lane 5 - L 35, Lane 6 - L45, Lane 7 L56 ,Lane 8 - B451

and administered to prawns . In all the cases except L10 the supernatant incorporated diets were found to be better supporting the survival of prawns on challenge with the virus. For L27 and L33 the performance were almost equal in both the cases. The control CL1 showed 100% mortality, and CL2 showed a survival rate of 2.27 %. Duncans multiple range analysis revealed that there was significant variation between the different supernatant treated groups , forming six groups. CL1 formed first group, L10 formed second group. L27 formed third group, L33 formed fourth group, L35 and L45 formed fifth group and L56 and B451 formed sixth group . Biomass treated groups formed four groups, group 1 consisted of L45, group 2 consisted of L35 and CL2 , group 3 consisted of L56 and group 4 consisted of L10, L27, L33 and B451. (*Fig.2.10*)



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

Supernatant			Biomass	
Culture No:	**Post challenge survival rate %**		**Culture No:**	**Post challenge survival rate %**
L10	21.33 ± 2.27		L10	46.05 ± 3.44
L27	48.21 ± 9.94		L27	53.31 ± 0.95
L33	53.33 ± 5.77		L33	56.05 ± 5.95
L35	57.90 ± 8.32		L35	26.46 ± 7.39
L45	60.00 ± 6.87		L45	13.90 ± 1.28
L56	62.00 ± 4.74		L56	39.46 ± 2.37
B451	66.60 ± 0.00		B451	51.06 ± 7.26
CL1	00.00 ± 0.00		CL2	21.33 ± 2.27
	(Mean ± S.D)			**(Mean ± S.D)**

Figure 2.10: Post challenge survival (%) of *P.monodon* fed on actinomycete culture supernatant and biomass incorporated diets and challenged with WSSV

2.6.5 Screening of Methanol Extract of Actinomycete Culture Supernatant

2.6.5.1 Virus Neutralization Test

Toxicity Test

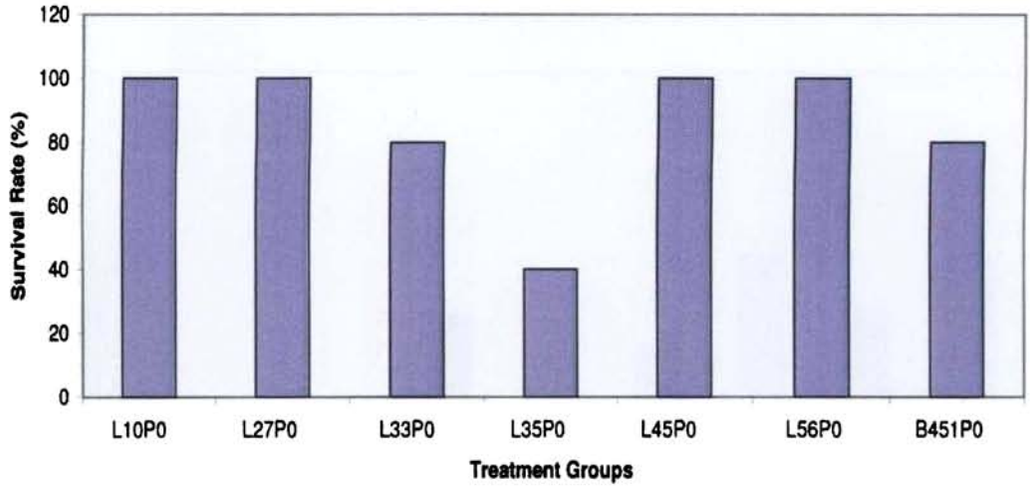
P.monodon treated with methanol extract concentrate of the selected seven isolates showed more than 80% survival except for L35. This confirms that the methanol extract concentrates is non toxic to *P.monodon* (*Fig.2.11*).

Infectivity Test

For confirmation of the antiviral property the methanol extract of actinomycete culture supernatant were mixed with white spot viral suspension and used for infecting the animals. More than 40 % survival could be obtained with L27, L35, L45, L56 and B451 treated groups whereas 100 % mortality was obtained for L10 and L33 treated groups as in the case of controls (*Fig.2.12*).

2.6.5.2 Administration of Medicated Diet (Methanol Extract)

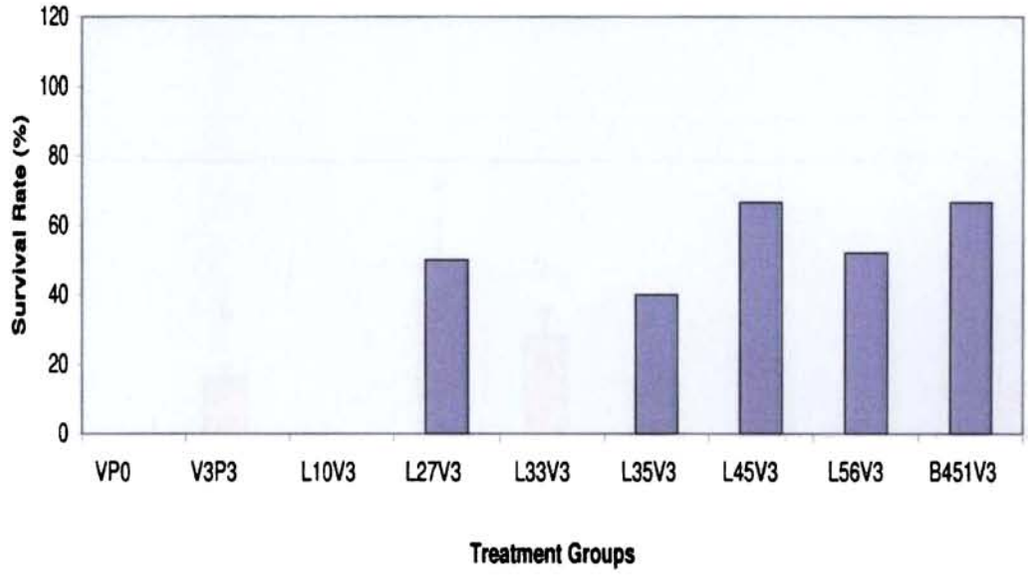
Medicated diet prepared from L35 supported the best survival (52.3%) followed by L45 (38.5%), B451(36.6%), L27(31.26%) and L56 (31%)). Cent percent mortality could be observed for L10 and L33 as in the case of control. There was significant variation between the different treatment groups, forming three groups. L10, L33, CL1 and CL2 formed first group, L27, L45, L56 and B451 formed second group and L35 formed third group (*Fig.5.21*)).



Serial No.	Code Used	Treatment Groups	Survival rate (%)
1	$L10P_0$	(Methanol extract of L10 +PBS at 0hr)	100
2	$L27P_0$	(Methanol extract of L27 +PBS at 0hr)	100
3	$L33P_0$	(Methanol extract of L33 +PBS at 0hr)	80
4	$L35P_0$	(Methanol extract of L35 +PBS at 0hr)	40
5	$L45P_0$	(Methanol extract of L45 +PBS at 0hr)	100
6	$L56P_0$	(Methanol extract of L56 +PBS at 0hr)	100
7	$B451P_0$	(Methanol extract of B451 +PBS at 0hr)	80

PBS – Phosphate Buffered Saline

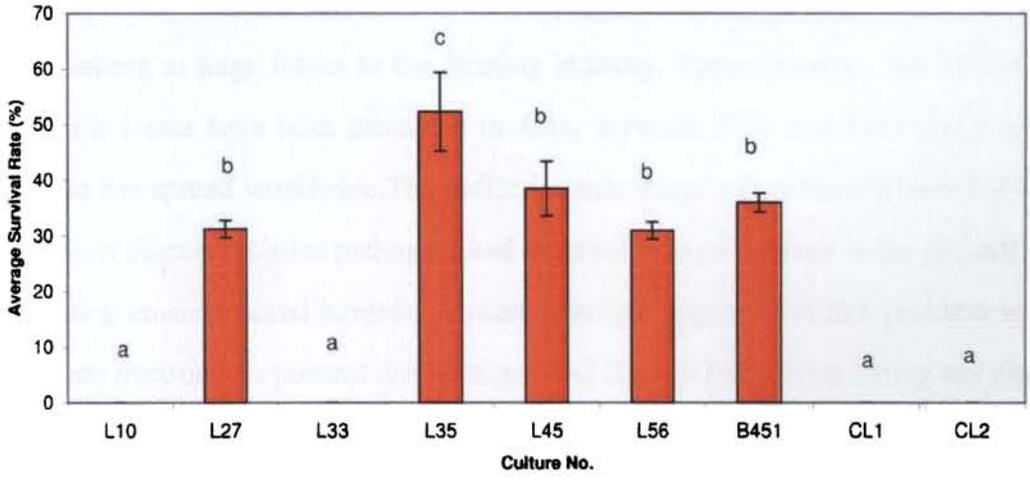
Figure 2.11: Toxicity test - Post challenge survival in *P.monodon* after injection with the methanol extract of selected actinomycete culture supernatant



Serial No.	Code Used	Treatment Groups	Survival rate (%)
1	VP_0	(Virus +PBS at 0hr)	0
2	V_3P_3	(Virus +PBS after 3 hr incubation)	0
3	$L10V_3$	(Methanol extract of L10 +PBS after 3 hr incubation)	0
4	$L27V_3$	(Methanol extract of L27 +PBS after 3 hr incubation)	50
5	$L33V_3$	(Methanol extract of L33 +PBS after 3 hr incubation)	0
6	$L35V_3$	(Methanol extract of L35 +PBS after 3 hr incubation)	40
7	$L45V_3$	(Methanol extract of L45 +PBS after 3 hr incubation)	66.6
8	$L56V_3$	(Methanol extract of L56 +PBS after 3 hr incubation)	52
9	$B451V_3$	(Methanol extract of B451+PBS after 3 hr incubation)	66.6

PBS – Phosphate Buffered Saline

Figure 2.12: Infectivity test for WSSV after neutralisation with methanol extract of the selected actinomycete culture supernatant - Post challenge survival in *P.monodon*



Culture No:	Post challenge survival rate %	Culture No:	Post challenge survival rate %
L10	0.00	L56	31.00 ± 1.55
L27	31.26 ± 1.57	B451	36.0 ± 1.7
L33	0.00	CL1	0.00
L35	52.3 ± 7.1	CL2	0.00
L45	38.5 ± 4.9		
	(Mean ± SD)		**(Mean ± SD)**

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

Figure 2.13: Post challenge survival (%) of *P.monodon* fed on medicated diet (methonol extract) and challenged with WSSV

2.7 Discussion

For the past few years, infectious and non-infectious diseases and environmental pollution have seriously affected shrimp farming industry (*Bachere*, 2000). White spot syndrome virus (WSSV) has been causing havoc by producing devastating epidemics in Asia since 1988 (*Primavera*, 1997). It causes 100 % mortality within 10 days in commercial shrimp farms, resulting in huge losses to the farming industry. Approximately, 4-6 billion US$ of economic losses have been estimated in Asia, between 1992 and 2001 and presently the disease has spread worldwide.The indiscriminate usage of antibiotics have led to the emergence of disease resistant pathogens and accumulation of residues in the animals apart from causing environmental hazards. A more practical approach to this problem will be to formulate measures to prevent the occurrence of disease rather than curing the disease. Conventional control strategies such as improvement of environmental conditions, stocking of specific pathogen free shrimp post-larvae and augmentation of disease resistance by oral immunostimulants, are currently employed to contain WSSV infections . A number of preventive approaches such as use of vaccines, immunostimulants and probiotics have been explored in order to reduce the losses by diseases. Vaccination using WSSV subunit vaccine has been reported in shrimps (Wetteveldt et al., 2004), but invertebrates lack an adaptive immune system and a defined immune memory and hence its effect is short- lived. Modular proteins in crustaceans can recognize various microbial cell wall components , resulting in enhanced fighting capabilities against invading pathogens.

Peptidogycans, β-glucans and lipopolysacccharides have been successfully used to initiate a series of non-specific defense activities in shrimps (*Soderhall and Smith* (1986); *Persson et al.* (1987)). Many reports had already been published on the potency of cell wall components in conferring protection against WSSV infection in shrimps. In one study, oral administration of 20 g LPS per kg shrimp body weight/day for 7 days

against penaeid acute viraemia resulted in 75 % survival (*Takahashi*, 2000). Dietary glucans have been shown to retard WSSV infection in *P.monodon* (*Chang et al.*, 1999). Other prophylactic components that could delay WSSV infection in *P.japonicus* were, peptidoglycan and lipopolysaccharides (LPS) (*Itami et al.*, 1998). In the present study, the application of marine actinomycetes was found to confer some protection against WSSV challenge in *Penaeus monodon*. The application of actinomycete whole broth administered through feed had significantly increased the survival rate of *Penaeus monodon* when challenged with white spot virus during primary and secondary screening. Seven isolates (L10 *Streptomyces sp.*, L27 *Brevibacterium linens*, L33 *Streptomyces flavidofuscus*, L35 *Nocardia nova*, L45 *Streptomyces grieus*, L56 *Streptomyces sp.*, B451 *Streptomyces fradiae*) which showed more than 40 % survival rate were selected for further study. This result clearly indicates that there is some antiviral property present in the actinomycete broths, that were incorporated in the feed. Better survival exhibited by prawns fed on certain strains of actinomycete-incorporated feeds maybe due to an antiviral compound or immunostimulant. The poor performance of other isolates could be due to the adverse effect of some components of the diet, which would have circumpassed the beneficial effects imparted by them. More works are to be carried out to prove the antiviral property of the actinomycete isolates. An antiviral agent if present prevents the viral attack either by preventing the attachment of the virus to the cell surface or by preventing uncoating of the virus envelope or acting at the transcriptional or translation level.

Both the biomass and supernatant of the selected seven isolates were also screened by incorporation into feed separately and it was found that the antiviral compound is released into the broth except for L10. The supernatant incorporated diet of L45, L56 and B451 showed a survival rate of 57.9 %, 60 %, and 62.08 % respectively . Accordingly, the supernatant of all the selected seven isolates was extracted using methanol as solvent, but

it was found that the antiviral property of the methanol extract was lesser when compared to whole broth. More than 40 % survival could be obtained with L27, L35, L45, L56 and B451 extracts. This implies that the extraction protocol has to be modified for effective recovery of the bioactive principle of other isolates.

Many antiviral compounds have been isolated from actinomycetes. Pentalactones from *Streptomyces*, pyrrole-2-carboxylic acid etc. have been reported to be active against several DNA viruses. The results obtained in the present study may also be considered in accordance with these findings. These isolates, when incorporated in the feeds, lowered WSSV infection in shrimps. Thus, it leads to the obvious conclusion that isolates of actinomycetes in the culture broth may have produced bioactive compounds that possess potent antiviral activities or else they could have boosted up the immunological parameters that could impart resistance on subsequent infection by the virus . Moreover, it is reasonable to state that these marine forms could be undoubtedly applied to penaeid shrimp culture systems which are restricted to brackish or seawater conditions.

There occurs a very few studies related to the dose or response of immunostimulants in shrimp. Unlike many chemotherapeutics, immunostimulants does not show a linear dose/effect relationship (*Bliznakov and Adler*, 1972). In fact they often show distinct maximum at a certain intermediate concentration and even a complete absence of effect or even adverse toxic effect at higher concentrations (*Floch et al.*, 1987). Since route of administration of a drug is of paramount importance in its efficacy, oral administration is the most preferred and practically feasible option in aquaculture. Many workers have reported that the oral administration of glucan improved the disease resistance of aquatic organisms, both fishes and shellfishes (*Raa* (1996) ; *Smith et al.* (2003)). Even though intraperitoneal injection of immunostimulants/bioactive compound enhances

the function of haemocytes and protection against pathogens, this method is labour intensive, time consuming and stressful to the animals. Immunostimulation via immersion treatment has also proved to be a successful method of application in aquatic larval systems.

Isolation of the bioactive compounds and characterization are essential to further the findings of this study, which would lead to the possibility of developing measures effective against white spot disease in shrimps. The present formulation is the first of its kind which has some prophylactic properties against WSSV in penaeid shrimps. There exist only very few reports on actinomycetes as source of antiviral agents against white spot viral disease in shrimps. Feeding experiments in *Penaeus monodon* post larvae using *Streptomyces pulveraceus* showed better survival when compared to control feed (*Mathew*, 2003). *Kumar et al.* (2006) have also reported on the anti-WSV properties of marine actinomycetes in *P.monodon*. The present formulation will be one among the very few reports proving marine actinomycetes as prophylactic agents against WSSV in penaeid shrimps. However, the study was carried out only under laboratory conditions and therefore, field trials have to be conducted to work out the effective dosage and frequency of application depending on the type of culture practice, density of stocking and intensity of infection.

CHAPTER 3

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

3.1 Introduction

The UN FAO estimates that half of the worlds seafood demand will be met by aquaculture in 2020, as wild capture fisheries are over exploited and in decline. Shrimp culture is widespread throughout the tropical world, and *Penaeus monodon*, the black tiger prawn is the most widely cultured species. However, the shrimp aquaculture industry is beset by disease, mostly due to bacteria, (especially the luminous *Vibrio harveyi*) and viruses. This has resulted in huge economic loss to the shrimp industry. The high density of animals in hatchery tanks and ponds is conducive to the spread of pathogens, and the aquatic environment, with regular applications of protein rich feed, is ideal for culturing bacteria

(*Moriarty*, 1997). Moreover, unlike land animals, aquatic animals are surrounded by a milieu that supports opportunistic pathogens independently of the host animal, and so the pathogens can reach high abundance around the animal.

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

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

Larval mortalities associated with the presence of *V.harveyi* have been reported in *Penaeus monodon* and *Penaeus vannamei* in Indonesia (*Sunaryanto and Mariam*, 1986),

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

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

In 1990, only 4 antibiotics were resistant to *Vibrio* spp., but 9 years later, the number of resistant antibiotics increased to 20 (*Eleonar and Leobert.*, 2001). The presence of antimicrobial agents at low concentration through leaching or continued usage may lead to the development of drug resistant strains and multiple antibiotic resistance (MAR) in

bacteria, which may result in resistance transfer to pathogenic bacteria. Hence developing alternative strategies to the use of antimicrobials in disease control is urgent. One of the successful methods to control vibriosis is the use of probiotics, which are applied in the feed or added to the culture tank or pond as preventive agents against infection by pathogens. Most probiotics proposed as biological control agents in aquaculture are lactic acid bacteria, (*Lactobacillus, Carnobacterium* etc) , non-pathogenic *Vibrio* (*Vibrio alginolyticus*,), *Bacillus* strains and *Pseudomonas* strains (*Verschuere et al.*, 2000), but few studies on actinomycetes as probiotics have been reported.

3.1.1 Probiotics

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

3.1.2 Probiotics in Aquaculture

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

In aquaculture systems, the probiotics are applied in two ways i.e.) as gut probiotics to maintain the microbial balance of the animal and thereby reduce the number of pathogenic species in the body and as pond probiotics applied to water / sediment , which will provide a healthy environment for the animals and help in the exclusion of pathogens (*Singh and Jayaprakash*, 2002). *Nogami and Maeda* (1992) isolated a bacterial strain from a crustacean culture pond, and was found to improve the growth of crab, *Portunus trituberculatus* larvae and repress the growth of vibrios. *Austin et al.* (1992) reported a kind of micro algae (*Tetraselmis suecica*) which can inhibit pathogenic bacteria of Atlantic salmon. *Smith and Davey* (1993) reported that fluorescent *pseudomonads* can competitively inhibit growth of fish pathogen *Aeromonas salmonicida*, by competing for free iron. *Garriques and Arevalo* (1995) reported that the use of *Vibrio alginolyticus* as a probiotic agent may increase survival and growth in *Penaeus vannamei* post larvae. *Douillet and Langdon* (1994) have reported that the addition of two probiotic bacteria

to oyster larval ponds had enhanced production by providing essential nutrients and digestive enzymes. *Pseudomonas* PS-102, isolated from a brackish water lagoon, showed antagonistic property to a wide range of pathogenic vibrios isolated from penaied and Macrobrachium larval rearing systems and it is proposed that the inhibitory activities is probably due to the ability of the strain to produce siderophores (*Vijayan et al.*, 2005).

3.1.2.1 Modes of Action of Probionts

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

Competitive Exclusion

Bacterial antagonism is a common phenomenon in nature; therefore, microbial interac-

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

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

Source of Nutrients and Hydrolytic Enzymes

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

Influence on Water Quality

They could also act by improving water quality. The rationale is that gram positive bacteria are better converters of organic matter back to CO_2 than gram negative bacteria. It has been reported that use of *Bacillus* sp. improved water quality, survival and growth rates and increased health status of juvenile *P.monodon* besides reducing the pathogenic vibrios (*Dalmin et al.*, 2001).

Enhancement of the Immune System

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

a level of 10^5 cfug^{-1} feed, stimulated the respiratory burst in rainbow trout (*Oncorhynchus mykiss*).

Colonisation in the Gastrointestinal Tract

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

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

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

gut when present in high numbers.

Antiviral Effects

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

3.1.3 Marine Actinomycetes as Probiotics in Shrimp Aquaculture

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

Actinomycetes have shown many interesting activities in water, such as degradation of starch and casein and production of antimicrobial agents against both gram negative and gram positive bacteria (*Barcina et al.* (1987) and *Pisano et al.* (1992)). Marine actinomycetes have also been proven to be antagonistic to *Vibrio* spp. pathogenic to shrimps (*You et al.*, 2005) .Marine actinomycetes exhibits a wide range of enzymatic activity, namely,

chitinolytic activity (*Pisano et al.* (1992) and *Mahendra et al.* (2002)) , cellulase activity by thermophilic actinomycete *Microbispora, Streptomyces lividans* (*Kluepfel et al.*, 1986) and lipase activity by *Streptomyces*,(*Large et al.* (1999) and *Gandolffi et al.* (2000)). With these bioactivities, actinomycetes could play an important role in the food webs of the marine environment.

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

3.2 Materials and Methods

3.2.1 Screening of Marine Actinomycetes for Antagonistic Property Against Shrimp Pathogens

3.2.1.1 Microorganisms

Actinomycetes isolates (99 Nos) isolated from the sediment samples collected from the West coast of India and South West coast of India were used to test the antagonistic potential against shrimp pathogens. Slant cultures of the different isolates grown on Marine Actinomycetes growth medium (MAG) (Table3.1) was used to inoculate 20 ml seed medium

(Marine Actinomycetes Growth medium) . The tubes were incubated on a reciprocal shaker at 100 rpm for 7 days at room temperature and were used for the study .

Starch	-	1g
Yeast Extract	-	0.4g
Peptone	-	0.2g
Agar	-	2g
Sea Water(15 ppt)	-	100 ml
pH	-	7

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

3.2.1.2 Pathogens

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

3.2.1.3 Antagonism Assay

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

3.2.2 Screening of Marine Actinomycetes for Hydrolytic Enzyme Production

3.2.2.1 Microorganisms

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

3.2.2.2 Amylase Test

The isolates were spot inoculated onto Starch Agar medium of *Harigan and Maccance* (1972) (Table3.2) and incubated for 4-5 days at room temperature (28 ± 2°C) .

Peptone	-	10g
Beef Extract	-	10g
Starch (Soluble)	-	5g
Agar	-	20g
Sea Water(50 %))	-	1L
pH	-	7.2

Table 3.2: Composition of Starch Agar Medium

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

3.2.2.3 Lipase Test

Production of lipase was tested on Tributyrin Agar medium (*Rhodes*, 1959) (Table3.3).

Peptone	-	5g
Beef Extract	-	3g
Tributyrin	-	10 ml
Agar	-	20 g
Seawater (50%)	-	1L
pH	-	7.2

Table 3.3: Composition of Tributyrin Agar medium

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

3.2.2.4 Gelatinase Test

Fraziers gelatin agar medium was used for detection of gelatinase activity (Table3.4).

Peptone	-	5g
Beef extract	-	3g
Gelatin	-	2g
Agar	-	20 g
Seawater (50%)	-	1L
pH	-	7.0

Table 3.4: Composition of Fraziers Gelatin Agar

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

3.2.2.5 Cellulase Test

The isolates were spot inoculated onto Cellulose Agar medium of (*Riviere*, 1961) and incubated for 4-5 days at room temperature (Table3.5). Cellulase production was detected by the appearance of halo zone around the colony. Zone of clearance was measured and recorded.

Cellulose powder	-	5g
NaNO3	-	1 g
Yeast extract	-	0.5g
Casein hydrolysate	-	0.5g
Agar	-	20g
Seawater (50 %)	-	1L
pH	-	6.8

Table 3.5: Composition of Cellulose Agar medium

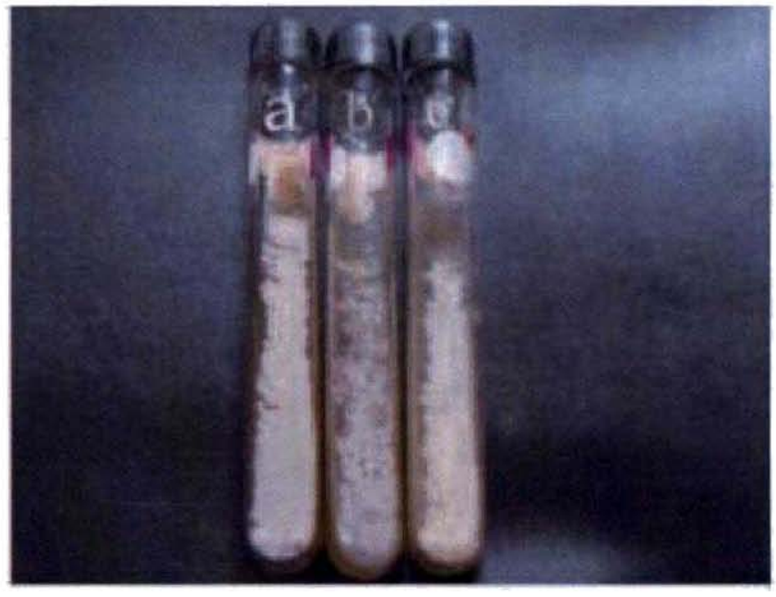
3.2.2.6 Chitinase Test

The isolates were spot inoculated onto Chitin Agar medium (*Holding and Collee*, 1971) and incubated for 4-5 days at room temperature (Table3.6). Chitinase production was detected by the appearance of halo zone around the colony. Zone of clearance was measured and recorded.

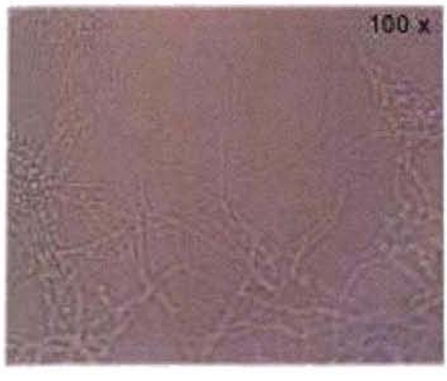
3.2.3 Screening of Marine Actinomycetes for Pathogenicity

3.2.3.1 Microorganisms Used

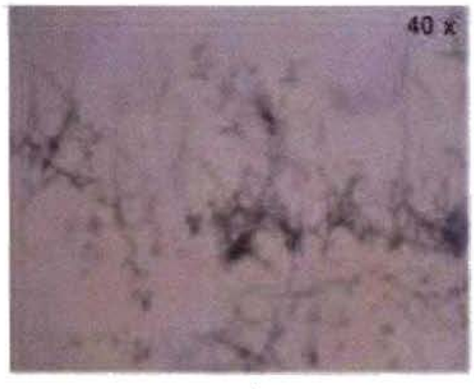
Based on the hydrolytic and antimicrobial activity three actinomycete strains (L18 , L39 , L45) (*Fig.3.1*) were selected for further study .



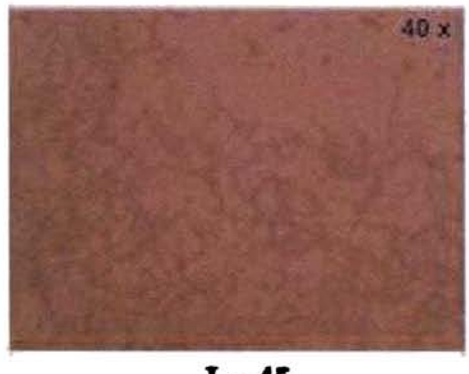
a. L-18 **b. L-39** **c. L-45**



L - 18



L - 39



L - 45

Figure 3.1: Slant and slide culture of the selected three strains

Peptone	-	5g
Beef extract	-	5g
Colloidal chitin	-	5g
Agar	-	20g
Seawater (50 %)	-	1L
pH	-	7.5

Table 3.6: Composition of Chitin Agar medium

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

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

3.2.3.3 Pathogenicity Test *in vitro*

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

Collection of Haemolymph

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

Haemolysis Assay

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

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

3.2.3.4 Pathogenicity Test *in vivo*

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

Preparation of Actinomycete Biomass

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

Experimental Animals

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

Experiment I - Challenge Via Rearing Water

Fifteen animals were stocked in Fibre Reinforced Plastic (FRP) tanks of 30 L capacity containing 20 L seawater (*Fig.2.2*) The experiments were done in triplicate for each treatment group and control. The actinomycete biomass was suspended in sterile PBS to OD_{600} of 1.0 , corresponding to 3 $x10^9$ cfu / ml. The prawns were challenged at 10^7 cfu / ml on alternate days for a period of 10 days .Water exchange was done on alternate days. Animals were observed for mortality upto 14 days.

Experiment II - Challenge Via Diet

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

3.2.4 Assessing the Colonisation Property of Actinomycetes in the Intestine of Shrimps

3.2.4.1 Preparation of Actinomycete Biomass

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

3.2.4.2 Experimental Animals

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

3.2.4.3 Experimental Setup

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

3.2.4.4 Evaluation of the Colonisation property of Actinomycetes

The harvested biomass of each actinomycete isolate (L18, L39, L45) was incorporated into feed at 1% (wet wt.) and administered once in a day for a period of seven days and the animals were maintained on control diet for the rest of the experimental period. For the recovery isolations, four animals were collected periodically on 8^{th}, 10^{th}, 15^{th} and 20^{th} day , intestine was removed asceptically, and then homogenized in sterile saline. Serial dilutions were performed and plating was done in triplicate on Marine Actinomycete growth Agar plates employing the pour plate and spread plate technique. Incubationr was done at 28±

2°C for 3-5 days. The characteristic actinomycete colonies on the plates were counted and the number was expressed as cfu/mg intestine of the animals.

3.2.5 Exclusion of Vibrios by Marine Actinomycetes

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

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

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

3.2.5.2 Exclusion of *Vibrio- in vivo* test

Experimental Animals

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

acclimatized to laboratory conditions. They were PCR screened for WSSV. These larvae were maintained on control diets for a period of one week.

Experimental Design

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

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

Experimental Animals

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

Experimental Design

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

Absolute Growth = Final Weight - Initial weight

3.3 Statistical Analysis

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

3.4 Results

3.4.1 Inhibition of Prawn Pathogens by Marine Actinomycetes

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

(*Fig.3.4*) and was inhibited by 17.4 %, followed by *V.parahaemolyticus* (14.1%), *V.fluvialis* (9.78%), *V.cholerae* (9.78 %) and *V.harveyi* (7.6%). L39 showed maximum inhibitory activity against *V.harveyi* (*Fig.3.5*).

3.4.2 Hydrolytic Enzyme Production by Marine Actinomycetes

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

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

Based on partial sequencing of 16S rDNA ,the actinomycete cultures were identified as follows (Fig.3.11 & Table3.8)

Sl.No.	Item	Genera/Species
1	L18	*Prauseria hordei*
2	L39	*Nocardia alba*
3	L45	*Streptomyces griseus*

Table 3.7: Actinomycete cultures identified by 16S rDNA sequencing

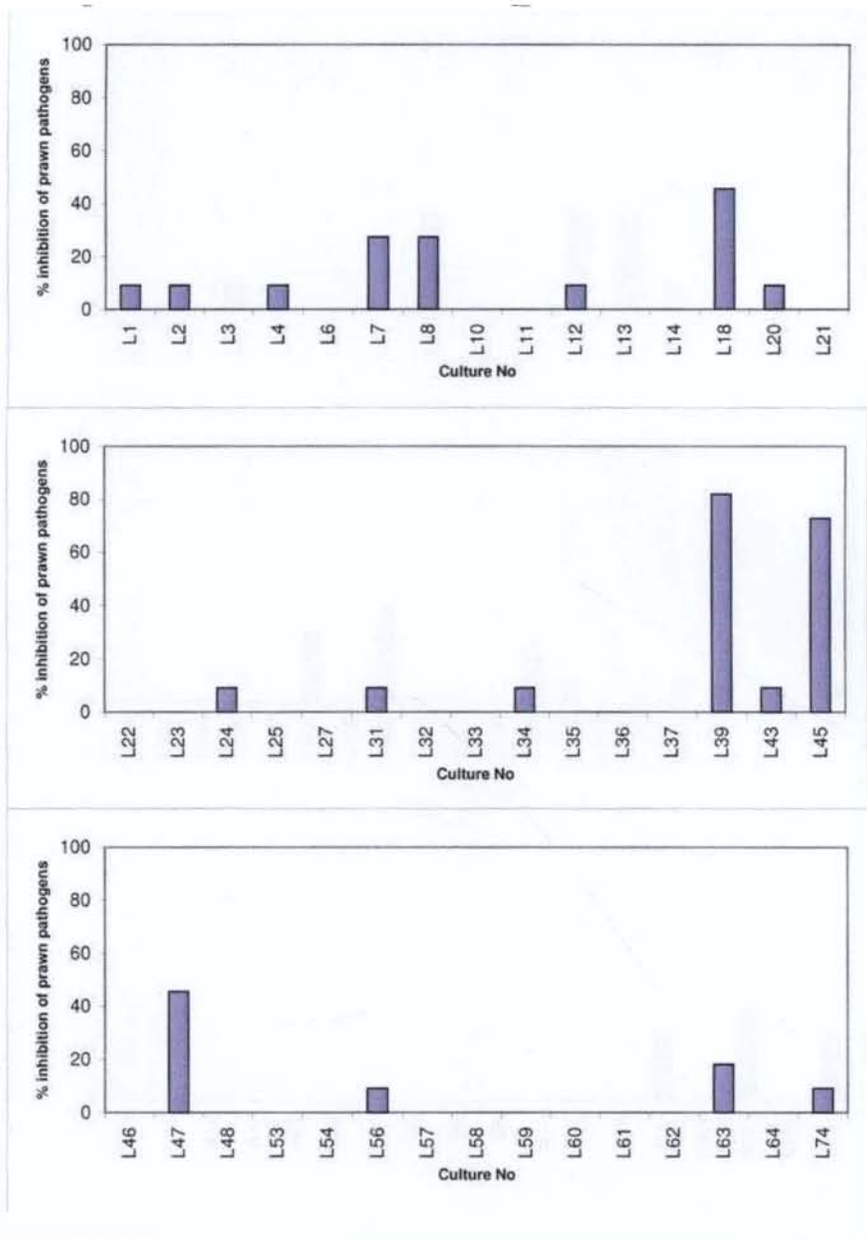


Figure 3.2: Inhibitory action of marine actinomycetes to prawn pathogens

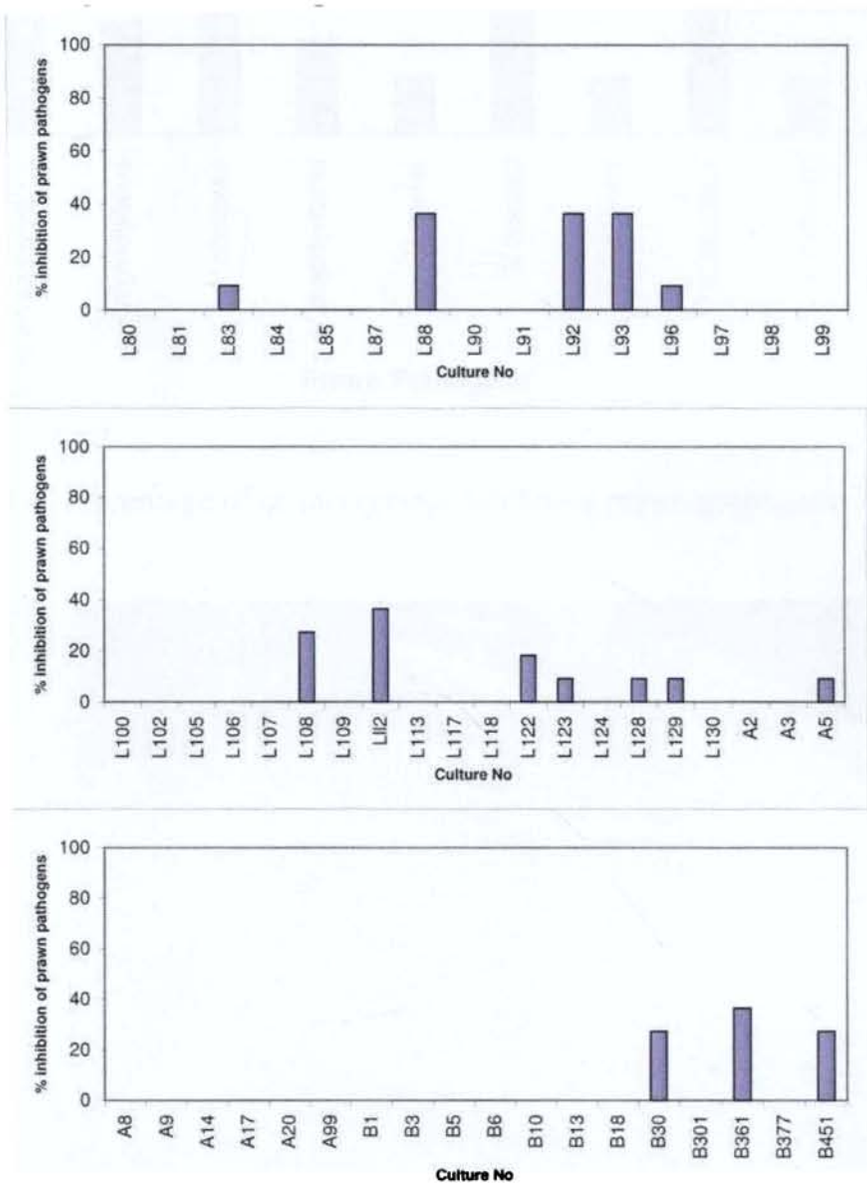


Figure 3.3: Inhibitory action of marine actinomycetes to prawn pathogens

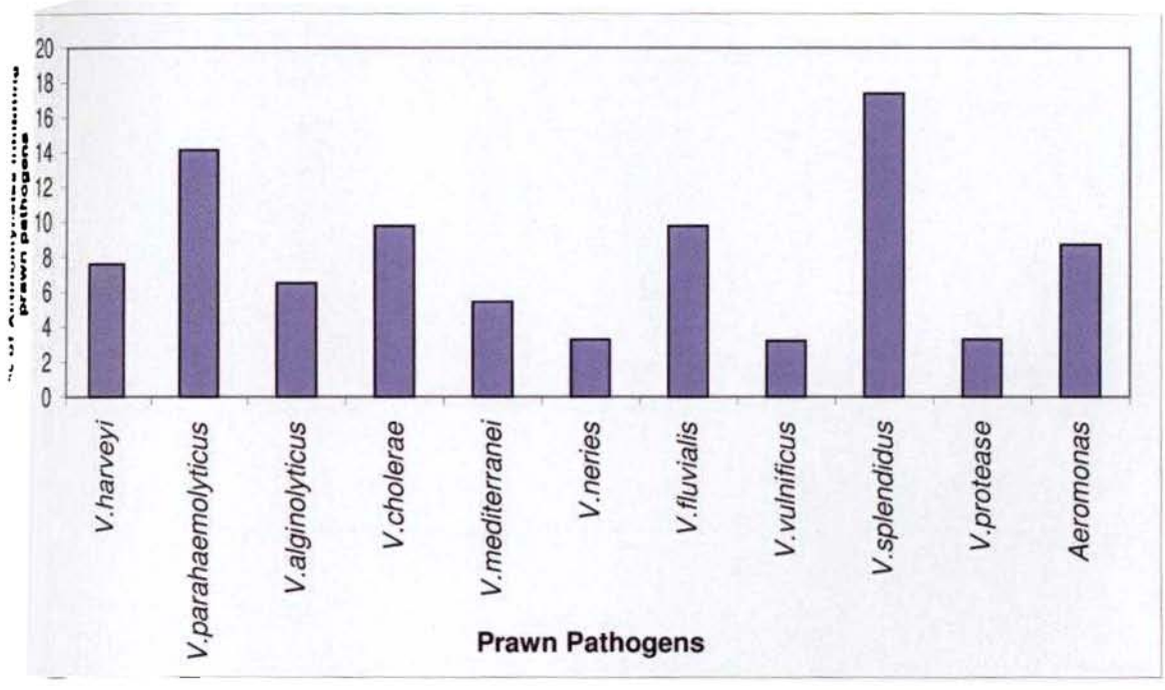


Figure 3.4: Percentage of actinomycetes inhibiting prawn pathogens

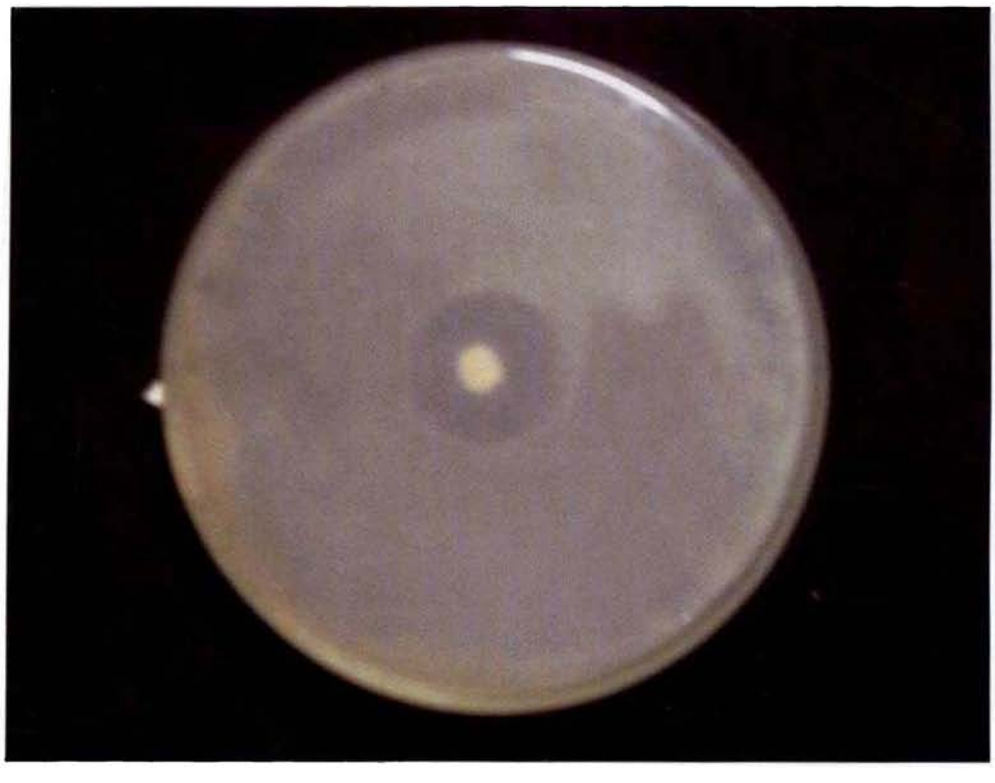


Figure 3.5: Inhibitory activity of L39 against *V.harveyi*

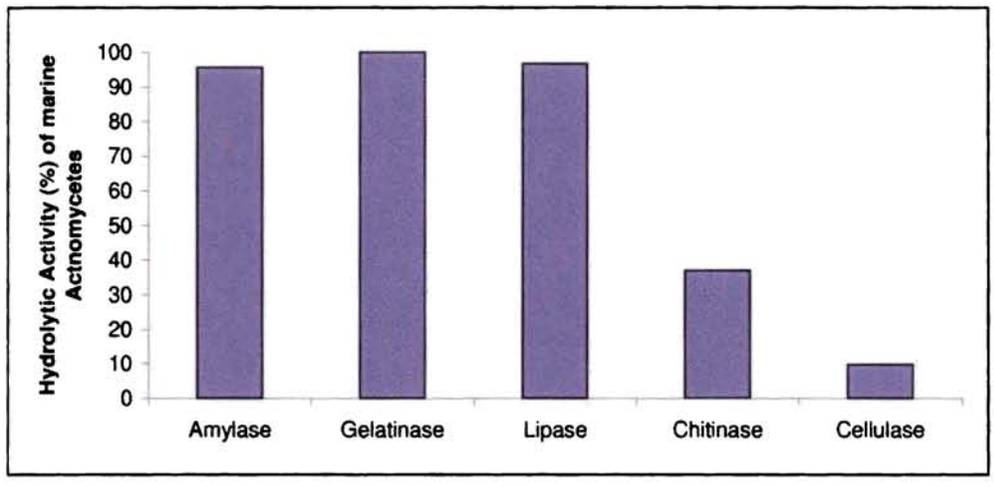


Figure 3.6: Hydrolytic enzyme activity (%) of marine actinomycetes

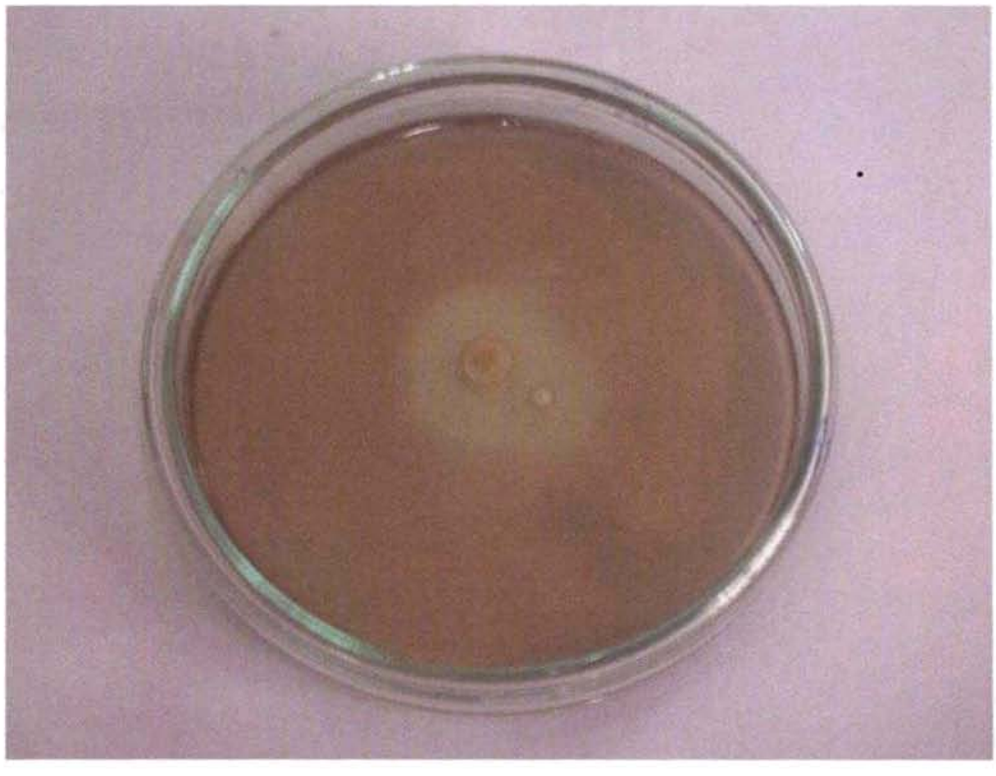


Figure 3.7: Amylase activity by L45

Figure 3.8: Gelatinase activity by L45

Figure 3.9: Lipase activity by L18

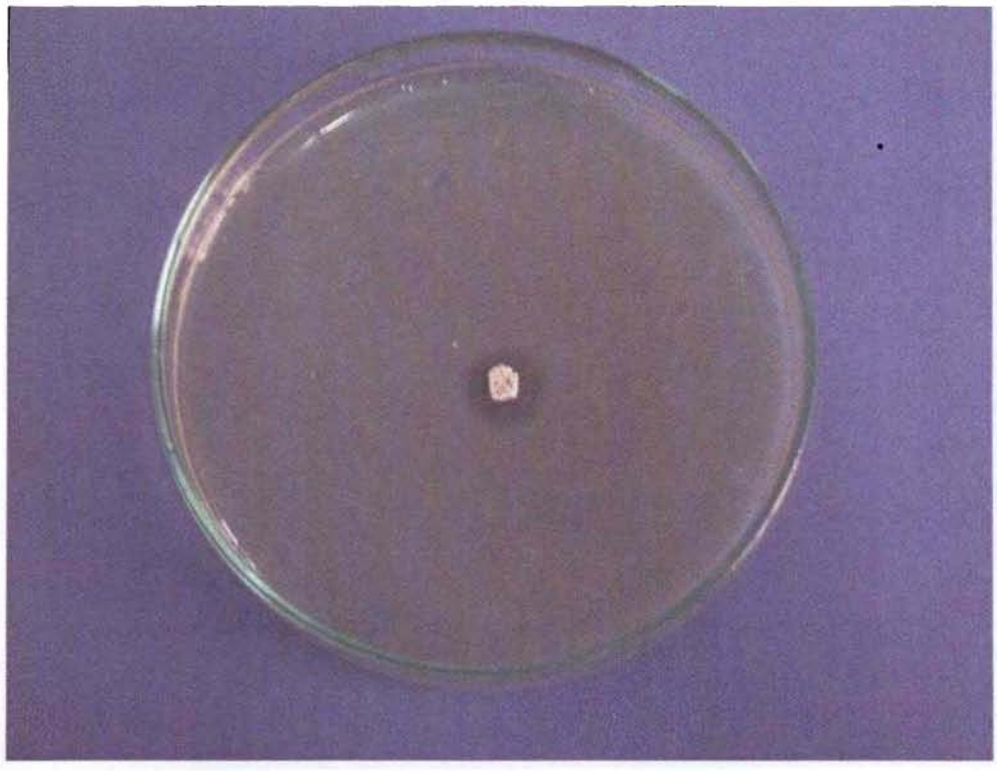


Figure 3.10: Chitinase activity by L18

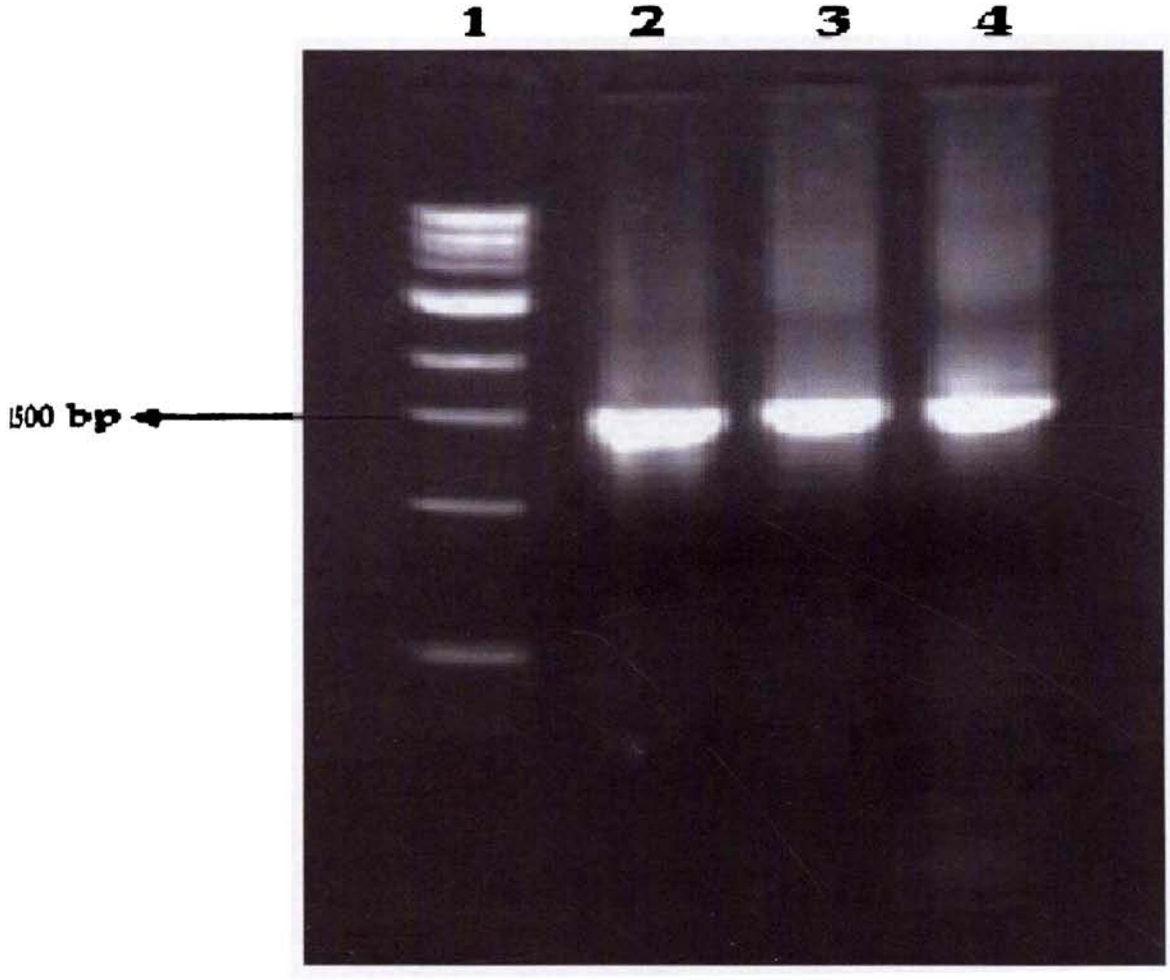
3.4.4 Screening of Marine Actinomycetes for Pathogenicity

3.4.4.1 Pathogenicity Test *in vitro* (Haemolytic Property on Prawn Blood Agar)

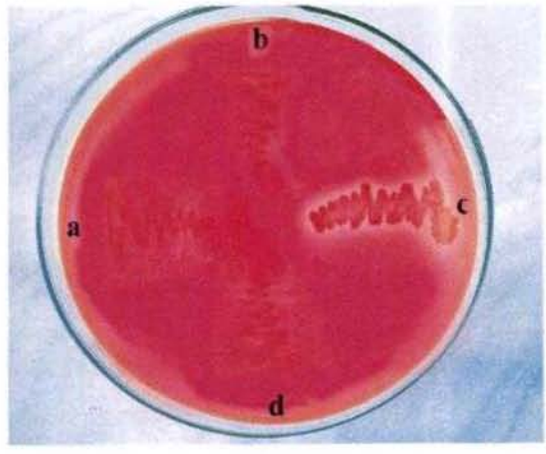
Based on the above results three isolates (L18, L39 and L45) were seleted for further study. *in vitro* pathogenicity test (Haemolytic Property on Prawn Blood Agar) showed that they were non haemolytic (*Fig.3.12*). *V.harveyi* used as a reference haemolytic strain showed haemolytic property on haemolymph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

3.4.4.2 Pathogenicity Test *in vivo* (Challenge via Rearing water and Diet)

No significant mortality of prawns could be observed by challenge via rearing water and diet. All the treatments showed above 90% survival as the control. The results confirmed the non-pathogenicity of the selected actinomycetes to *Penaeus monodon* post larvae (*Fig.3.13*). Even better survival could be recorded in animals maintained on actinomycete



gure 3.11: Gel Photograph of 16s rDNA amplification of selected three Actinomycetes ne l-DNA Ladder (1Kb) , Lane 2 - L18, Lane 3 - L 39, Lane 4 - L45



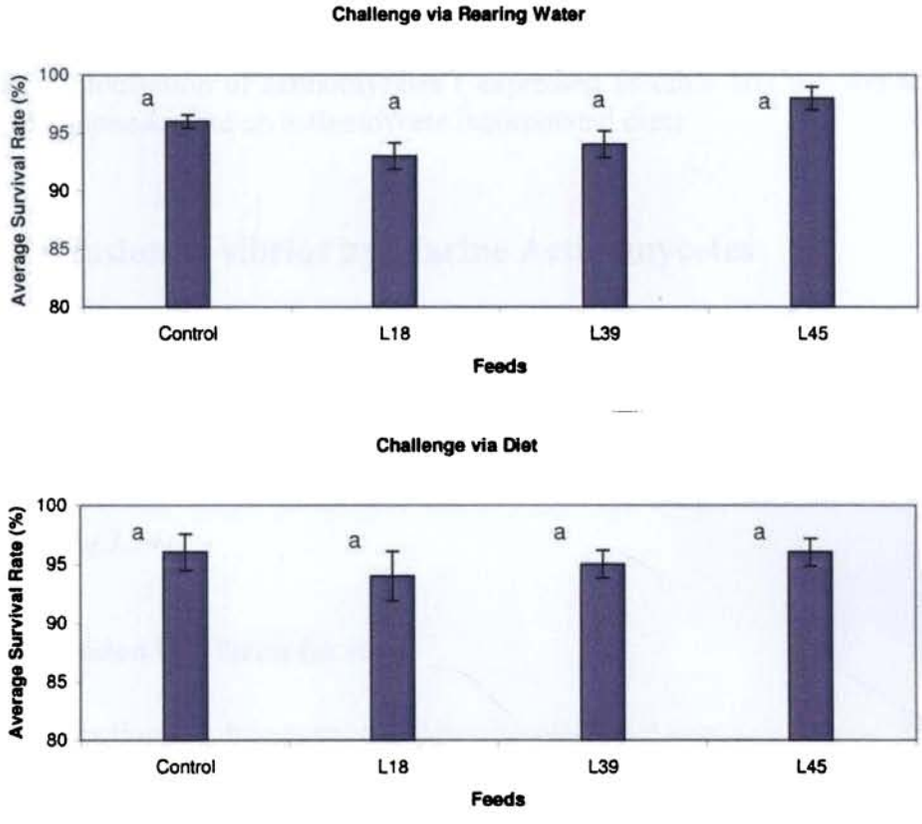
a - L18 b – L39 c – *Vibrio harveyi* d – L45

Figure 3.12: Haemolytic assay of marine actinomycetes using prawn blood agar

diets. Duncun's multiple range analysis of variance showed no significant variations between different groups when challenged through water and diet.

3.4.5 Colonisation Capacity of Marine Actinomycetes in the Intestine of Shrimps

Typical colonies of selected actinomycetes were not found on marine actinomycete agar plates during the recovery isolations. This confirms that these isolates could not colonise the intestine of shrimps (*Table.3.8*).



Challenge via Rearing Water		Challenge via Diet	
Treatments	Average Survival Rate (%)	Treatments	Average Survival Rate (%)
Control	96 ± 0.58	Control	96 ± 1.53
L18	93 ± 1.15	L18	94 ± 2.08
L39	94 ± 1.15	L39	95 ± 1.15
L45	98 ± 1.00	L45	96 ± 1.15
	Mean ± SD		M ean ± SD

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

Figure 3.13: Percentage survival of *P.monodon* post larvae after exposure to actinomycetes strains (Through Rearing water and Diet)

Time of Isolation	L18	L39	L45
8th day	10	12	10
10th day	3	1	3
15th day	-	-	-
20th day	-	-	-

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

3.4.6 Exclusion of vibrios by Marine Actinomycetes

3.4.6.1 Co-Culture Experiments (*in vitro*)

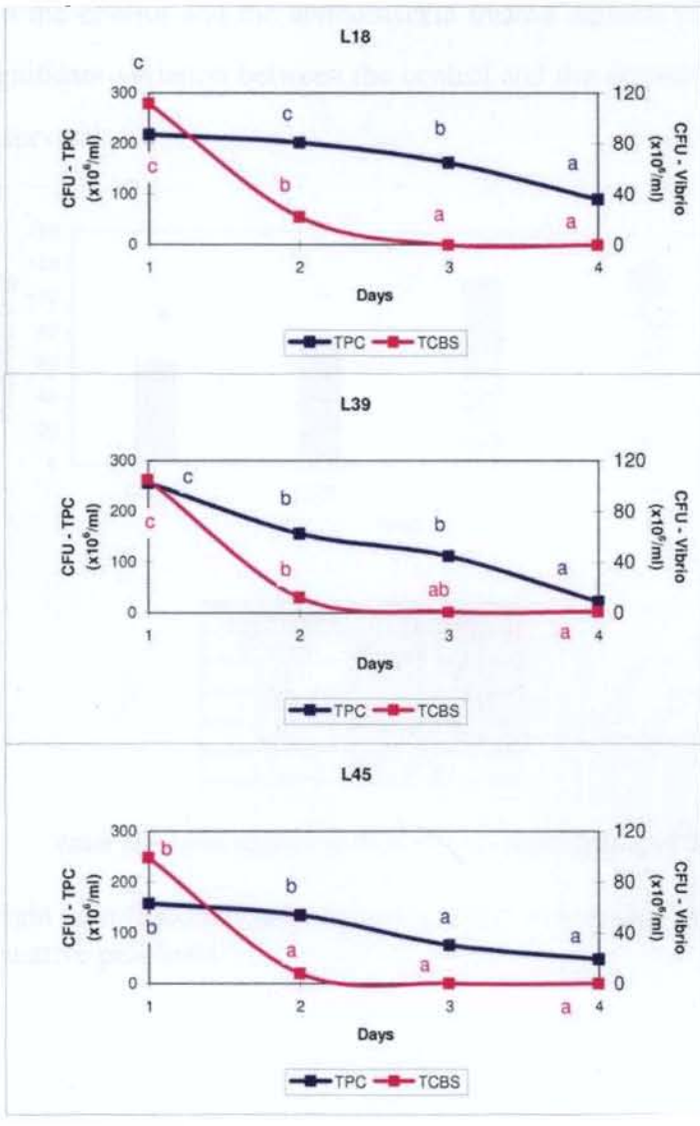
In co-culture experiments with actinomycetes and *V.harveyi*, a gradual decline in vibrio count was observed for all co-cultures, and it became undetectable on the 3^{rd} day for all co-cultures (*Fig.3.14*).

3.4.6.2 Exclusion Of vibrios (*in vivo*)

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

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

The individual increase in weight (production) was more for prawns reared with actinomycete as probionts compared to control (6.033%), in the order L18 (7.14%), L45 (9.83%), & L39 (10.916%) (*Fig.3.15*).The survival rate was also more for actinomycete fed prawns compared to control (77.5%) in the order L18 (85.51%), L45 (86.63%)& L39 (87.58%) (*Fig.3.16*). (*Fig.3.17*). Duncun's multiple range analysis of variance showed significant



TPC

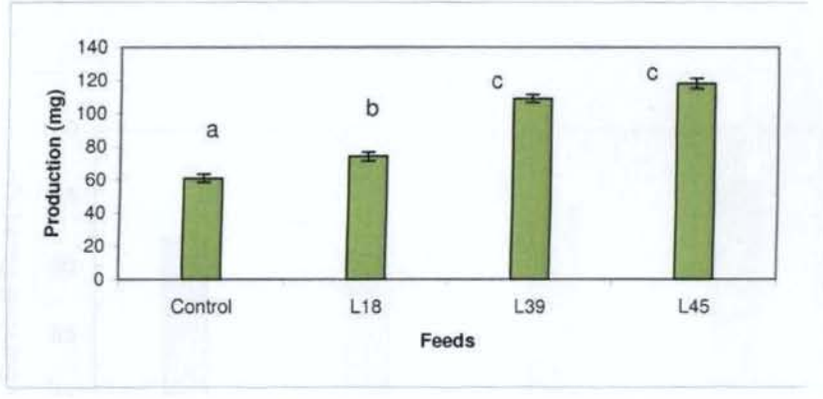
Co-culture	1st day	2nd day	3rd day	4th day
L18 + *V.harveyi*	218 ± 0.05	202 ± 0.01	102 ± 0.02	90 ± 0.01
L39 + *V.harveyi*	256 ± 0.08	156 ± 0.02	112 ± 0.06	22 ± 0.09
L45 + *V.harveyi*	158 ± 1.23	135 ± 0.07	76 ± 0.07	48 ± 0.06

Vibrio Count

Co-culture	1st day	2nd day	3rd day	4th day
L18 + *V.harveyi*	112 ± 0.06	22 ± 0.15	0 ± 0.08	0 ± 0.00
L39 + *V.harveyi*	105 ± 0.05	12 ± 0.06	1 ± 0.06	1 ± 0.06
L45 + *V.harveyi*	99 ± 0.08	8 ± 1.43	0 ± 0.02	0 ± 0.05

Figure 3.14: Total plate count (TPC) and vibrio count for co-culture of various actinomycetes with *V.harveyi*

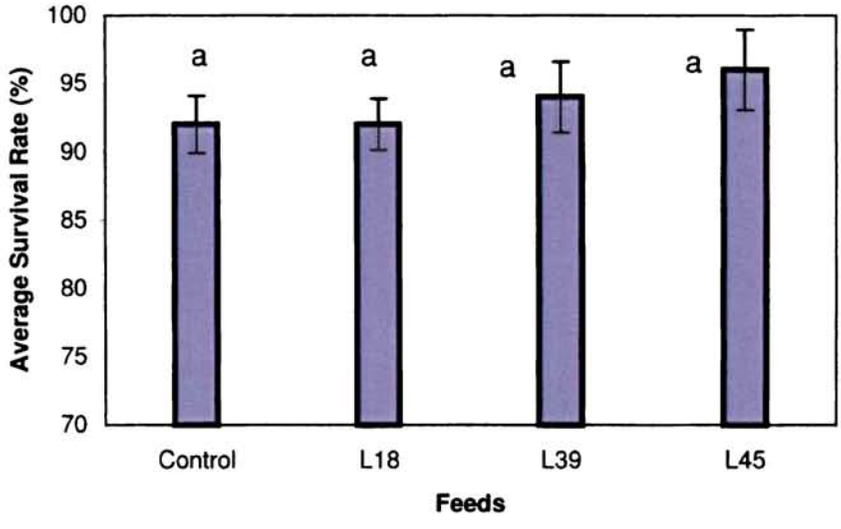
variation between the control and the actinomycete treated animals in terms of growth. There was no significant variation between the control and the actinomycete treated animals in terms of survival.



Treatments	Production
Control	061.00 ± 2.5
L18	074.16 ± 2.74
L39	109.16 ± 2.39
L45	118.33 ± 3.1
	Ave ± SD

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

Figure 3.15: Weight gain (Production in mg) obtained in *P.monodon* post larvae reared in the presence of putative probionts



Treatments	Average Survival Rate (%)
Control	92 ± 2.1
L18	92 ± 1.87
L39	94 ± 2.6
L45	96 ± 2.95

(Ave ± SD)

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

Figure 3.16: Average survival obtained in *P.monodon* post larvae reared in the presence of putative probionts

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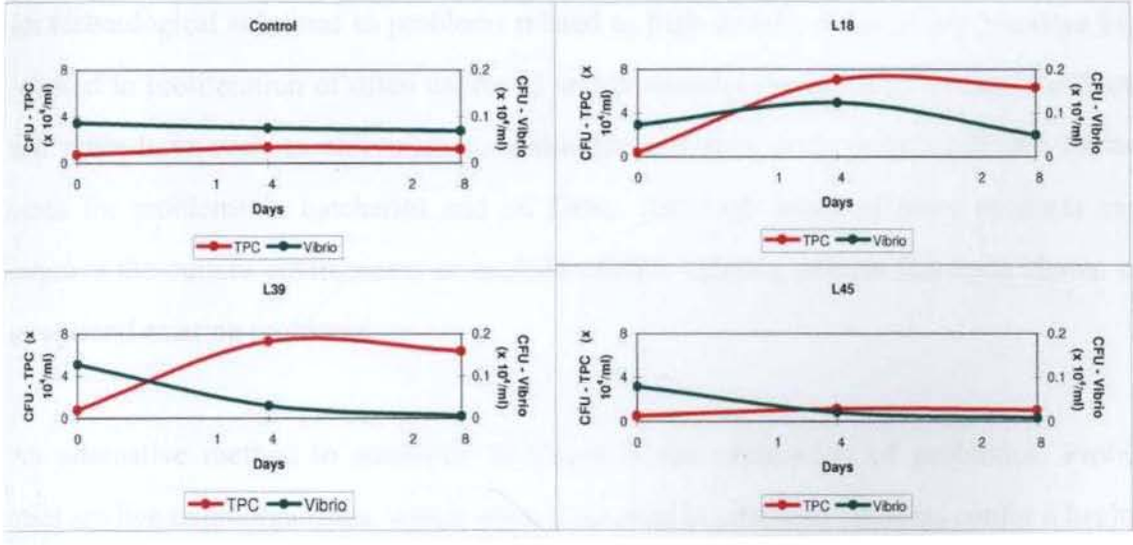


TPC			
Treatments	Initial	14th day	28th day
Control	0.72 ± 0.05^{Ca}	1.132 ± 0.10^{Ab}	1.180 ± 0.05^{Ab}
L18	0.33 ± 0.08^{Aa}	6.07 ± 1.43^{Bb}	5.819 ± 0.76^{Bb}
L39	0.78 ± 0.05^{Ca}	6.70 ± 0.82^{Bb}	5.800 ± 0.79^{Bb}
L45	0.508 ± 0.04^{Ba}	1.105 ± 0.04^{Ab}	1.112 ± 0.12^{Ab}
	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)

Vibrio Count			
Treatments	Initial	14th day	28th day
Control	0.075 ± 0.016^{Aa}	0.066 ± 0.014^{Aa}	0.065 ± 0.007^{Ca}
L18	0.67 ± 0.08^{Aa}	0.067 ± 0.08^{Aa}	0.036 ± 0.021^{BCa}
L39	0.107 ± 0.029^{Ab}	0.024 ± 0.008^{Aa}	0.004 ± 0.001^{Aa}
L45	0.071 ± 0.012^{Ab}	0.017 ± 0.004^{Aa}	0.007 ± 0.001^{ABa}
	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)

Capital superscript indicates group variance with respect to treatments
Small superscript indicates group variance with respect to time

Figure 3.17: Total plate count (TPC) and vibrio count in the rearing water of *P.monodon* post larvae bioassay system in the presence of actinomycetes as probiotics

3.5 Discussion

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

An alternative method to antibiotic treatment is the application of probiotics. Probiotics are live microorgainsms, which when consumed in adequate amounts confer a health benefit to the host, and can be administered as a food supplement or as additive to the water (*Moriarty*, 1998). The present study was undertaken to evaluate the effects of marine actinomycetes as probiotics in *Penaeus monodon* culture systems. Its hydrolytic, antibacterial, pathogenicity and colonisation properties were also investigated. Actinomycetes, although proven to be a rich source of hydrolytic enzymes and antibiotics had rarely been applied in aquaculture systems as prophylactic agents. Although literature pertaining to probiotic effects of actinomycetes in aquaculture are scanty, there are reports showing marine actinomycetes inhibiting *Vibrio* pathogens of shrimp (*You et al.*, 2005) and fish pathogens (*Patil et al.*, 2001).*Mathew* (2003), have reported on the efficacy of marine actinomycetes in reducing the vibrio counts and improving water quality in *P.monodon* rearing tanks

In the current study, 99 isolates of actinomycetes were screened for hydrolytic and antagonistic properties against prawn pathogens. Production of inhibitory substances help in the exclusion of of pathogens from culture systems. *Maeda and Nogami* (1989) reported that bacterial strains possessing vibriostatic activity, could improve the growth of prawn and crab larvae. *Vaseeharan and Ramasamy* (2003) reported the effect of *Bacillus subtilis* BT23 in controlling the growth of pathogenic *Vibrio harveyi* in *Penaues monodon* under *in vitro* and *in vivo* conditions. By applying these bacteria equilibrium between beneficial and deleterious microorganisms can be produced and the results show that the population of *Vibrio* sps., were decreased. In this study, the percentage of actinomycetes inhibitory to prawn pathogens were found to be less. However, a few actinmoycetes exhibited good inhibitory property against prawn pathogens. Maximum inhibition was exhibited by L39 (81.81%) followed by L45 (72.7%) and L18 (45.45%). The antagonistic property of these actinomycetes can be exploited for the control of vibrios in culture systems, the most prevalent pathogen in penaied prawns. This is particularly important in the context of ban of many of the antibiotics in aquaculture. Best choice is bioaugmentation , ie , application of beneficial microbes for the maintenance of desirable microflora and thereby the health of the ecosystem.

All actinomycete isolates possessed proteolytic activity, lipase activity was shown by 96 % followed by amylase activity (95 %) , chitinase (36 %) & cellulase (9.8 %) activity. *Grey and Williams* (1971) reported that actinomycetes could efficiently degrade high molecular weight polymers. Microbes act as source of enzymes which help in digestion thus promoting growth as already reported by *Prieur et al.* (1990). Prawns being detritus feeders, the diet will consist of components like starch, cellulose, chitin etc. Microbes capable of digesting these high molecular weight compounds will definitely contribute to the nutritional enrichment in the intestine . Water quality improvement is

another criteria of importance for as good probiont. Organic load in culture ponds form faecal matter and uneaten food is a major problem. Actinomycetes are gifted with the specific property of degrading high molecular compounds. Hence these microbes are of importance in water quality improvement. In the present study, few actinomycetes were found to produce all the enzymes tested. These strains can very well be used for cleaning the pond water and maintain water qualities at the desired level.

Probiotics selected should be non-pathogenic. Both *in vitro* and *in vivo* studies revealed that the tested actinomycetes (3 Nos.) were non-pathogenic to shrimps and can be safely applied in culture systems . These isolates were obtained from the marine sediments and there are no reports of marine actinomycetes as pathogens in culture systems. The study on colonisation of actinomycetes in the gut revealed that they were not able to colonise the intestine. *Skjermo and Vadstein* (1999) pointed out that bacterial colonisation depends on several factors such as adhesion properties, bacterial attachment site, stress factors, diet and environmental factors. Colonisation of intestine and other tissues of marine larvae take place during early feeding stages and the diversity of ambient flora determines the microbial diversity in the animal (*Hansen and Olafsen*, 1999). If opportunists are dominant, in ambient water, then they will colonise and proliferate. Therefore, it has been suggested that probiotic treatment at early stages significantly impacts survival positively preventing opportunists from making deleterious effect (*Ringo and Vadstein*, 1998). The intestinal flora in prawns depend on the ambient flora of the culture environment and especially the pond bottom since they are detritus feeders. The diet remain in the intestine only for a few hours and therefore the intestinal flora being a direct reflection of the ambient flora and is transient in nature and depends on the feed intake.

Co-culture experiments with *Bacillus subtilis* BT23 and *V. harveyi* revealed that the growth of *V. harveyi* was inhibited by *B.subtilis* at an initial cell density of 10^5-10^9 cfu/ml (*Vaseeharan and Ramasamy*, 2003) .They suggest that the antagonist must be present at significantly higher levels than the pathogen and the degree of inhibition increased with the level of the antagonist. A similar trend was observed in the co-culture experiments with *Pseudomonas* MCCB103 and *V.harveyi* were a cell density greater than 10^5 cfu / ml and 10^6 cfu / ml could significantly eliminate the pathogen (*Pai*, 2006). In the co-culture experiment study with actinomycetes and *V.harveyi* (at a cell density of 10^8 cfu / ml and 10^3 cfu / ml respectively), a gradual decline in *Vibrio harveyi* count was observed for all co-cultures and became undetectable on the third day of incubation. Application of actinomycetes under rearing conditions also revealed that there was a reduction in *Vibrio harveyi* count while the total bacterial count was more or less steady throughout the experimental period. This suggest that actinomycetes could inhibit the proliferation of *vibrios* in aquaculture systems. The actinomycetes also enhanced the survival and production of the larvae showing that these probiotics can significantly improve yield.

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

CHAPTER 4

Immunological Profile of *Penaeus monodon* Against White Spot Virus on Administration of the Selected Probionts

4.1 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 occur as a result of environmental deterioration and stress associated with intensification of shrimp farming. (*Tseng and Chen*, 2004). Many shrimp farms have been particularly affected by epidemics of viruses like white spot syndrome virus (WSSV), Monodon baculovirus (MBV) , Yellow head virus (YHV) , and Taura syndrome virus (TSV) (*Lightner et al.* (1987), *Wang et al.* (2000) & *Smith et al.* (2003)). Several disease outbreaks have also been

associated with vibriosis-causing bacteria like *Vibrio harveyi, Vibrio damsela and Vibrio alginolyticus* (*Song et al.* (1993) & *Lee and Wickins* (1992)). A few diseases had fungal and protozoan aetiologies. (*Destomieux et al.*, 2000).

Disease in the aquatic environment is the end result of a complex interaction among the host, the pathogen and the environment. Though disease prevention is the ultimate goal, it may often be difficult to achieve and therefore, therapeutic measures play a very important role in disease management. Antibiotics, fungicides and parasiticides have been used extensively in aquaculture. (*Aoki*, 1992) indicated that not less than 38 antimicrobials and 22 pesticides are used in Asian aquaculture. 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 of disease management. The need for alternate methods for regulating the number of pathogenic bacteria and also detrimental effects of viruses in aquaculture, have led researchers to turn to different methods of treatments such as probiotics and immunostimulants.

4.1.1 Immunostimulants in Aquaculture

Immunostimulants comprises compounds that enhance the non-specific defence mechanisms and provide resistance against the invading pathogenic microorganisms. The application of antibiotics have resulted in undesirable risk factors, and recently there has been growing interest in finding ways to protect the stock prophylactically in a manner conceptually equivalent to the use of vaccines now routine for humans, farmed fish etc. Perusal of the literature indicated that immunostimulants are proven very successfully in treating / preventing microbial diseases in cultured shellfishes. 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) & *Persson et al.* (1987)).

The immune system in crustaceans includes cellular factors, proPO system, haemolymph coagulation, agglutinins and hemolytic molecules (*Muta and Iwanaga* (1996) & *Vargas-Albores* (1995)). Shrimp immune system has a strong proteinic base. Proteins are involved in recognizing foreign components through lipopolysaccharide binding protein (LPSBP) and β-glucan binding protein (BGBP) (*Vargas-Albores*, 2000). Studies involving stimulation of the immune response help to identify the events or specific metabolites that could serve as physiological parameters for detection of some pathological states, as well as gene expressed under specific conditions. (*Mialhe et al.*, 1995). Immunostimulants that are reported to be effective in fish and shell fish includes synthetic chemicals like levamisole, FK-565, muramyl dipeptide and biological substances like β-glucan , chitin, levamisole, Vitamins C and E, interferon, prolactin etc.

Vitamin C plays a role as immunostimulant, as evidenced by the ability of *P.monodon* post larvae and juveniles to avoid Baculovirus and to resist *V.harveyi* and saline shock (*Catacutan and Lavilla-Pitogo* (1994) & *Merchie et al.* (1998a)). Dietary administration of sodium alginate could enhance ability of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus* (*Cheng et al.*, 2005). Administration of hot water extract from several species of red algae including *Porphyra yezoensis* and *Gloiopeltis furcata* have been reported to increase the resistance of *Cyprinus cyprinus* against *Edwardsiella tarda*, and yellowtail *Seriola quinqueradiata* against *Streptococcus* infection. *Sargassum fusiforme* polysaccharide extracts had enhanced vibriosis resistance and immune activity of *Fenneropenaeus chinensis* (*Huang et al.*, 2006). Oral administration

of crude fuciodan extracted from *Sargassum polycystum* reduced the impact of WSSV infection in *P.monodon.*(*Chotigeat et al.*, 2004).

Administration of chitin by immersion and orally has been reported to increase both humoral and cellular immune responses of gilt head seabream, *Sparus aurata.* Gram-negative Gram-positive (*Soderhall and Duvic*, 1990) bacteria have also been used for activating some factors of immune system in crustaceans (*Johansson and Soderhall* (1989) & *Soderhall et al.* (1990).

Administration of β-1,3-1,6-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). Plant extract from *Lantana camera, Cyanodon dactylon, Aegle marmelos, Ocimum sanctum, Mimosa pudica, Circuma longa* and *Allium sativum* were proved to have prophylactic and therapeutic properties against WSSV in penaeid shrimps *Achuthankutty and Desai* (2004) .Shrimp fed with peptidoglycan responds to an immunostimulant in the same manner as to a microbe aggression. Oral administration of peptidoglycan derived from *Bifidobacterium thermophilum* was found to enhance disease resistance against WSSV in *P.japonicus 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). This chapter is focused on testing the potential of the actinomycete probionts as immunostimulants in *P.monodon.*

4.2 Materials and Methods

4.2.1 Microorganisms Used

Three putative actinomycete probionts (L18 (*Prauseria hordei*), L39 (*Nocardia alba*), L45 *(Streptomyces griseus*)) were used for the study.

4.2.2 Preparation of Actinomycete Biomass

The selected actinomycete cultures (48 hour old) were inoculated onto Marine Actinomycete Agar plates (Starch-1g, Yeast Extract-0.4 g, Peptone-0.2 g, Agar-2g, Seawater -100 ml, pH-7) , incubated at room temperature for 48 hrs and harvested with 0.5 % sterile saline. The cell suspension was centrifuged at 10,000 rpm for 15 minutes (Remi C-30) and the actinomycete biomass was stored at 4°C until used.

4.2.3 Experimental Animals and Rearing Conditions

Adult *Penaeus monodon* (mean body weight,25-30 g) obtained from a commercial farm located at Kannamali, Kochi were used as experimental animals in the present study (*Fig.4.1*). They were transported to the laboratory within 1 hour of capture. Shrimps were reared in rectangular concrete tanks containing 15ppt seawater and allowed to acclimate for a week. Continous aeration was provided and shrimps were fed on a commercial shrimp diet *ad libitum* (Higashimaru, Kochi). Physico-chemical parameters of the rearing water were monitored regularly and salinity, NH_3-N, NO_2-N, NO_3-N and dissolved oxygen were estimated as per APHA (1995) and maintained at optimal level The faecal matter and left over feed were removed daily by siphoning. About 30-40% water exchange was done on alternate days. Biological filter was set up to maintain the appropriate levels of water qual-

parameters. After acclimation for a period of 7 days , the immunological profile was corded from a group of shrimps (n=5) as the baseline data.

Figure 4.1: Experimental Animal (*Penaeus monodon*)) adult used for the study

2.4 Experimental Design

rimps were distributed in the experimental tanks of 500 L capacity (n=30 / tank). There re three treatment groups and one control group. Salinity of all tanks were maintained at ppt throughout the experiment (*Fig.4.2*).

2.5 Experimental Diets

commercial diet (Higashimaru) was used as the basal diet in the study. Three groups of perimental diets were prepared by coating 1% actinomycete biomass (wet wt.) of L18, 39 and L45 (freshly prepared) into the basal diet. Incorporation was done using binder index gel) and stored (4°C) in a refrigerator until use.

Figure 4.2: Bioassay system used to study the efficacy of marine actinomycetes (putative probionts) as immunostimulant to *Penaeus monodon*

4.2.6 Feeding Experiment

Four groups of experimental animals were maintained- Group I-Control, and other groups maintained on different actinomycete biomass incorporated feeds, Group II- L18, Group III- L39 and Group IV-L45. The prawns were fed twice daily, morning 10 A.M and evening 8 P.M, at a rate of 10-15% of their body weight The feeding experiments were carried for a period of 14 days. Physico-chemical parameters of the rearing water were monitored regularly.

4.2.7 WSSV Challenge

After 14 days of feeding experiment the animals were challenged with White Spot Syndrome virus (WSSV) via oral administration of white spot virus infected prawn flesh at the rate of 1g of infected flesh / shrimp. The animals were starved for 12 hrs before the challenge to ensure the intake of infected prawn flesh. 24 hours after challenge, animals were maintained on test feeds . Shrimps were sampled (n=5) at the beginning of the feeding

experiment, just before challenge (15th day) , after 48 hr (post challenge day2, PCD2) and 120 hr of challenge (post challenge day 5, PCD5). Post challenge survival was recorded for a period of seven days with dead animals removed promptly. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of infected shrimps.

4.2.8 Assay of Immunological Parameters

4.2.8.1 Extraction of Haemolymph

An anticoagulant for extraction of haemolymph was prepared (Glucose-2 %, Sodium chloride-2 %, 0.5% EDTA in 0.05 M Tris HCl, pH-7.6) , autoclaved at 10 lbs for 10 minutes and stored at 4°C. Haamolymph was withdrawn aseptically from rostral sinus using specially designed sterile capillary tubes of diameter 0.5 mm , rinsed thoroughly with pre-cooled anticoagulant. The samples were transferred to sterile eppendorf vials containing pre-cooled anticoagulant. Haemolymph collected from five shrimps (n=5) of each treatment group was analysed separately.

4.2.8.2 Total Haemocyte Count

Total haemocyte count (THC) was taken by using a Neubauer improved haemocytometer and expressed as THC ml^{-1} haemolymph.

4.2.8.3 Phenoloxidase (PO) Activity

Phenoloxidase activity of haemolymph was measured by using L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate according to *Soderhall and Unestam* (1979). Briefly 10μl of haemolymph was incubated with 10μl of 1% SLS (Sodium Lauryl Sulphate) for half an hour at 20°C. 200μl of substrate L-DOPA (in 0.05 M Tris- HCL , pH

7) was added using a multichannel pipette and the absorbance at 490 nm was recorded for every 30 seconds for a period of 3 minutes in Tecan Infinite microplate reader. Enzyme activity was expressed as increase in absorbance per minute per 100 μl haemolymph. L-DOPA with distilled water was used as blank.

4.2.8.4 Superoxide Anion (NBT Reduction) Assay

This test allows to indirectly assess the intracellular superoxide anion levels. Nitroblue tetrazolium was used as substrate that gives a blue formazan colour by O_2^- produced during phagocytosis of haemocytes. 70μl haemolymph was taken in an eppendorf tube. NBT solution (2 mg / ml) prepared in 0.05 M Tris HCl buffers (pH 7.6) containing 2 % NaCl was added to the haemolymph and incubated for 1 hr in 10°C. The samples were then centrifuged at 5000 rpm for 5 minutes in a cooling centrifuge. Discarded the supernatant and stopped the reaction by adding 500μl absolute methanol followed by incubation for 10 minutes. Spun down the tubes again, discarded the supernatant and left the tubes for air drying for 30 minutes. The eppendorf tubes was then rinsed with 500μl 50% methanol thrice to fix the residue. This residue was then allowed to solubilize with 120μl of 2M KOH. 140μl DMSO (Dimethyl sulphoxide) was added and mixed well. 1 ml of distilled water added to this colored solution to make it to a readable volume. 200μl from this was transferred to microtitre plate and optical density was read at 620 nm in Tecans Infinite 200 microplate reader against a blank. The blank was prepared using all the above reagents in the same volume with equal volume of distilled water instead of haemolymph. NBT activity was expressed per 100μl haemolymph.

4.2.8.5 Alkaline Phosphatase Assay

Alkaline phosphatase catalyse the hydrolytic cleavage of phosphoric acid esters and their pH optima lie in the alkaline pH range of 9.0. The procedure was carried out according to *Romey et al.* (1999). 10μl of heamolymph was added to 1.1 ml of p-nitrophenyl phosphate substrate solution (2 % in Glycine- NaOH buffer, pH 9.0). The mixture was incubated at 37°C for half an hour. At the end of the reaction period the enzyme reaction was terminated by adding 2 ml of 0.1N NaOH. The yellow coloured solution was read against a blank at 405 nm after transferring 200μl to microtiter plate. The blank was prepared by incubating a mixture of 2 ml of 0.1 N NaOH and 1.1 ml of substrate solution to which 10 μl distilled water was added.

4.2.8.6 Acid Phosphatase Assay

Acid phosphatases were assayed according to *Reichardt et al.* (1967) using p-nitrophenyl phosphate , a colourless substrate that produces a colorimetric end product p-nitrophenol. The buffer substrate mixture for the assay was prepared by dissolving 2% in citrate buffer. The mixture was incubated at 37°C for 30 minutes. The incubation period of enzyme reaction was terminated by adding 2 ml of 0.1 N NaOH. The yellow coloured solution was read against blank at 405 nm using Tecans Infinite 200 microplate reader.

4.3 Statistical Analysis

In order to determine significant difference if any, in immunological parameters between the different treatment groups, the results were analyzed using one way analysis of variance (ANOVA) and Duncans multiple comparison of the means by using SPSS 14.0 for windows. The level of significance was set at $p< 0.05$.

4.4 Results

4.4.1 Total Haemocyte Count

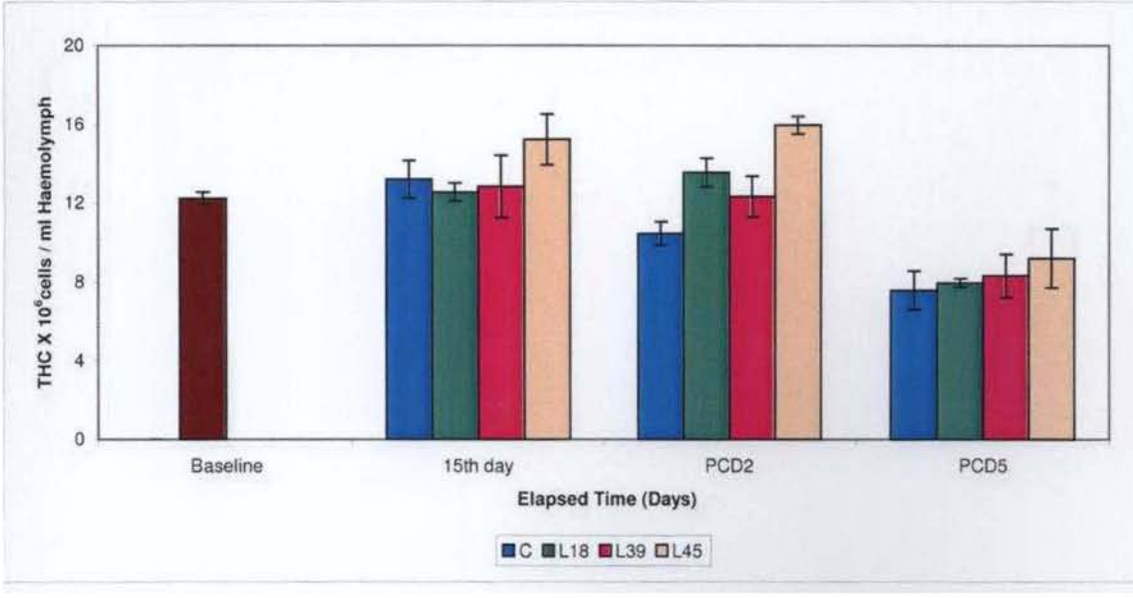
Shrimps fed with actinomycete incorporated feeds showed a higher number of total haemocytes when compared to control. There was no significant variation in the total haemocyte count among various treatment groups. THC was comparatively high in L45 fed animals (15.959 ± 0.444 x10^6 cells/ml) followed by L39, L18 and control (13.2 ± 0.958 x 10^6 cells/ml). There was no significant variation in THC before and after challenge. A substantial increase in THC was observed for L18 and L45 fed animals on post challenge day 2 and a sudden decrease in THC was observed on post challenge day 5 for all treatment groups. (*Fig.4.3*).

4.4.2 Phenoloxidase (PO) Activity

Phenol oxidase activity was higher in the haemolymph of shrimps fed with actinomycete incorporated feeds when compared to control There was significant variation ($p< 0.05$) in the phenol oxidase activity between the different treatment groups. PO activity was significantly high in L45 fed animals (0.709 ± 0.099) followed by L39, L18 and control fed animals ((0.345 ± 0.021). A substantial increase in PO was observed for control and L18 on post challenge day 2 and a sudden decrease on post challenge day 5 for all treatment groups . (*Fig.4.4*).

4.4.3 Superoxide Anion (NBT reduction) Activity

The intracellular superoxide anion production was higher in shrimps fed with actinomycete incorporated feeds when compared to control There was no significant variation in the NBT value among various treatment groups. NBT value was significantly high in L45 fed

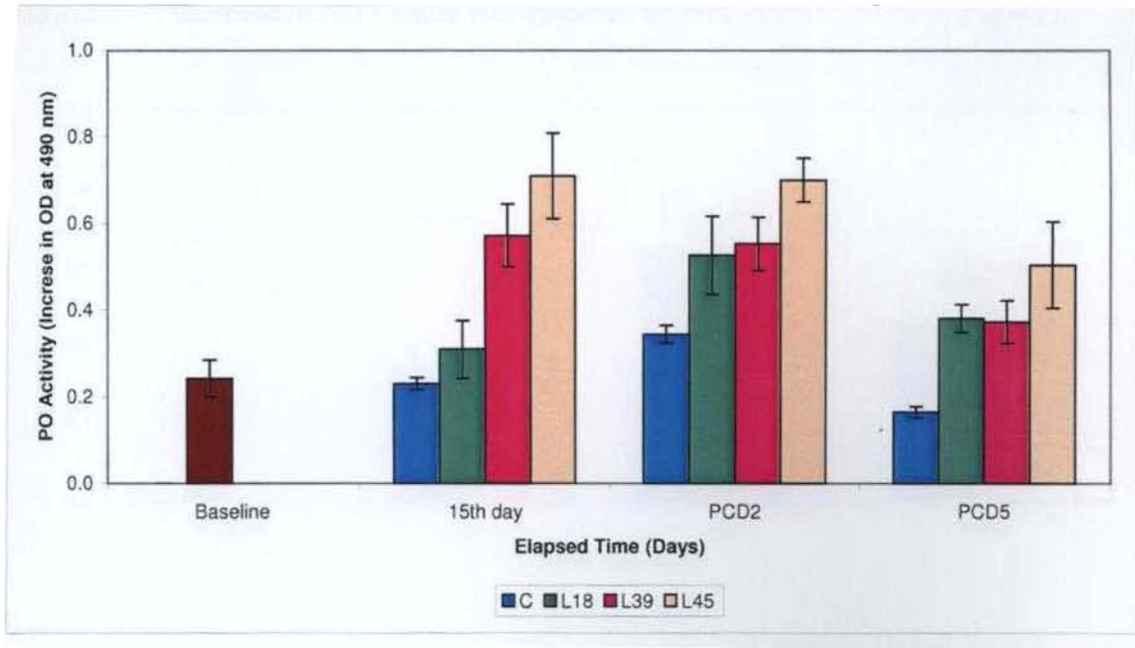


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	12.237 ± 0.327	13.200 ± 0.958 ^{Aa}	10.452 ± 0.594 ^{Aa}	7.571 ± 0.976 ^{Aa}
L18	12.237 ± 0.327	12.546 ± 0.454 ^{Aa}	13.543 ± 0.730 ^{Aa}	7.947 ± 0.217 ^{Aa}
L39	12.237 ± 0.327	12.834 ± 1.583 ^{Aa}	12.327 ± 1.040 ^{Aa}	8.312 ± 1.103 ^{Aa}
L45	12.237 ± 0.327	15.224 ± 1.290 ^{Aa}	15.959 ± 0.444 ^{Aa}	9.194 ± 1.497 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 4.3: (Mean ±SD) THC of *P.monodon* fed on various actinomycete diets and then challenged with WSSV



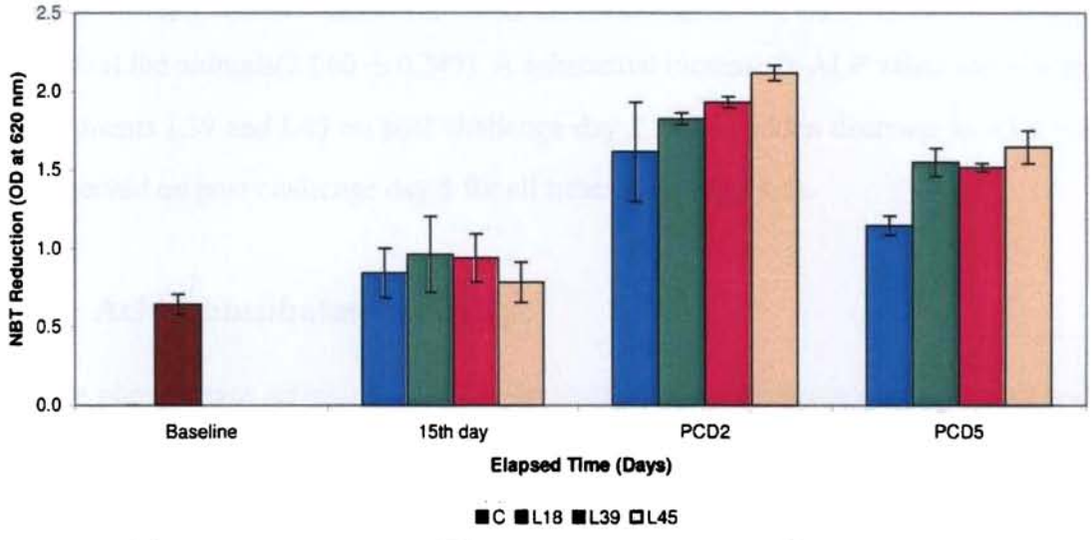
PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	0.242 ± 0.043	0.230 ± 0.014 ^{Aa}	0.345 ± 0.021 ^{Aa}	0.165 ± 0.013 ^{Aa}
L18	0.242 ± 0.043	0.310 ± 0.067 ^{ABa}	0.526 ± 0.090 ^{ABa}	0.381 ± 0.032 ^{Ba}
L39	0.242 ± 0.043	0.572 ± 0.072 ^{ABa}	0.553 ± 0.061 ^{BCa}	0.373 ± 0.049 ^{Ba}
L45	0.242 ± 0.043	0.709 ± 0.099 ^{Ba}	0.700 ± 0.051 ^{Ca}	0.504 ± 0.100 ^{Ca}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 4.4: (Mean ±SD) PO value of *P.monodon* fed on various actinomycete diets and then challenged with WSSV

animals (2.117 ± 0.048) followed by L39, L18 and control fed animals (1.613 ± 0.316). There was no significant variation ($p<0.05$)in NBT value before and after challenge. A substantial increase in NBT value was observed for all treatments on post challenge day 2 and a sudden decrease in NBT value was observed on post challenge day 5.(*Fig.4.5*).



PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	0.643 ± 0.065	0.844 ± 0.157 ^{Aa}	1.613 ± 0.316 ^{Aa}	1.144 ± 0.061 ^{Aa}
L18	0.643 ± 0.065	0.961 ± 0.243 ^{Aa}	1.825 ± 0.038 ^{Aa}	1.547 ± 0.090 ^{Aa}
L39	0.643 ± 0.065	0.940 ± 0.152 ^{Aa}	1.931 ± 0.035 ^{Aa}	1.515 ± 0.026 ^{Aa}
L45	0.643 ± 0.065	0.784 ± 0.128 ^{Aa}	2.117 ± 0.048 ^{Aa}	1.644 ± 0.105 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 4.5: (Mean ±SD) NBT value of *P.monodon* fed on various actinomycete diets and then challenged with WSSV

4.4.4 Alkaline Phosphatase Acitivty

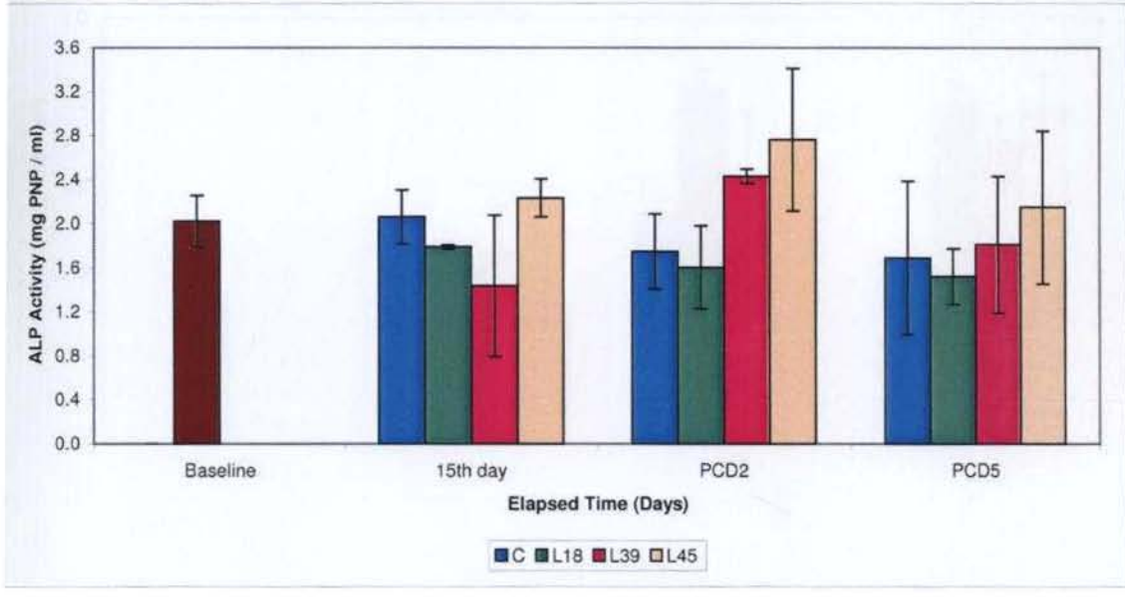
Alkaline phosphatase activity was higher in shrimps fed with actinomycete incorporated feeds when compared to control. There was significant variation in alkaline phosphatase activity among treatment groups except on post challenge day 2. Alkaline phosphatase activity was significantly high in L45 fed animals (2.763 ± 0.648) followed by L39, L18 and control fed animals(2.060 ± 0.243). A substantial increase in ALP value was observed for treatments L39 and L45 on post challenge day 2 and a sudden decrease in ALP value was observed on post challenge day 5 for all treatments. (*Fig.4.6*).

4.4.5 Acid Phosphatase Activity

Alkaline phosphatase activity was higher in shrimps fed with actinomycete incorporated feeds when compared to control . There was no significant variation among the various treatment groups. Acid phosphatase activity was significantly high in L18 fed animals followed by L45, L39 and control fed animals. Acid phosphatase activity was significantly high for L45 and L39 on PCD5. The activity increased significantly post challenge in all the treatment groups. (*Fig.4.7*).

4.4.6 Effect of Actinomycete Incorporated Diet on the Susceptibility of *Penaeus monodon* to White Spot Virus

The effect of actinomycete incorporated diet on susceptibility of *P.monodon* to white spot virus was tested. Mortality was not reported during the fourteen days of the experimental period. However, 100% mortality was reported for control on the 6^{th}day of challenge. Shrimps fed with L18, L39 and L45 incorporated diets showed 20%, 50% and 62.5% survival respectively on 7^{th} day post challenge (*Fig.4.8*).

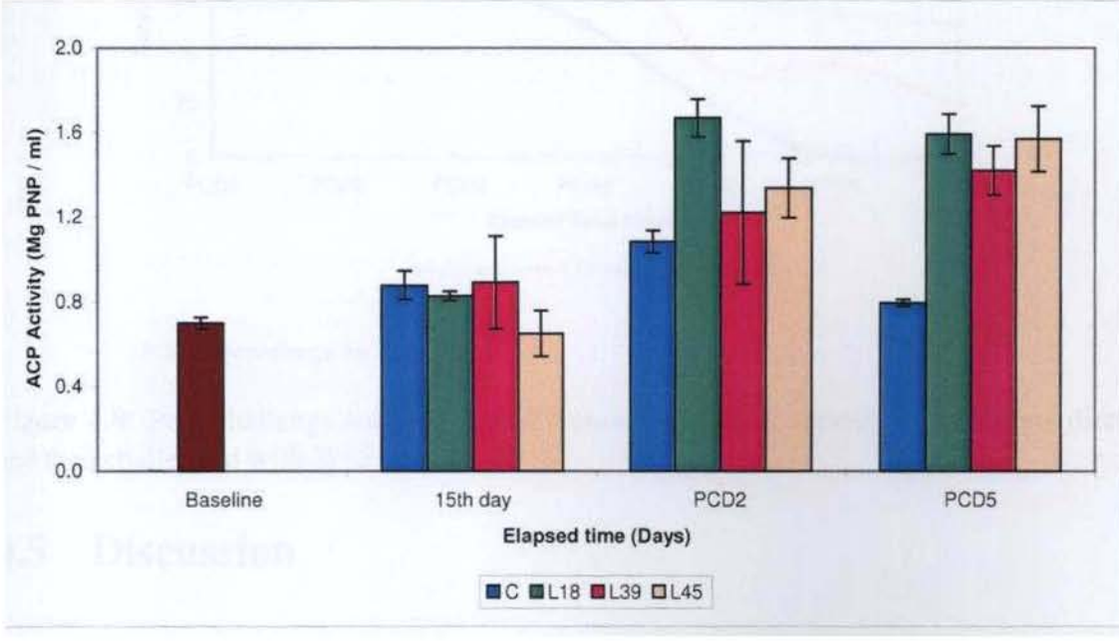


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	2.020 ± 0.234	2.060 ± 0.243 ^{ABa}	1.747 ± 0.341 ^{Aa}	1.690 ± 0.696 ^{ABa}
L18	2.020 ± 0.234	1.788 ± 0.019 ^{Aa}	1.603 ± 0.376 ^{Aa}	1.520 ± 0.253 ^{ABa}
L39	2.020 ± 0.234	1.435 ± 0.639 ^{Ba}	2.430 ± 0.065 ^{Aa}	1.808 ± 0.623 ^{Aa}
L45	2.020 ± 0.234	2.231 ± 0.172 ^{ABa}	2.763 ± 0.648 ^{Aa}	2.148 ± 0.694 ^{Ba}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 4.6: (Mean ±SD) ALP value of *P.monodon* fed on various actinomycete diets and then challenged with WSSV

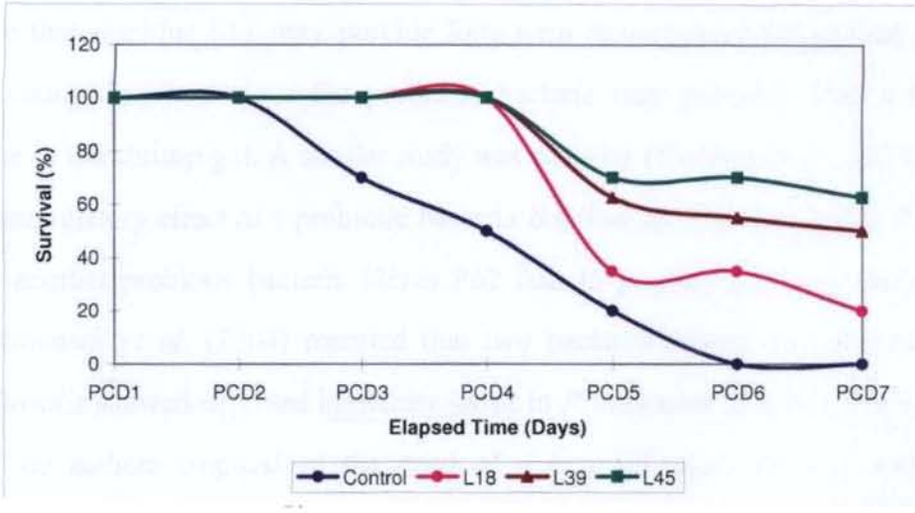


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	0.700 ± 0.026	0.878 ± 0.068 ^{Aa}	1.083 ± 0.052 ^{Aa}	0.795 ± 0.016 ^{Aa}
L18	0.700 ± 0.026	0.827 ± 0.022 ^{Aa}	1.667 ± 0.090 ^{Aa}	1.593 ± 0.095 ^{Aa}
L39	0.700 ± 0.026	0.891 ± 0.218 ^{Aa}	1.220 ± 0.338 ^{Aa}	1.421 ± 0.116 ^{Aa}
L45	0.700 ± 0.026	0.650 ± 0.108 ^{Aa}	1.337 ± 0.141 ^{Aa}	1.571 ± 0.155 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 4.7: (Mean ±SD) ACP value of *P.monodon* fed on various actinomycete diets and then challenged with WSSV



PCD - Post challenge day

Figure 4.8: Post challenge survival (%) of *P.monodon* fed on various actinomycete diets and then challenged with WSSV

4.5 Discussion

Reports on enhanced survival and evidence of immunostimulation due to purified components like β-glucan and LPS are more numerous when compared to whole cells. In this study the immunostimulatory potential of whole actinomycete was compared with the control feed. Of the three test feeds, L45 (*Streptomyces griseus*) showed better immunostimulation during the feeding experiment before and after challenge.

A few workers have reported on the ability of live microbial culture in enhancing the disease resistance of shrimp. *Rengipipat et al.* (2000) reported greater immunity indices (total haemocyte count, phagocytic activity, phenol oxidase activity and serum antibacterial activity) in *Penaeus monodon* fed with live bacterium *Bacillus* S11. *Bacillus* S11 surface antigens or their metabolites might have acted as immunogens for shrimp immune defense. *Bacillus* S11 peptidoglycan might elicit an immune function in shrimps (*Itami et al.*, 1998) by acting on granulocytes for higher phagocytic activity. The authors

speculate that *Bacillus* S11 may provide long term immunostimulation than glucan or other immunostimulants since the probiotic bacteria may probably find a long term resistance in the shrimp gut. A similar study was done by (*Gullian et al.*, 2003) in which immunostimulatory effect of a probiotic bacteria *Bacillus* sp. was reported in *P.vannamei* whereas another probiotic bacteria *Vibrio* P62 fails to produce any immunostimulatory effect. *Alavandi et al.* (2004) reported that two bacterial strains *Pseudomonas* sp.and *Vibrio fluvialis* showed deprived immunity index in *P. monodon* in spite of their probiotic nature. The authors emphasized the need of a new selection protocol incorporating immunostimulation potential for identifying and employing probiotics for penaeid culture systems.

The total hemocyte count in the group fed with actinomycete incorporated diets was higher when compared to control. THC was significantly high in L45 fed animals followed by L39, L18 and control fed animals. A substantial increase in THC was observed for L18 and L45 treatments on day 2 post challenge and a sudden decrease in THC was observed on post challenge day 5 for all treatment groups. Even though there is a marginal drop in total hemocyte count after WSSV infection, L45 maintained a higher TPC value till the end of the experiment. *Tsing* and *Braak et al.* (2002) reported that an increase in circulation of young and immature haemocyetes might have been an indicator of an intense proliferation of haematopoetic tissue. A slight drop in the THC just after challenge was noticed in all the test groups including control, but during the subsequent days it regained. So this may be due to the mobilization or proliferation of haemocytes on recognizing the pathogen. A probable explanation for the slight decrease in total haemocyte count at the beginning of infection could be the infiltration of haemocytes, especially the semigranualr cells to connective tissue ,stomach and gills in the WSSV infection as reported by (*Munoz et al.*, 2002).

Prophenol oxidase, the key enzyme in the synthesis of melanin, occurs in hemolymph as an inactive pro-enzyme. Prophenol is activated to form phenol oxidase when it reacts with cell wall components. PO activity was significantly high in L45 fed animals followed by L39 , L18 and control groups. A substantial increase in PO was observed for L18 and control on day 2 post challenge and a sudden decrease in PO was observed for all treatment groups on post challenge day 5. NBT level was significantly high in L45 fed animals followed by control, L39 , L18 and control groups. A substantial increase in NBT level was observed for all treatments on day 2 post challenge and a sudden decrease in NBT level was observed on post challenge day 5. A similar type of enhancement in immune responses to WSSV infection was observed on post challenge day two and post challenge day three in *Fenneropenaeus indicus* fed on marine yeast (10 %) incorporated diets (*Sajeevan et al.*, 2006). Generally, there is a gradual increase in NBT level post challenge, and this can be attributed to an increase in phagocytosis resulting in the production of more super oxide anions for checking infection.

Results from several experiments have implied that apart from their role in melanization, components of the putative proPO activating system stimulate several cellular defence reactions, including phagocytosis, nodule formation, encapsulation, and hemocyte loco-motion. Shrimps fed with β-glucan diets showed a significantly higher ($P< 0.001$) proPO concentration than the control diet. After viral challenging the value initially dropped and reached a maximum level at the 9^{th} day (*Chang et al.*, 2003). The total haemocyte count (THC) and O_2^- production also started recovering after 6 days post challenge. A similar result in proPO and O_2^- levels was obtained for the herbal extracts administered to *Penaeus monodon* challenged with WSSV. Increasing proPO and O_2^- production with the herbal extracts seem to act as a promoter of shrimp immune system against the WSSV infection. Phagocytes have a unique membranous enzyme, NADPH oxidase, capable of one-electron

reduction of molecular oxygen into superoxide anion (O_2^-) during a process known as the respiratory burst. Being the first product released during the respiratory burst, O_2^- concentration is widely accepted as an accurate measure quantifying the intensity of a respiratory burst .

Phosphatases play an important role in acute energy crisis in aquatic organisms and are involved in cytolysis and differentiation processes. In addition, phosphatases is the most important element of lysozomal enzymes in crustacean cells, which perform the double function of digestion and defence *Jiang and Mu* (1999). Both acid and alkaline phosphatases are composed of many kinds of phosphomonoesterases, which is considered to be very important in crustacean immune system. Alkaline phosphatase is an intrinsic plasma membrane enzyme of almost all animal cells . ACP is released during phagocytosis, nodule and capsule formation to hydrolyse phosphate groups of the invaders. Alkaline phosphatase (ALP) directly take part in the transfer of phosphate groups and metabolism of calcium and phosphates. In the present study, alkaline phosphatase activity was significantly high in L45 fed animals followed by L39, L18 and control groups. Acid phosphatase activity was significantly high ($p< 0.05$) in L18 fed animals followed by L45,L39 and control groups. The activity increased significantly post challenge in all the treatment groups .The survival rate of actinomycete fed shrimps was comparatively greater than the controls. 100 % mortality was observed for control while shrimps fed on L18, L39 and L45 incorporated diets showed 20%, 50 % and 62.5% survival respectively on post challenge day 7.

The route of choice may be an important factor in determining the success of the treatment in enhancing protection against disease, although the diversity of agents used, species tested and methods of study has made general recommendations difficult to

establish (*Raa* (1996) & *Sakai* (1999)). Even though many authors have reported that administration by injection is the most efficacious (*Sakai*, 1999), it is extremely labour intensive and costly. Further, it often represents an additional stress to the cultured stock. Consequently, administration by immersion or as a dietary component has proved to be the preferred option within commercial shellfish farms.

In conclusion, the present study documents that actinomycete incorporated diet increased the immunity of *P.monodon* by increasing its phenol oxidase activity, and production of superoxide radicals together with an increase in resistance against WSSV . Moreover, the halo tolerant property of marine actinomycetes is an added advantage as it can be applied to the penaied shrimp culture systems which is practiced in brackish or seawater conditions with salinities ranging from 15 ppt to 35 ppt. However, the frequency and dosage of administration of actinomycetes are vital for a positive and long lasting effect.

CHAPTER 5

Antioxidant Profile of *Penaeus monodon* Against White Spot Virus on Administration of the Selected Probionts

5.1 Introduction

Evidences indicate that the health of aquatic organisms might be linked to the deleterious consequences of oxygen utilization (*Giulio et al.* (1989) & *Livingston et al.* (2000)) . Aquatic life is dependant on molecular oxygen (O_2) for provision of energy through coupling of oxidation to energy transfer via phosphorylation of ADP. This process is handled by mitochondrial electron transport chain in which oxygen undergoes a concerted four electron reduction to water (*Sole et al.*, 1996) . As a consequence of concerted reduction, partially reduced oxygen results and various reactive oxygen species (ROS) or oxyradicals are produced. Approximately, 0.1-0.2% of the oxygen consumed by aerobic

cells is converted to reactive oxygen species in the step-wise reduction of O_2 during cell respiration (*Fridovich*, 2004). Reactive oxygen species (ROS), including free radicals that have an unpaired electron in their outer orbit, are highly reactive molecules. These can damage cellular macromolecules, such as membrane lipids, proteins, carbohydrates and nucleotides (*Halliwell and Gutteridge*, 2001). ROS induced damage in aerobic animals and humans are related to many diseases, such as atherosclerosis, ischemia-reperfusion, stroke, nutritional encephalomalacia, dietary hapatosis and is known to accumulate with age (*Reiter* (1995) & *Chihuailaf et al.* (2002)).

Chemical compounds and reactions that are involved in generation of free radicals or reactive oxygen species are referred to as prooxidants. Compounds that dispose off these species, scavenging them or suppressing them are called as antioxidants. In a normal cell, there is an appropriate prooxidant ; antioxidant balance. If balance shifts towards prooxidants, it creates oxidative stress. Oxidative stress has been defined as a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses. When an organism is subjected to stresses such as chemical, physical, biological (i,e. pathogen infection) , abnormal oxidative reactions in the aerobic metabolic pathway results in the formation of excess amounts of singlet oxygen and subsequently generates radicals (or free radicals). Radical damage can be significant because it can proceed as a chain reaction. Consequently, mortality can occur due to severe destruction by massive oxyradicals .

Naturally occurring substances that neutralize the potential ill effects of singlet oxygen and radicals are generally grouped into the antioxidant defense system (*Yu*, 1994).The antioxidant defense mechanism comprises first line, second line and third line defenses. Several vitamins, micronutrients, enzymes such as superoxide dismutase (SOD), glu-

tathione peroxidase (GPX), catalase (CAT) constitute first line of defense. SOD act by quenching superoxide anions (O_2^-) and converts it to H_2O_2 , GPX a selenium containing enzyme catalyses reduction of H_2O_2 and lipid hydroperoxide to H_2O using reduced glutathione (GSH) as substrate, and catalase acts by decomposing H_2O_2 to H_2O and O_2. In cytosolic and mitochondrial compartments the oxidised glutathione (GSSH) is reduced at the expense of NADPH by the ubiquitous flavin containing enzyme glutathione reductase. Glutathione - S-Transferase (GST) are a group of detoxifying enzymes that catalyse the conjugation of reduced glutathione with a variety of compounds bearing suitable electrophilic centers in them (*Boyland and Chasseaud*, 1969). The second line of defense include glutathione (GSH) , Vitamin C, uric acid, albumin, bilirubin, Vitamin E (α- tocopherol), carotenoids, flavanoid and ubiquinol. Glutathione is the most abundant non-protein thiol, synthesized in the liver and acts as a substrate for glutathione peroxidase enzyme. This also serves to protect the cell from oxidative damage by reducing the disulphide groups of proteins and other cellular molecules and also scavenge different free radicals leading to the formation of oxidized glutathione and other disulphides (*Kosower and Kosower*, 1978). Similarly β-carotene (pro-vitamin A), Vitamin C and Vitamin E are some important scavenging antioxidant vitamins which cannot be synthesized by most animals. The third line of defense comprises a group of enzymes for repair of damaged DNA, damaged protein, oxidized lipids and peroxides and also to stop chain propagation of peroxyl lipid radical. Example-lipase, protease, DNA repair enzymes, transferase and sulphoxide reductase besides methionine.

Exposure to environmental stressors is an inescapable aspect of an aquatic organism's life. These stresses include temperature, pH, light, oxygen, salt, pollutants, infection and so on. Under natural conditions, oxidative stress will increase the activities of detoxifying enzymes. But when the organism is under pathogenic stress (bacterial, fungal

and viral infection) there is an increased level of lipid peroxidation, followed by a substantial decrease in the activity of antioxidant enzymes. Virus induced oxidative stress associated with the activation of phagocytosis is reported to be responsible for the release of ROS (Reactive oxygen species) (*Schwarz*, 1996). In *Penaeus monodon*, the phagocytic activity of haemocytes is less efficient in oxygen depleted shrimp (*Direkbusarakom and Danayadol*, 1998). ROS are known to be important components of the defense responses of crustaceans to invading microorganisms like bacteria, fungi and viruses (*Munoz et al.*, 2000). ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen and hydroxyl radical (OH^-) are highly microbicidal (*Bachere et al.* (1995) & *Munoz et al.* (2000)).

Biological antioxidants are compounds that protect biological systems against harmful effects of free radicals (*Palozza and Krisky*, 1992).The effect of biological antioxidants like Vitamin E and carotenoids are well studied. Blue green algae contain a significant amount of carotenoids namely β-carotene, lycopene and lutein. By their quenching action on reactive oxygen species, antioxidants carry intrinsic anti-inflammatory properties. β-carotene is recognized as a lipid antioxidant, a free radical trap and quencher of singlet oxygen. Phycocyanin, was shown to inhibit inflammation in mouse ears and prevent acetic acid induced colitis in rats. This was attributed to the reduced formation of leukotriene B4 , an inflammatory metabolite of arachidonic acid (*Romay et al.*, 1999).

In *P.monodon* culture, administration of astaxanthin increased survival during culture (*Thongrod et al.*, 1995). *Chein et al.* (1999) demonstrated that under the stress of oxygen depletion tiger prawn juveniles fed a diet containing a high level of astaxanthin had higher survival than that of control animals. An enhancement of resistance to salinity stress in penaeid shrimp post larvae was associated with an increase in dietary and

body astaxanthin (*Chein et al.*, 1999). In those studies, a close relationship between the antioxidant properties of astaxanthin and the stress resistance was indicated by an increase in prawn survival. The administration of Vitamin C, an antioxidant had enhanced the resistance of *P.monodon* post larvae to WSSV and *V.harveyi* infections (*Catacutan and Lavilla-Pitogo* (1994) & *Merchie et al.* (1998b)). *Litopennaeus 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* and showed a lower GPX activity when compared to control. (*Cheng et al.*, 2005). The effect of supplemental vitamin C, in the form of L-ascorbyl-2-polyphosphate in enriched live food dietary (*Artemia*) on oxyradicals and antioxidant enzyme activities in *Litopenaeus vannamei* exposed to ammonia-N were studied and found that the antioxidant activities (SOD, CAT, GPX, GST and GR) increased in shrimps fed with enriched Artemia when compared to shrimps fed with starved *Artemia* diet (*Wang et al.*, 2006).

In immune defense research of crustaceans, a thorough understanding of haemocyte function is important, but has not been studied in detail, particularly the capacity to generate oxidative and antioxidant responses (*Roch*, 1999). Superoxide dismutase (SOD) is one of the main antioxidant pathways in response to oxidative stress (*Fridovich*, 1995). Increased SOD activity in *Litopenaues vannamei*, after challenge, appeared earlier in haemocyetes and muscle. In addition, SOD activity decreased in haemocytes after 48h exposure to immunostimulants (*Campa-Cordora et al.*, 2002) similar to the enzymatic activity reported in *Palaemonetes argentius* shrimp related to oxidative stress (*Kosower and Kosower*, 1978). *Downs et al.* (2001) has reported increased levels of Mn-SOD, glutathione, heat shock proteins and ubiqiutin in grass shrimp *Palaemonetes pugio* after heat stress, specifically in response to increased protein synthesis and denaturation (*Ellis*, 1996), indicating that mitochondria is experiencing and responding to oxidative stress.

Antioxidants are potential indicators of oxidative stress in marine organisms (*Agius et al.* (1998), *Kosower and Kosower* (1978) & *Downs et al.* (2001)). White spot syndrome virus (WSSV) is a pathogen of major economic importance in shrimp aquaculture throughout the world. The activities of lipid peroxidation, total reduced glutathione, SOD, CAT, glutathione peroxidase, glutathione-S-Transferase, glutathione reductase and membrane bound ATPase are analyzed in WSSV-infected and uninfected tissues of *Penaeus monodon.* It has been reported that when *P.monodon* was challenged with WSSV, lipid peroxidation increased significantly in all tissues whereas the activity of antioxidants and membrane bound enzymes declined sharply (*Rameshthangam and Ramasamy*, 2005).Viruses can affect the host cell pro-antioxidant balance by increasing cellular pro-oxidants such as iron and nitric oxide and also by inhibiting the synthesis of antioxidant enzymes (*Moriarty*, 1998).

Against this background, there is an urgent need to develop a strategy that could enhance the activity of antioxidant enzymes or could themselves act as antioxidants scavenging the oxyradicles so as to protect shrimps from the deleterious consequences of infection.The aim of this study was to assess the effect of actinomycetes on antioxidant responses of *Penaeus monodon* to white spot viral infection. Antioxidant enzymes such as gutathione peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and total reduced glutathione and malondialdehyde in various tissues (haemolymph, muscle, gills and hepatopancreas) were determined. The concentration of enzymes and glutathione were estimated and expressed per milligram of protein in the corresponding tissues and therefore protein content of the tissues were determined.

5.2 Materials and Methods

5.2.1 Microorganisms Used

Actinomycetes (3 Nos.), (L18 (*Prauseria hordei*), L39 (*Nocardia alba*), L45 *(Streptomyces griseus*)) were selected for studying its efficacy as source of antioxidants in *Penaeus monodon* .

5.2.2 Preparation of Actinomycete Biomass

The selected actinomycete cultures (48 hour old) were inoculated onto Marine Actinomycete Agar plates (Starch-1g, Yeast Extract-0.4 g, Peptone-0.2 g, Agar-2g, Seawater -100 ml), pH-7) , incubated at room temperature for 48 hrs and harvested with 0.5% sterile saline. The cell suspension was centrifuged at 10,000 rpm for 15 minutes (Remi C-30) and the actinomycete biomass was stored at 4°C until used.

5.2.3 Experimental Animals and Rearing Conditions

Adult *Penaeus monodon* (mean body weight, 25-30 g) obtained from a commercial farm located at Kannamali, Kochi were used as experimental animals in the present study.They were transported to the laboratory within 1 hour of capture. Shrimps were reared in rectangular concrete tanks containing 15ppt seawater and allowed to acclimate for a week. Continous aeration was provided and shrimps were fed on a commercial shrimp diet *ad libitum* (Higashimaru, Kochi). Physico-chemical parameters of the rearing water were monitored regularly and salinity, NH_3-N, NO_2-N, NO_3-N and dissolved oxygen were estimated as per APHA (1995) and maintained at optimal level . The faecal matter and left over feed were removed daily by siphoning. About 30-40% water exchange was done on alternate days. Biological filter was set up to maintain the appropriate levels of water quality parameters.

After acclimation for a period of 7 days , the antioxidant profile was obtained from a group of shrimps (n=5) as the baseline data.

5.2.4 Experimental Design

Shrimps were distributed in the experimental tanks of 500 L capacity (n=30 / tank). There were three treatment groups and one control group. Salinity of all tanks were maintained at 15ppt throughout the experiment (*Fig.4.2*).

5.2.5 Experimental Diets

A commercial diet (Higashimaru) was used as the basal diet in the study. Feeding experiments were done for each actinomycete isolate (L18, L39,L45) incorporated diet along with a control diet (normal feed) for a period of 14 days.Three groups of experimental diets were prepared by coating 1% actinomycete biomass of L18, L39 and L45 into the basal diet. Incorporation was done using binder (Bindex gel) and dried at room temperature (28 ± 2°C).

5.2.6 Feeding Experiment

Four groups of experimental animals were maintained- Group I-Control, and other groups maintained on different actinomycete biomass incorporated feeds namely, Group II- L18, Group III- L39 and Group IV-L45. The prawns were fed twice daily, morning 10 A.M and evening 8 P.M, at a rate of 10-15% of their body weight The feeding experiments were carried for a period of 14 days. Physico-chemical parameters of the rearing water were monitored regularly and salinity, NH_3-N, NO_2^-N, NO_3-N and dissolved oxygen were estimated as per APHA (1995) and maintained at optimal level.

5.2.7 WSSV Challenge

After 14 days of feeding experiment the animals were challenged with White Spot Syndrome virus (WSSV) via oral administration of white spot virus infected prawn flesh at the rate of 1g of infected flesh / shrimp. The animals were starved for 12 hrs before the challenge to ensure the intake of infected prawn flesh. 24 hours after challenge, animals were maintained on test feeds . Shrimps were sampled (n=5) at the beginning of the feeding experiment, just before challenge (15th day) , after 48 hr (post challenge day 2, PCD2) and 120 hr of challenge (post challenge day 5, PCD5). Infection with WSSV was confirmed by examining the carapace of dead animals for white spots on it. Post challenge survival was recorded for a period of seven days with dead animals removed promptly.

5.2.8 Extraction of Heamolymph

An anticoagulant for extraction of heamolymph was prepared (Glucose-2%, Sodium chloride-2%, 0.5% EDTA in 0.05 M Tris HCl, pH-7.6) , autoclaved at 10 lbs for 10 minutes and stored at 4°C. Heamolymph was withdrawn aseptically from rostral sinus using specially designed sterile capillary tubes of diameter 0.5 mm , rinsed thoroughly with pre-cooled anticoagulant. The samples were transferred to sterile eppendorf vials containing pre-cooled anticoagulant. 300 μl of heamolymph was diluted to 1.75 ml using 0.1 M Tris-HCl buffer, pH 7.5 and the samples stored at -20°C. Heamolymph collected from five shrimps (n=5) of each treatment group was analysed separately.

5.2.9 Preparation of Tissue Homogenate

The gill, muscle, hepatopancreas and heamolymph of *Penaeus monodon* adult was taken for the study. 0.1 g of gill , muscle tissue and hepatopancreas were homogenized in 3.0 ml 0.1 M Tris HCl buffer, pH 7.5. Gill and muscle homogenate was diluted to 5 ml, while

hepatopancreas was diluted to 11 ml. The homogenate was centrifuged at 7000 rpm for 10 minutes and the supernatant was collected and stored at -20°C for further analysis.

5.2.10 Antioxidant Assays

Antioxidant enzymes such as gutathione peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and the total reduced glutathione content and malondialdehyde (byproduct of lipid peroxidation) in various tissues were determined. The concentration of enzymes and glutathione were estimated and expressed as per milligram of protein in the corresponding tissues and therefore protein content of the tissues were determined. Protein determination was done by employing the Bradford method using Coomassie Brilliant Blue G-250 (OD at 595 nm).

5.2.10.1 Estimation of Protein

To 100 μl heamolymph and tissue sample 900 μl 95% ethanol was added and centrifuged. The supernatant was discarded and the residual pellet consisting of precipitated protein was dissolved in 1N NaOH and used for protein estimation by Bradford method (*Bradford*, 1976).

5.2.10.2 Estimation of Catalase (CAT)

Catalase in different tissues was determined using method of *Machly and Chance* (1955). The estimation was done following decrease in absorbance at 230 nm. The reaction mixture contained 30 mM H_2O_2 in 0.01 M phosphate buffer and the extract from homogenized tissue. Specific activity expressed as International Units (IU) / mg protein. 1 IU = Change in absorbance / min / extinction coefficient (0.021).

5.2.10.3 Estimation of Superoxide Dismutase (SOD)

Superoxide dismutase was estimated by the method of Kakkar (*Kakkar et al.*, 1984). Assay mixture contained 240 μl sodium pyrophosphate buffer (pH 8.3) , 20 μl PMS, 80μl NBT, 60 μl NADH, 240 μl distilled water and 60 μl of sample. Tubes were incubated at 30°C for 2.5 minutes and reaction was stopped by addition of 200 μl glacial acetic acid. Reaction mixture shaken vigorously with 800 μl n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged . Upper butanol layer was removed . Absorbance of chromogen in butanol was measured at 560 nm against n-butanol blank. A reagent blank without sample served as control. One unit of enzyme activity is defined as enzyme concentration required to inhibit chromogen production by 50% in one minute under assay conditions and specific activity is expressed as units / mg protein. 1 Unit - Amount of enzyme which gives 50% inhibition of formazan formation / minute.

5.2.10.4 Estimation of Glutathione Peroxidase (GPX)

Glutathione peroxidase in different tissues were estimated by the method of Rotruck (*Rotruck et al.*, 1973). 250 μl of tissue homogenate was taken in an eppendorf and 100 μl of 0.4 M Tris buffer, 100μl of 0.4mM Ethylene Diamine Tetra Acetic Acid (EDTA) and 50μl of 10mM Sodium Azide was added and mixed well. To this mixture 100μl reduced glutathione followed by 50μl H_2O_2 were added. The contents were mixed well and incubated at 37°C for 10 minutes along with a control containing all reagents except tissue homogenate. After 10 minutes the reaction was arrested by the addition of 250μl of 10% trichloroacetic acid (TCA) . Tubes were centrifuged and the supernatant was collected. 100μl supernatant was transferred to microtitre plate and to this 100μl Na_2HPO_4 and 50μl dithio bis nitro benzoic acid (DTNB) were added. The optical density was read at 412 nm. The values were expressed as μg of GSH / min / mg protein.

5.2.10.5 Estimation of Glutathione-S-Transferase (GST)

Glutathione-S-Transferase in different tissues was determined using the method of Beutler (*Beutler*, 1986). The reaction mixture containing 500 μl of 0.5M phosphate buffer, 50 μl 1-chloro 2, 4 dinitrobenzene (CDNB) and 850 μl of tissue homogenate were taken in an eppendorf tube. The tubes were incubated at 37°C for 5 minutes. After the incubation 180 μl of the reaction mixture was transferred to microtitre plate and 20 μl of reduced glutathione was added. The increase in absorbance was noted at 340 nm for 3 minutes. Values were expressed in μmole of CDNB complexed / min / mg protein. The extinction coefficient between CDNB-GSH conjugate and CDNB is 9.6 mM^{-1} cm^{-1}.

5.2.10.6 Estimation of Total Reduced Glutathione (GSH)

Glutathione was measured by its reaction with Di Thio bis nitro benzoic acid (DTNB) to give a compound that absorbs at 412 nm as per Ellmans method (*Ellman*, 1959). 250 μl of tissue homogenate was mixed with 1 ml of metaphosphoric acid. The precipitate was removed by centrifugation. 100 μl of supernatant was taken in the microtitre plate and 100 μl of 0.4 M disodium hydrogen phosphate and 50 μl DTNB were added. The absorbance was read within 2 minutes at 412 nm. The amount of glutathione was expressed as μg / mg protein.

5.2.10.7 Estimation of Malondialdehyde (MDA)

Malondialdehyde was estimated by the method of Nihaeus and Samuelson (*Nihaus and Samuelson*, 1968). The tissue homogenate of different tissues were prepared in Tris HCl buffer and was combined with thiobarbituric acid and mixed thoroughly and heated for 15 minutes in a bioling water bath. It was then cooled and centrifuged for 10 minutes at 3000 rpm for 10 minutes. The absorbance was read at 535 nm. The values were expressed as millimoles of MDA per 100 g wet weight of tissue.

5.3 Statistical Analysis

In order to determine significant difference if any, in immunological parameters between the different treatment groups, the results were analyzed using one way analysis of variance (ANOVA) and Duncans multiple comparison of the means by using SPSS 14.0 for windows. The level of significance was set at $p< 0.05$.

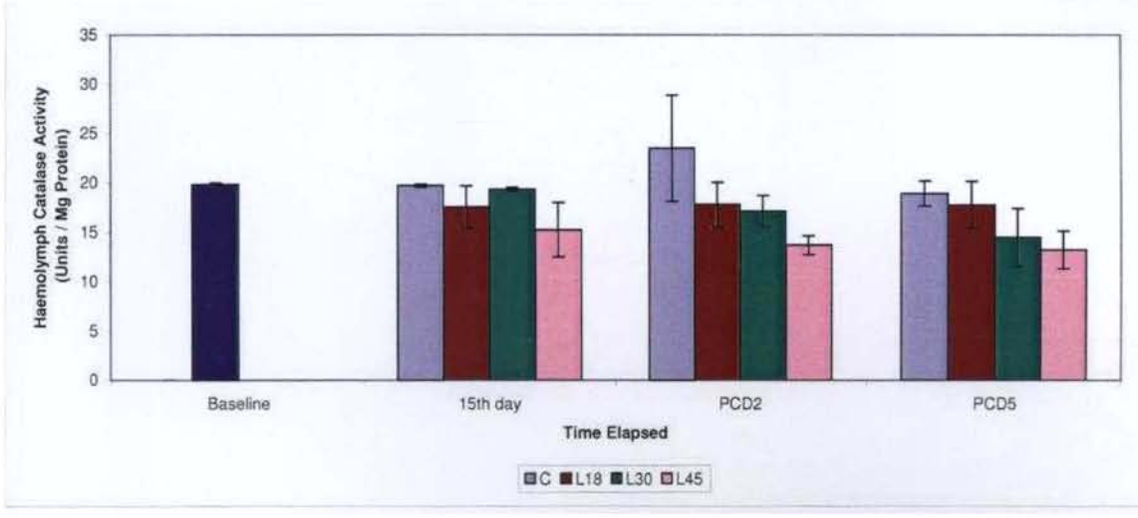
5.4 Results

5.4.1 Catalase Activity

In the present study catalase activity was significantly high in control when compared to actinomycete fed diets .There was not much significant variation ($p< 0.05$) among various treatment groups except in gills. In actinomycete fed diets maximum activity was shown by L18, followed by L39 and L45. There was significant variations in CAT activity of various tissues with haemolymph showing high activity followed by hepatopancreas, gills and muscles. A substantial increase in CAT activity was observed for all treatments on post challenge day 2 and a sudden decrease in CAT activity was observed on post challenge day 5 . (*Figs.5.1, 5.2, 5.3, & 5.4*) .

5.4.2 Super Oxide Dismutase Activity

Superoxide dismutase activity was significantly high in control followed by L39, L18 and L45 . There was significant variation ($p< 0.05$) between control and actinomycete fed groups in hepatopancreas. Maximum SOD activity was observed in hepatopancreas followed by haemolymph , muscle and gills. SOD activity lowered significantly post challenge except in haemolymph *Figs. 5.5, 5.6, 5.7 & 5.8*).

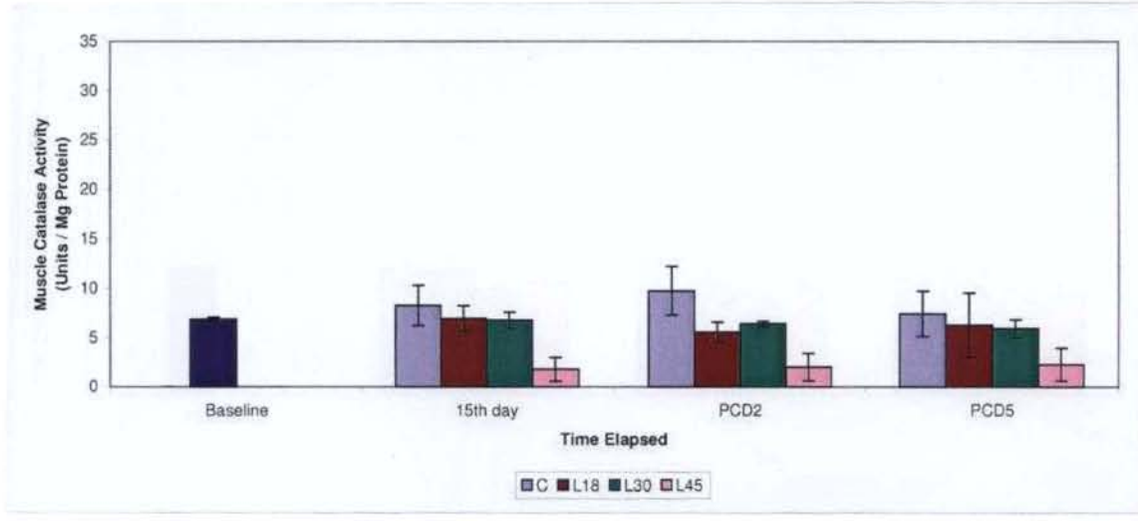


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	19.850 ± 0.150	19.714 ± 0.149 ^{Ba}	23.504 ± 5.386 ^{Ba}	18.942 ± 1.266 ^{Ba}
L18	19.850 ± 0.150	17.549 ± 2.147 ^{Ba}	17.796 ± 2.256 ^{Ba}	17.765 ± 2.391 ^{Ba}
L39	19.850 ± 0.150	19.356 ± 0.181 ^{Bb}	17.142 ± 1.598 ^{Aa}	14.485 ± 2.897 ^{Aa}
L45	19.850 ± 0.150	15.258 ± 2.748 ^{Aa}	13.689 ± 0.965 ^{Aa}	13.213 ± 1.918 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.1: Catalase activity (Mean±SD) in Haemolymph of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

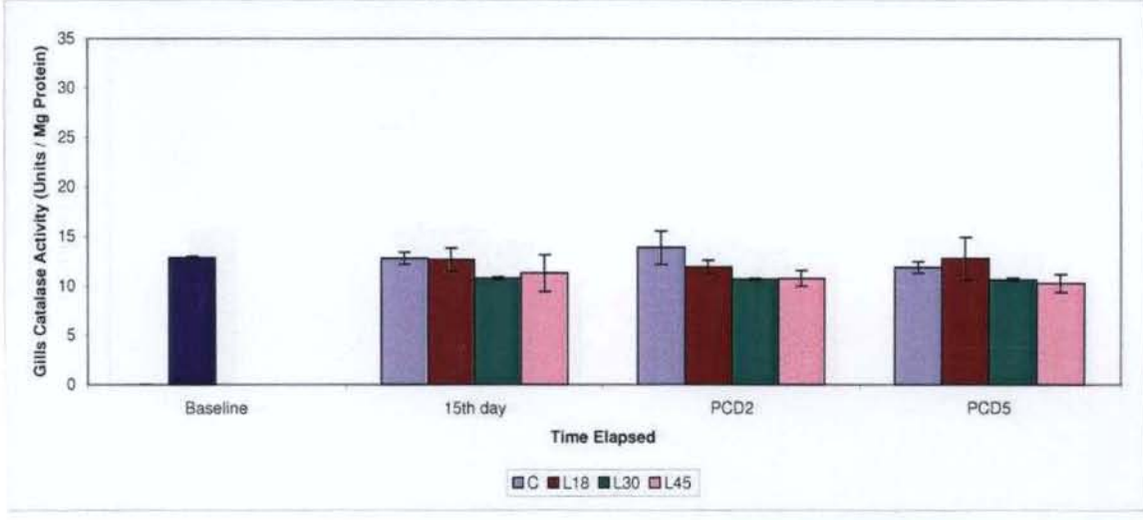


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	6.890 ± 0.115	8.267 ± 2.040 ^{Ba}	9.733 ± 2.458 ^{Ca}	7.414 ± 2.313 ^{Ba}
L18	6.890 ± 0.115	6.931 ± 1.300 ^{Ba}	5.583 ± 0.993 ^{Ba}	6.286 ± 3.245 ^{Ba}
L39	6.890 ± 0.115	6.749 ± 0.842 ^{Ba}	6.384 ± 0.302 ^{Ba}	5.920 ± 0.929 ^{Ba}
L45	6.890 ± 0.115	1.763 ± 1.214 ^{Aa}	1.999 ± 1.389 ^{Aa}	2.266 ± 1.665 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.2: Catalase activity (Mean±SD) in Muscle of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

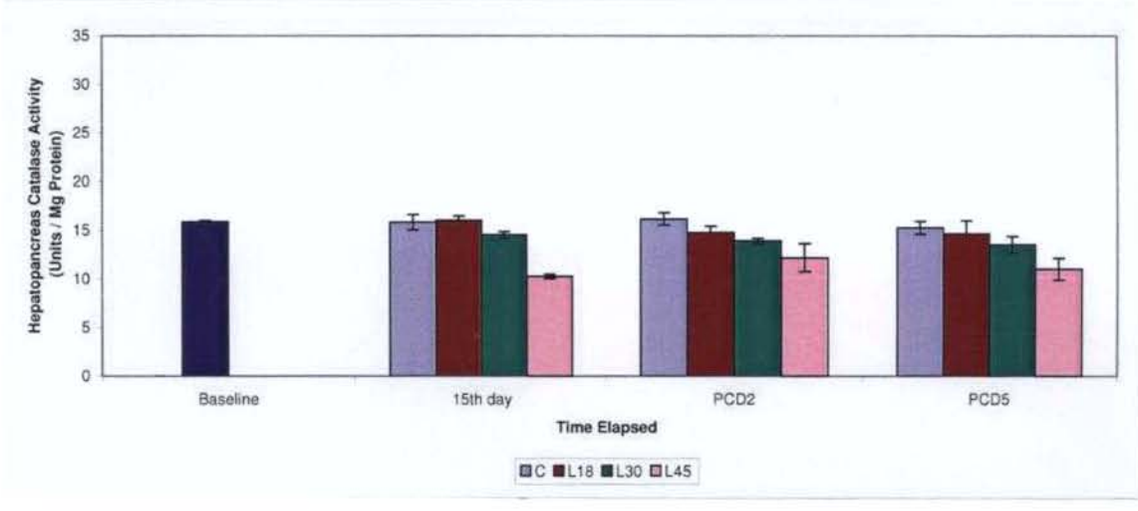


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	12.860 ± 0.143	12.774 ± 0.608 ^{Ba}	13.853 ± 1.679 ^{Ba}	11.870 ± 0.584 ^{ABa}
L18	12.860 ± 0.143	12.647 ± 1.159 ^{Ba}	11.910 ± 0.659 ^{Aa}	12.762 ± 2.172 ^{Ba}
L39	12.860 ± 0.143	10.767 ± 0.154 ^{Aa}	10.675 ± 0.094 ^{Aa}	10.617 ± 0.177 ^{ABa}
L45	12.860 ± 0.143	11.282 ± 1.841 ^{Aa}	10.745 ± 0.793 ^{Aa}	10.257 ± 0.919 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.3: Catalase activity (Mean±SD) in Gills of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

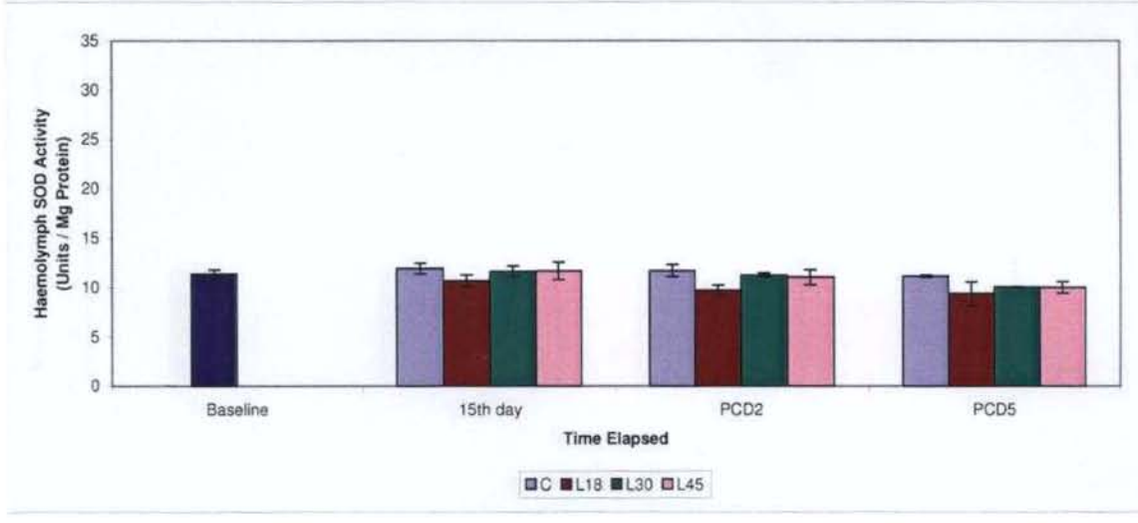


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	15.850 ± 0.431	15.816 ± 0.782 ^{Ca}	16.178 ± 0.623 ^{Ca}	15.273 ± 0.672 ^{Ba}
L18	15.850 ± 0.431	16.023 ± 0.472 ^{Ca}	14.759 ± 0.674 ^{Ba}	14.660 ± 1.343 ^{Ba}
L39	15.850 ± 0.431	14.541 ± 0.339 ^{Ba}	13.900 ± 0.295 ^{Ba}	13.524 ± 0.873 ^{Ba}
L45	15.850 ± 0.431	10.258 ± 0.224 ^{Aa}	12.194 ± 1.449 ^{Ab}	11.014 ± 1.122 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.4: Catalase activity (Mean±SD) in Hepatopancreas of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

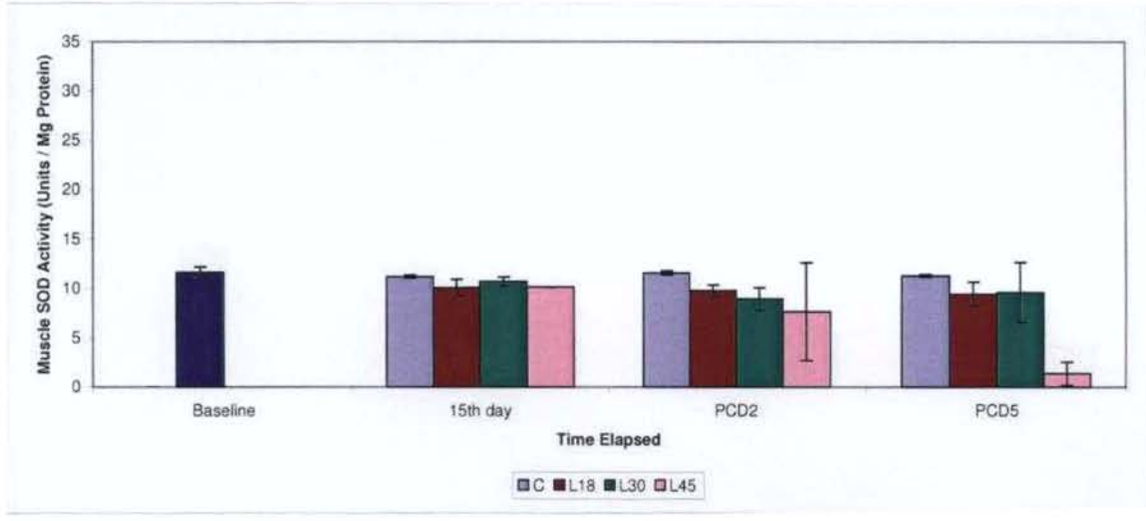


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	11.360 ± 0.408	11.934 ± 0.551 ^{ABab}	11.700 ± 0.608 ^{Bb}	11.134 ± 0.114 ^{Ca}
L18	11.360 ± 0.408	10.658 ± 0.627 ^{Aa}	9.711 ± 0.520 ^{Aa}	9.376 ± 1.182 ^{Ba}
L39	11.360 ± 0.408	11.638 ± 0.546 ^{Bb}	11.246 ± 0.216 ^{Bab}	10.032 ± 0.047 ^{Ba}
L45	11.360 ± 0.408	11.679 ± 0.891 ^{ABb}	11.039 ± 0.772 ^{Bb}	9.993 ± 0.595 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.5: Super Oxide Dimutase activity (Mean±SD) in Haemolymph of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

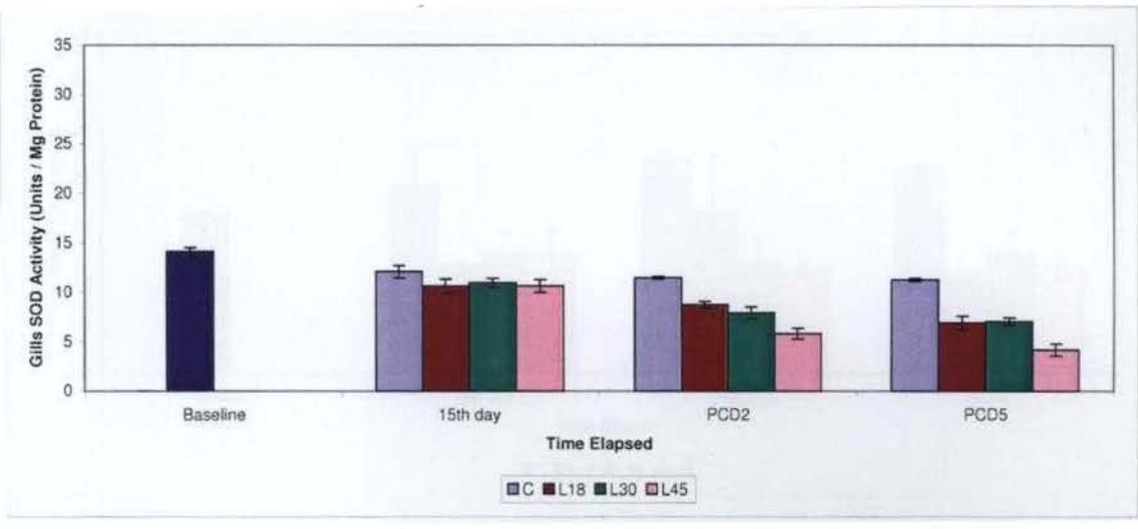


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	11.650 ± 0.520	11.193 ± 0.143 ^{BCa}	11.578 ± 0.215 ^{Bb}	11.282 ± 0.148 ^{Bab}
L18	11.650 ± 0.520	10.086 ± 0.833 ^{Bb}	9.781 ± 0.581 ^{Aa}	9.422 ± 1.219 ^{Ba}
L39	11.650 ± 0.520	10.673 ± 0.486 ^{Ca}	8.946 ± 1.155 ^{Aa}	9.592 ± 3.023 ^{Ba}
L45	11.650 ± 0.520	10.133 ± 0.005 ^{Ab}	7.643 ± 4.961 ^{Ab}	1.376 ± 1.179 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.6: Super Oxide Dimutase activity (Mean±SD) in Muscle of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

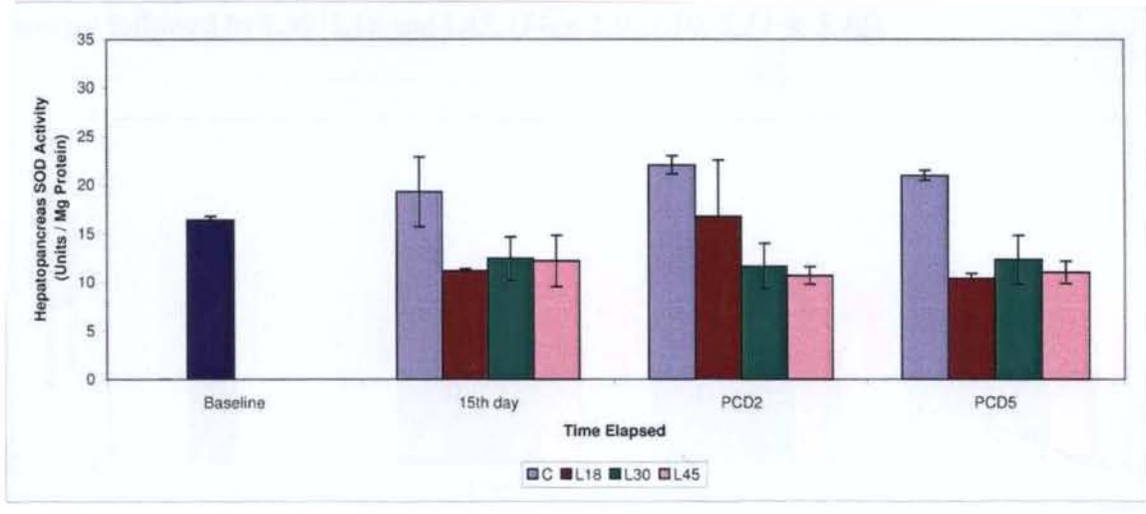


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	14.110 ± 0.600	12.088 ± 0.638 ^{Aa}	11.476 ± 0.145 ^{Ca}	11.242 ± 0.143 ^{Ca}
L18	14.110 ± 0.600	10.636 ± 0.710 ^{Ac}	8.747 ± 0.334 ^{Bb}	6.897 ± 0.693 ^{Ba}
L39	14.110 ± 0.600	10.945 ± 0.484 ^{Ab}	7.919 ± 0.602 ^{Ba}	7.037 ± 0.388 ^{Ba}
L45	14.110 ± 0.600	10.636 ± 0.654 ^{Ac}	5.811 ± 0.551 ^{Ab}	4.158 ± 0.624 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.7: Super Oxide Dimutase activity (Mean±SD) in Gills of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge



PCD - Post challenge day

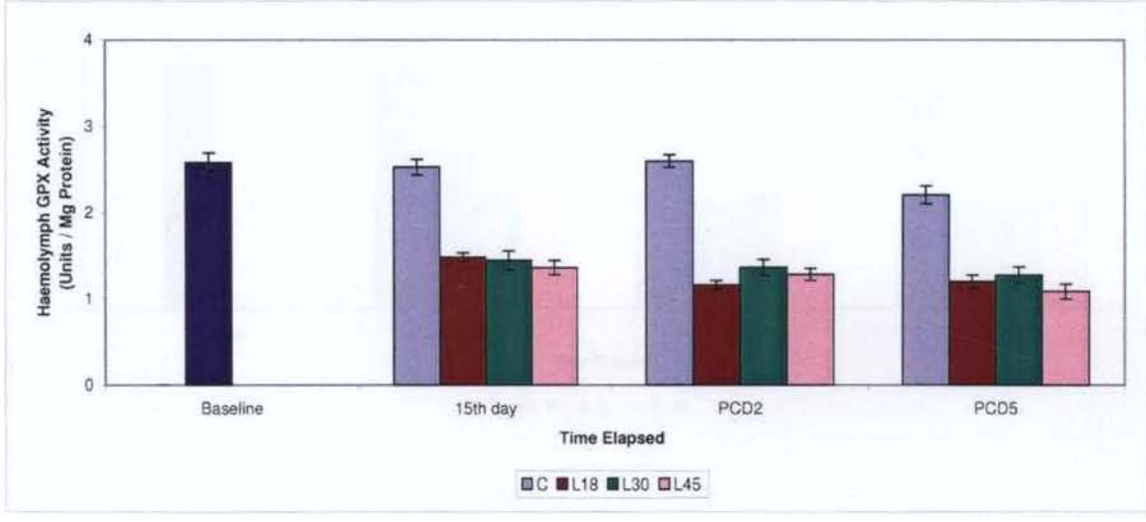
Feeds	Baseline	15th day	PCD2	PCD5
Control	16.390 ± 0.660	19.290 ± 3.594 ^{Ba}	22.043 ± 0.957 ^{Bb}	20.966 ± 0.519 ^{Ba}
L18	16.390 ± 0.660	11.177 ± 0.235 ^{Aa}	16.783 ± 5.771 ^{Aa}	10.371 ± 0.548 ^{Aa}
L39	16.390 ± 0.660	12.435 ± 2.209 ^{Aa}	11.648 ± 2.327 ^{Aa}	12.315 ± 2.493 ^{Aa}
L45	16.390 ± 0.660	12.182 ± 2.632 ^{Aa}	10.674 ± 0.903 ^{Aa}	11.007 ± 1.148 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.8: Super Oxide Dimutase activity (Mean±SD) in Hepatopancreas of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

5.4.3 Glutathione Peroxidase Activity

Glutathione peroxidase activity showed significant variation in GPX activity ($p< 0.05$) between control and actinomycete fed diets. There was no significant variations could be observed among various actinomycete fed diets. Maiximum GPX activity was observed for control followed by L39, L18 and L45. (*Figs. 5.9, 5.10, 5.11 & 5.12*).

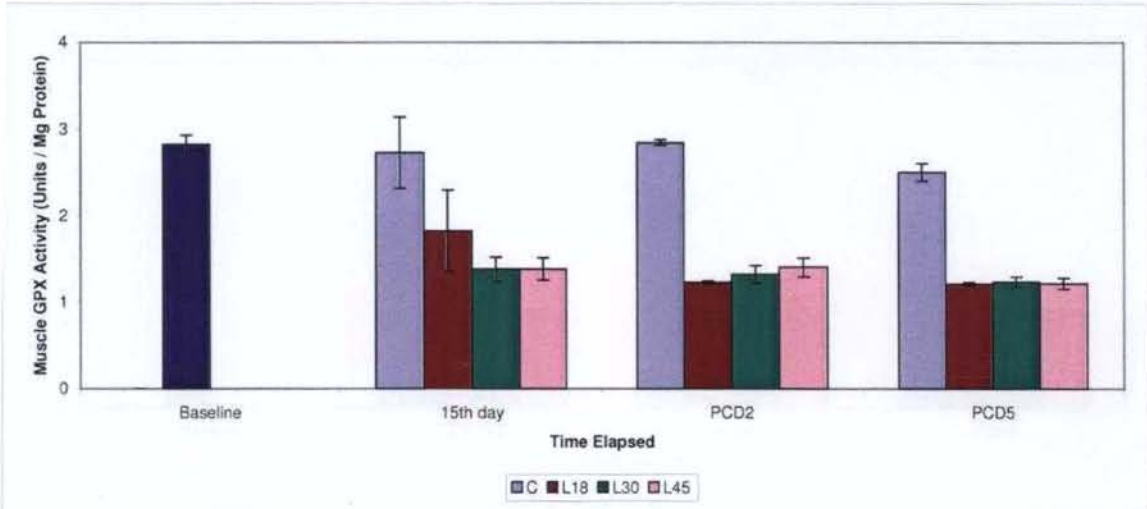


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	2.580 ± 0.108	2.527 ± 0.091 ^{Bb}	2.597 ± 0.072 ^{Cb}	2.206 ± 0.104 ^{Ba}
L18	2.580 ± 0.108	1.484 ± 0.050 ^{Ab}	1.159 ± 0.052 ^{Aa}	1.200 ± 0.074 ^{Aa}
L39	2.580 ± 0.108	1.447 ± 0.113 ^{Aa}	1.364 ± 0.093 ^{Ba}	1.274 ± 0.097 ^{Aa}
L45	2.580 ± 0.108	1.363 ± 0.083 ^{Ab}	1.283 ± 0.071 ^{Ab}	1.086 ± 0.087 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.9: Glutathione Peroxidase activity (Mean±SD) in Haemolymph of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

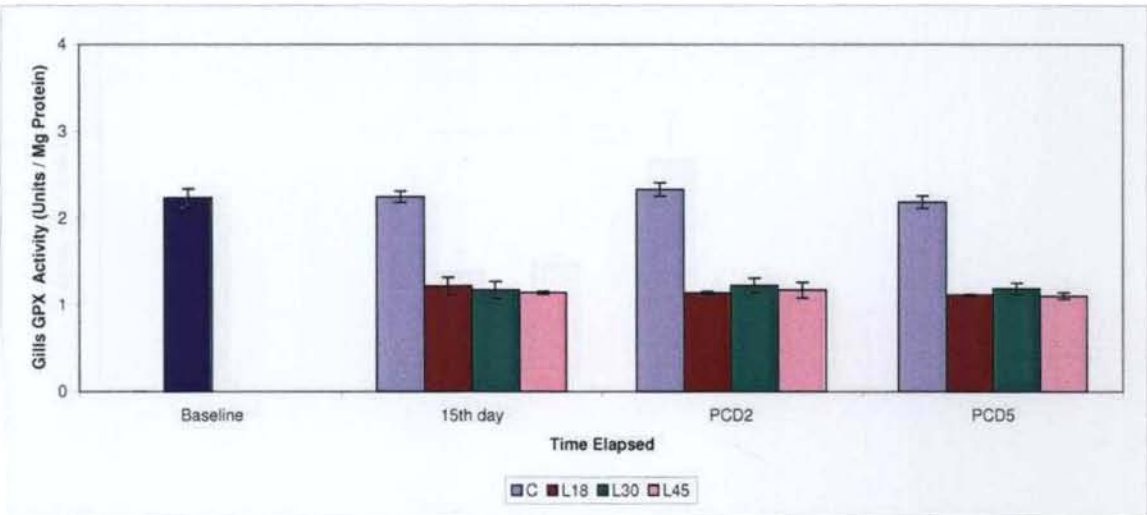


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	2.824 ± 0.115	2.729 ± 0.412 ^{Cb}	2.844 ± 0.033 ^{Cb}	2.504 ± 0.100 ^{Ba}
L18	2.824 ± 0.115	1.827 ± 0.473 ^{Bb}	1.230 ± 0.020 ^{Aa}	1.214 ± 0.015 ^{Aa}
L39	2.824 ± 0.115	1.382 ± 0.140 ^{Aa}	1.324 ± 0.102 ^{ABa}	1.236 ± 0.058 ^{Aa}
L45	2.824 ± 0.115	1.386 ± 0.130 ^{Aba}	1.407 ± 0.111 ^{Ba}	1.217 ± 0.063 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.10: Glutathione Peroxidase activity (Mean±SD) in Muscle of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

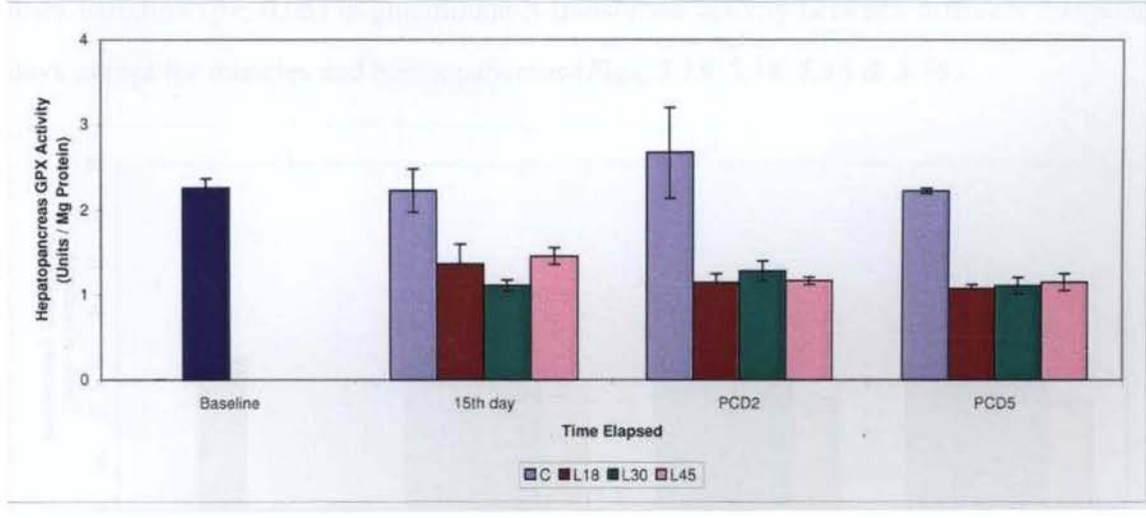


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	2.235 ± 0.104	2.250 ± 0.067 ^{Bab}	2.332 ± 0.079 ^{Bb}	2.187 ± 0.070 ^{Ba}
L18	2.235 ± 0.104	1.219 ± 0.103 ^{Aa}	1.137 ± 0.022 ^{Aa}	1.116 ± 0.010 ^{Aa}
L39	2.235 ± 0.104	1.173 ± 0.102 ^{Aa}	1.224 ± 0.084 ^{Aa}	1.187 ± 0.066 ^{Aa}
L45	2.235 ± 0.104	1.141 ± 0.017 ^{Aa}	1.171 ± 0.089 ^{Aa}	1.105 ± 0.038 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.11: Glutathione Peroxidase activity (Mean±SD) in Gills of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge



PCD - Post challenge day

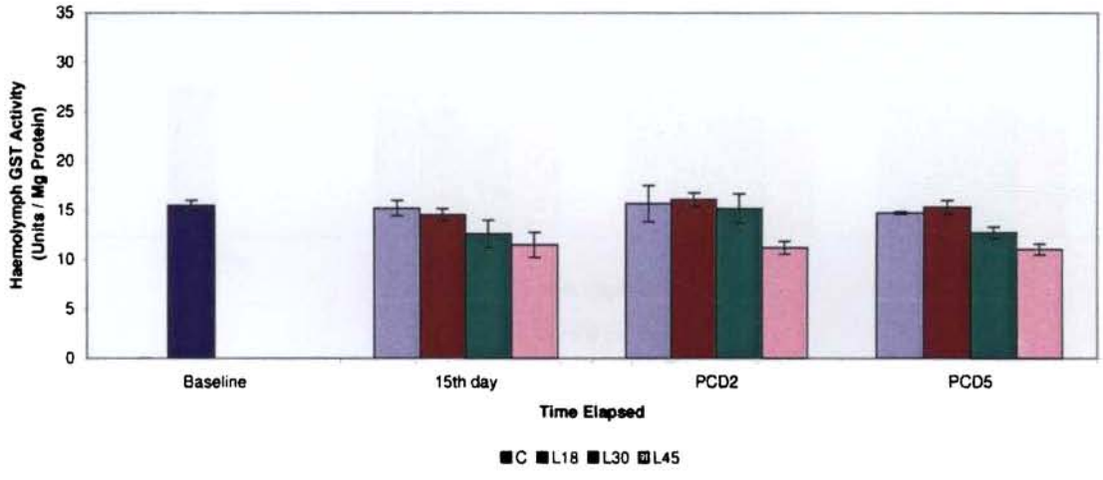
Feeds	Baseline	15th day	PCD2	PCD5
Control	2.258 ± 0.043	2.227 ± 0.253 ^{Ca}	2.670 ± 0.530 ^{Ab}	2.226 ± 0.029 ^{Ba}
L18	2.258 ± 0.043	1.361 ± 0.234 ^{Ab}	1.142 ± 0.105 ^{Aab}	1.076 ± 0.045 ^{Aa}
L39	2.258 ± 0.043	1.111 ± 0.064 ^{Aa}	1.282 ± 0.116 ^{Aa}	1.109 ± 0.095 ^{Aa}
L45	2.258 ± 0.043	1.455 ± 0.096 ^{Bb}	1.166 ± 0.043 ^{Aa}	1.150 ± 0.099 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.12: Glutathione Peroxidase activity (Mean±SD) in Hepatopancreas of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

5.4.4 Glutathione-S-Transferase (GST) Activity

Glutathione-S-transferase activity was high in control when compared to actinomycete fed diets. There was significant variation among treatment groups. Maximum GST activity was observed in haemolymph followed by hepatopancreas, muscles and gills. There was significant variation ($p < 0.05$) in glutathione-S-transferase activity between different sampling days except for muscles and hepatopancreas.(*Figs. 5.13, 5.14, 5.15 & 5.16*).

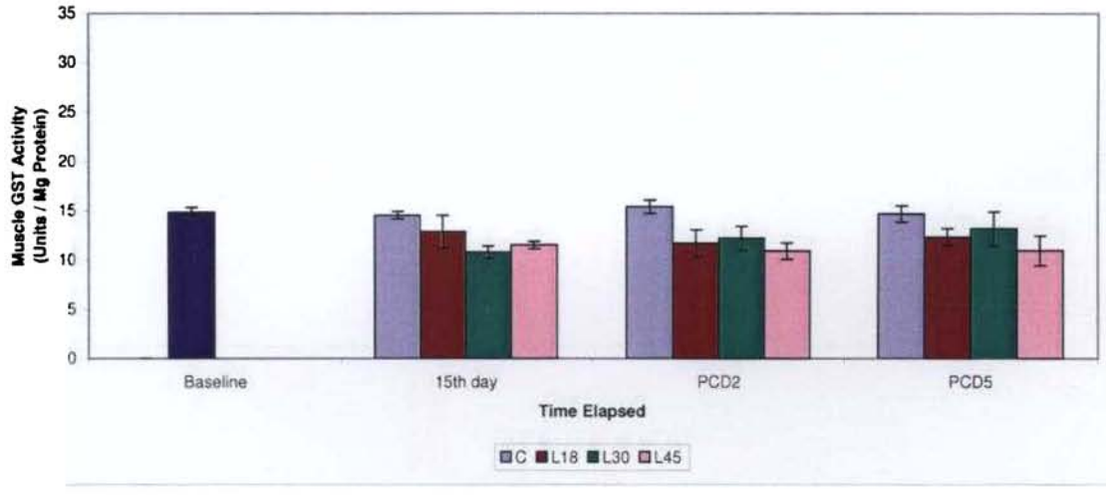


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	15.480 ± 0.502	15.200 ± 0.785 ^{Ca}	15.657 ± 1.856 ^{Ba}	14.729 ± 0.129 ^{Ca}
L18	15.480 ± 0.502	14.533 ± 0.594 ^{BCa}	16.078 ± 0.683 ^{Bb}	15.295 ± 0.691 ^{Cab}
L39	15.480 ± 0.502	12.587 ± 1.385 ^{ABa}	15.157 ± 1.485 ^{Bb}	12.718 ± 0.590 ^{Ba}
L45	15.480 ± 0.502	11.472 ± 1.277 ^{Aa}	11.183 ± 0.651 ^{Aa}	11.024 ± 0.555 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.13: Glutathione-S-Transferase activity (Mean±SD) in Haemolymph of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

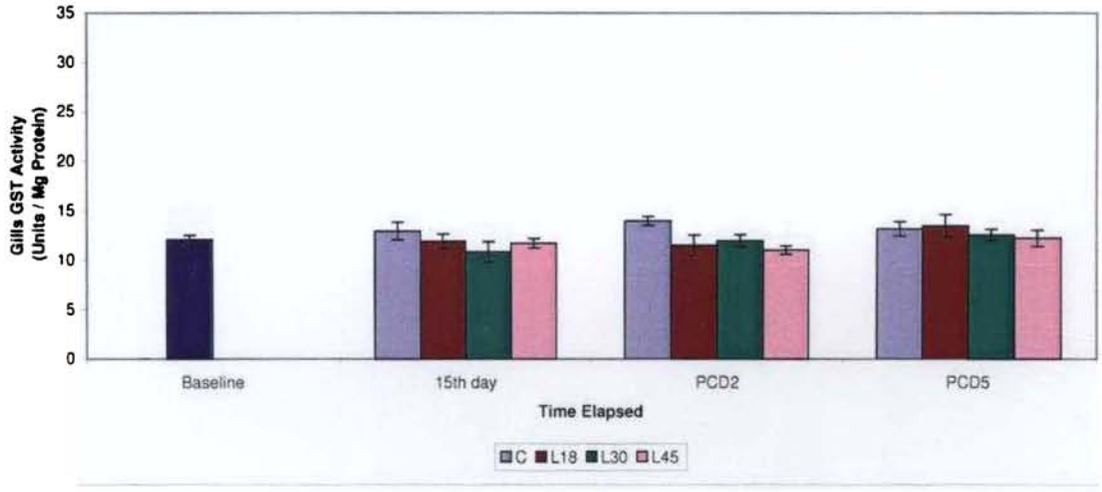


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	14.820 ± 0.012	14.553 ± 0.390 ^{Ba}	15.403 ± 0.687 ^{ba}	14.682 ± 0.831 ^{Ba}
L18	14.820 ± 0.012	12.882 ± 1.653 ^{Aa}	11.694 ± 1.371 ^{Aa}	12.320 ± 0.871 ^{ABa}
L39	14.820 ± 0.012	10.795 ± 0.620 ^{Aa}	12.216 ± 1.235 ^{Aa}	13.184 ± 1.685 ^{ABa}
L45	14.820 ± 0.012	11.546 ± 0.386 ^{Aa}	10.922 ± 0.825 ^{Aa}	10.951 ± 1.514 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.14: Glutathione-S-Transferase activity (Mean±SD) in Muscle of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

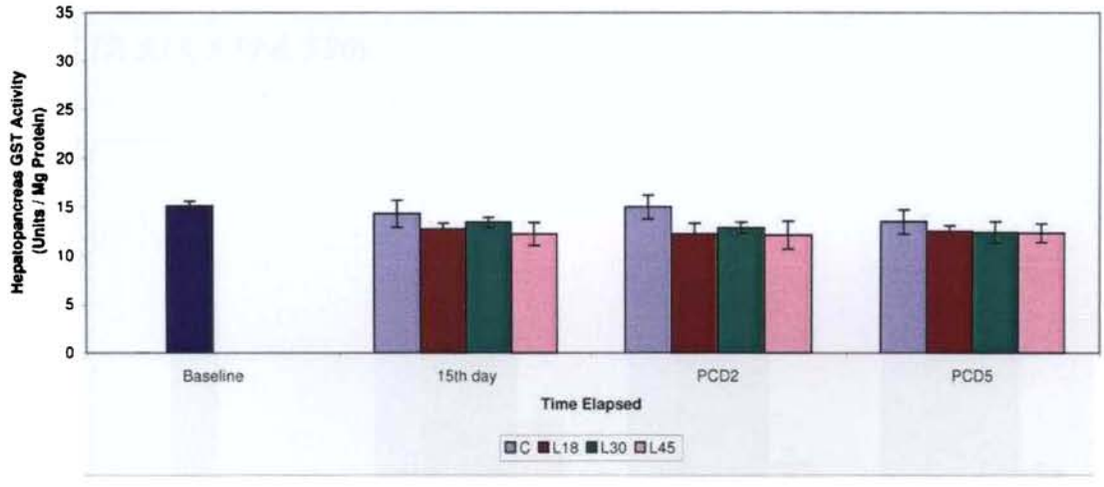


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	12.030 ± 0.010	12.936 ± 0.901 ^{Ca}	13.968 ± 0.467 ^{Ba}	13.160 ± 0.734 ^{Aa}
L18	12.030 ± 0.010	11.911 ± 0.725 ^{BCa}	11.510 ± 1.047 ^{Aa}	13.468 ± 1.126 ^{Aa}
L39	12.030 ± 0.010	10.834 ± 1.011 ^{Aa}	11.966 ± 0.619 ^{Aa}	12.535 ± 0.587 ^{Aa}
L45	12.030 ± 0.010	11.702 ± 0.479 ^{ABab}	11.006 ± 0.405 ^{Aa}	12.201 ± 0.820 ^{Ab}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.15: Glutathione-S-Transferase activity (Mean±SD) in Gills of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge



PCD - Post challenge day

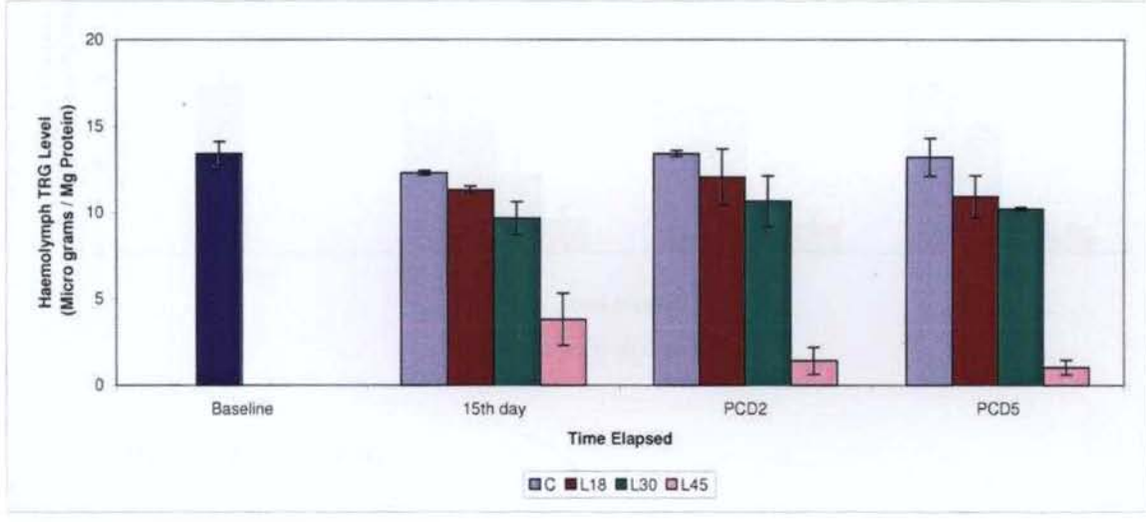
Feeds	Baseline	15th day	PCD2	PCD5
Control	15.130 ± 0.043	14.320 ± 1.392 ^{Ba}	15.023 ± 1.234 ^{Ba}	13.492 ± 1.255 ^{Aa}
L18	15.130 ± 0.043	12.751 ± 0.600 ^{ABa}	12.259 ± 1.095 ^{Aa}	12.540 ± 0.556 ^{Aa}
L39	15.130 ± 0.043	13.432 ± 0.537 ^{ABa}	12.872 ± 0.575 ^{Aa}	12.419 ± 1.109 ^{Aa}
L45	15.130 ± 0.043	12.242 ± 1.185 ^{Aa}	12.130 ± 1.424 ^{Aa}	12.336 ± 0.950 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.16: Glutathione-S-Transferase activity (Mean±SD) in Hepatopancreas of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

5.4.5 Total Reduced Glutathione (GSH)

Total reduced glutathione level was significantly high in control followed by L18, L39 and L45 and there was significant variation ($p< 0.05$) between the various treatment groups. Maximum TRG level was observed in hepatopancreas followed by haemolymph, gills and muscle. There was no significant variation in the level between the different sampling days. (*Figs. 5.17, 5.18, 5.19 & 5.20*).

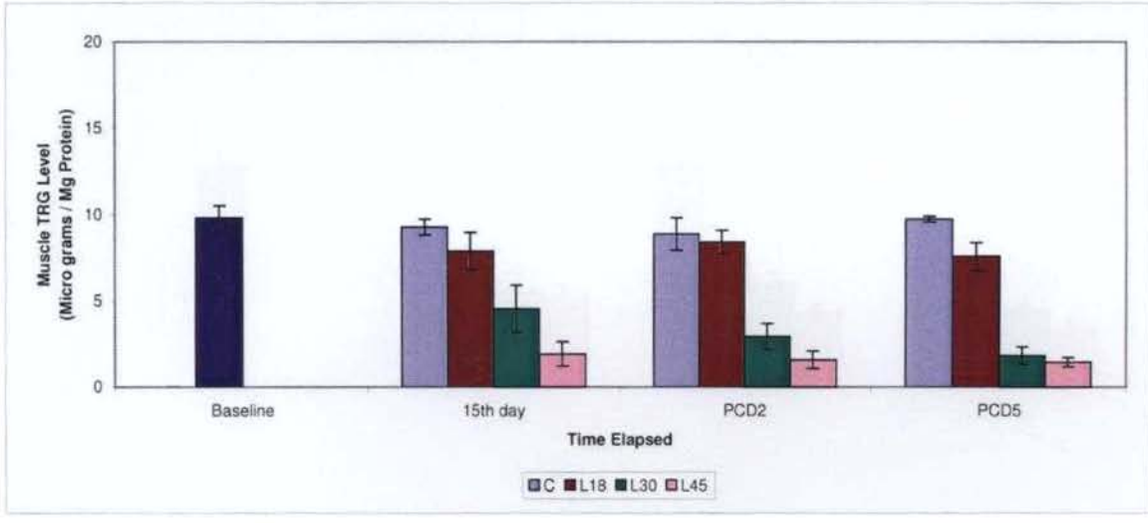


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	13.410 ± 0.700	12.305 ± 0.131 ^{Ba}	13.409 ± 0.178 ^{Ba}	13.198 ± 1.095 ^{Ba}
L18	13.410 ± 0.700	11.320 ± 0.206 ^{Ba}	12.064 ± 1.618 ^{ABa}	10.929 ± 1.223 ^{Ba}
L39	13.410 ± 0.700	9.668 ± 0.963 ^{Bb}	10.659 ± 1.473 ^{Ab}	10.205 ± 0.099 ^{Aa}
L45	13.410 ± 0.700	3.802 ± 1.512 ^{Aa}	1.403 ± 0.769 ^{Aa}	1.015 ± 0.419 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.17: Glutathione content (Mean±SD) in Haemolymph of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

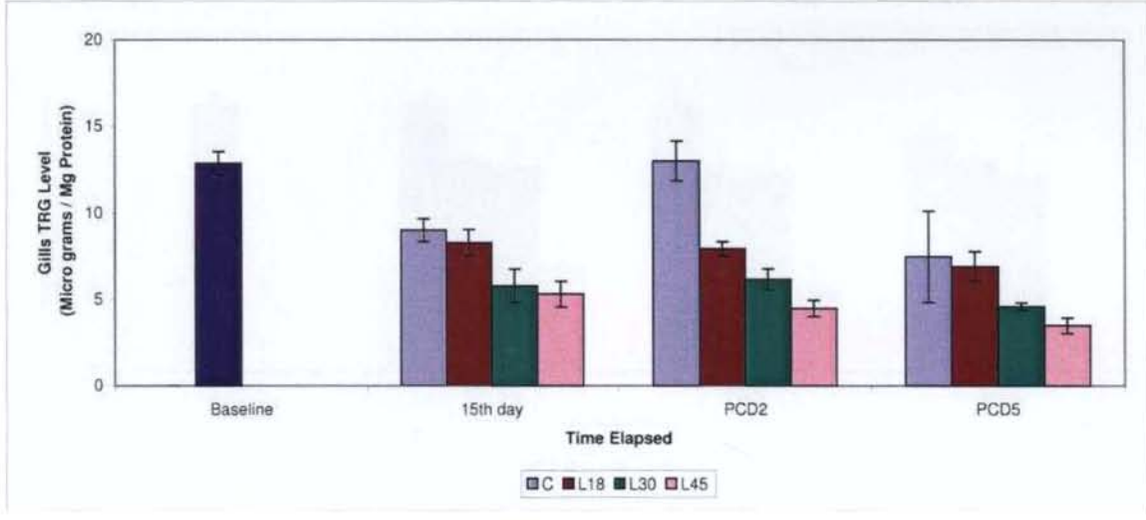


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	9.820 ± 0.700	9.271 ± 0.470 ^{Ba}	8.865 ± 0.949 ^{Ca}	9.742 ± 0.158 ^{Ba}
L18	9.820 ± 0.700	7.882 ± 1.082 ^{Ba}	8.401 ± 0.692 ^{Ba}	7.564 ± 0.811 ^{Ba}
L39	9.820 ± 0.700	4.544 ± 1.366 ^{Ba}	2.931 ± 0.752 ^{Ba}	1.817 ± 0.515 ^{Ba}
L45	9.820 ± 0.700	1.905 ± 0.718 ^{Aa}	1.582 ± 0.512 ^{Aa}	1.451 ± 0.277 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.18: Glutathione content (Mean±SD) in Muscle of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

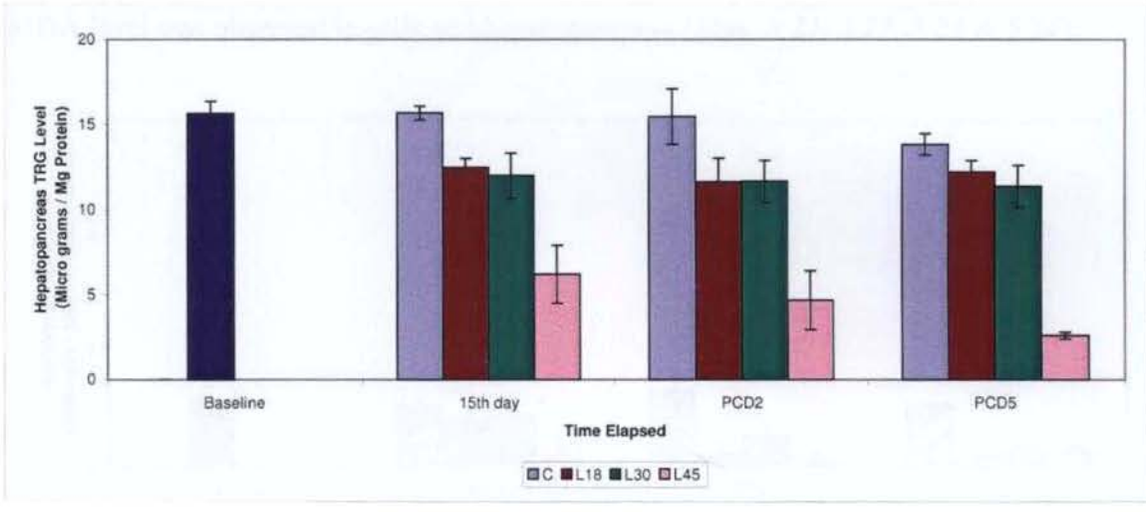


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	12.840 ± 0.700	9.000 ± 0.663 ^{Ba}	12.980 ± 1.152 ^{Ba}	7.465 ± 2.645 ^{Aba}
L18	12.840 ± 0.700	8.267 ± 0.756 ^{Ba}	7.923 ± 0.416 ^{Aa}	6.897 ± 0.864 ^{Ba}
L39	12.840 ± 0.700	5.764 ± 0.970 ^{Aa}	6.164 ± 0.596 ^{Aa}	4.583 ± 0.208 ^{Aba}
L45	12.840 ± 0.700	5.290 ± 0.756 ^{Aa}	4.477 ± 0.462 ^{Aa}	3.484 ± 0.451 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.19: Glutathione content (Mean±SD) in Gills of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge



PCD - Post challenge day

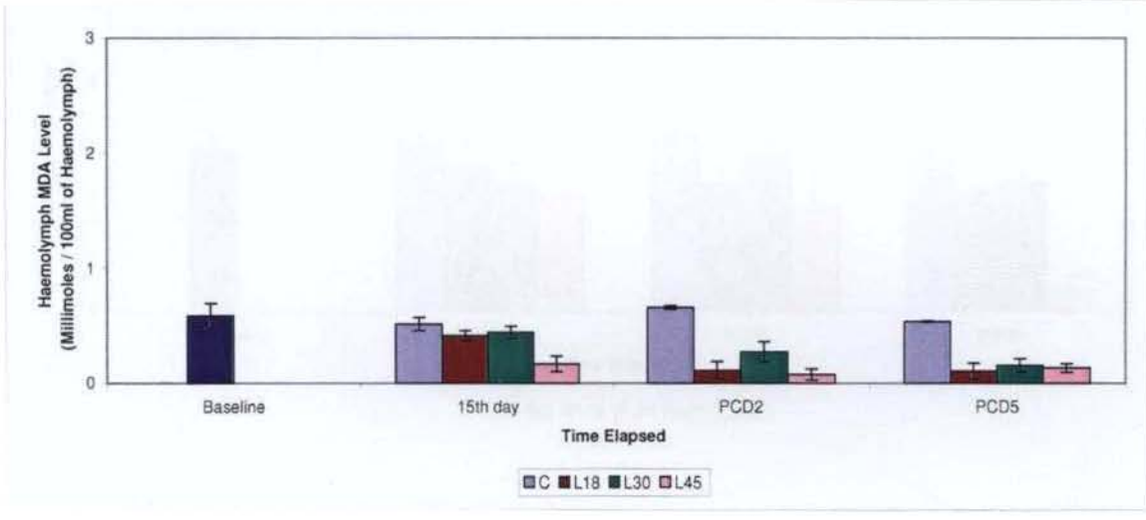
Feeds	Baseline	15th day	PCD2	PCD5
Control	15.640 ± 0.700	15.668 ± 0.404 ^{Ca}	15.449 ± 1.630 ^{Ca}	13.823 ± 0.619 ^{Ba}
L18	15.640 ± 0.700	12.468 ± 0.514 ^{Ca}	11.620 ± 1.392 ^{Ba}	12.200 ± 0.669 ^{Ba}
L39	15.640 ± 0.700	11.990 ± 1.324 ^{Ba}	11.650 ± 1.228 ^{Ba}	11.345 ± 1.254 ^{Ba}
L45	15.640 ± 0.700	6.190 ± 1.705 ^{Aa}	4.682 ± 1.730 ^{Ab}	2.602 ± 0.193 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.20: Glutathione content (Mean±SD) in Hepatopancreas of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

5.4.6 Malondialdehyde (MDA)

Malondialdehyde level was significantly high in control followed by L39, L18 and L45. Significant variation ($p < 0.05$) was observed for different treatment groups. . Considerable variation in MDA content was observed between haemolymph and muscle. Maximum MDA level was observed in gills and hepatopancreas (*Figs. 5.21, 5.22, 5.23 & 5.24*).

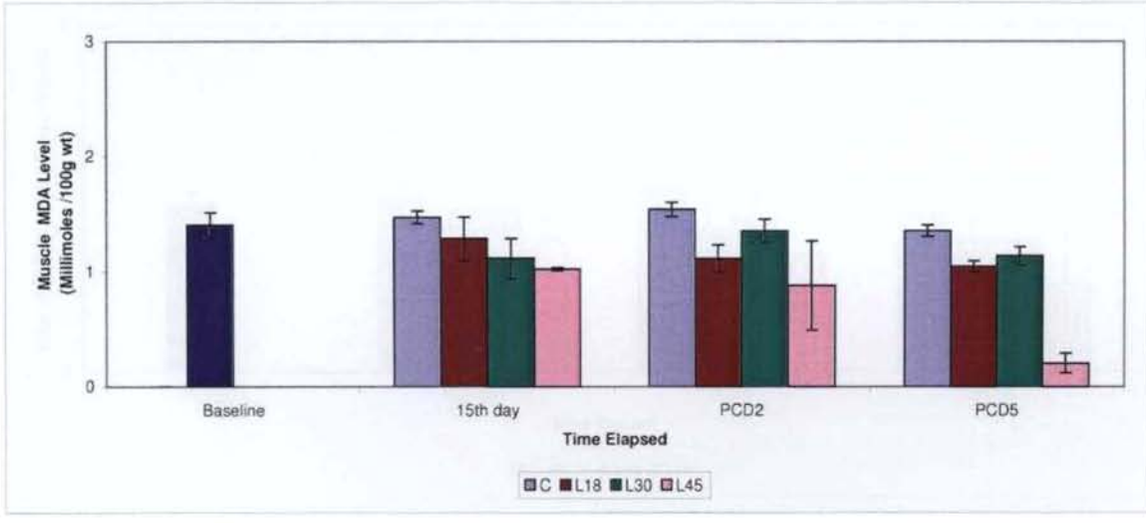


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	0.585 ± 0.108	0.516 ± 0.057 ^{Ca}	0.657 ± 0.017 ^{Cb}	0.536 ± 0.005 ^{Ba}
L18	0.585 ± 0.108	0.414 ± 0.042 ^{Bb}	0.111 ± 0.078 ^{Aa}	0.104 ± 0.071 ^{Aa}
L39	0.585 ± 0.108	0.442 ± 0.052 ^{Bb}	0.273 ± 0.088 ^{Bab}	0.155 ± 0.059 ^{Aa}
L45	0.585 ± 0.108	0.168 ± 0.069 ^{Ab}	0.074 ± 0.048 ^{Aa}	0.132 ± 0.038 ^{Aab}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.21: Malondialdehyde content (Mean±SD) in Haemolymph of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

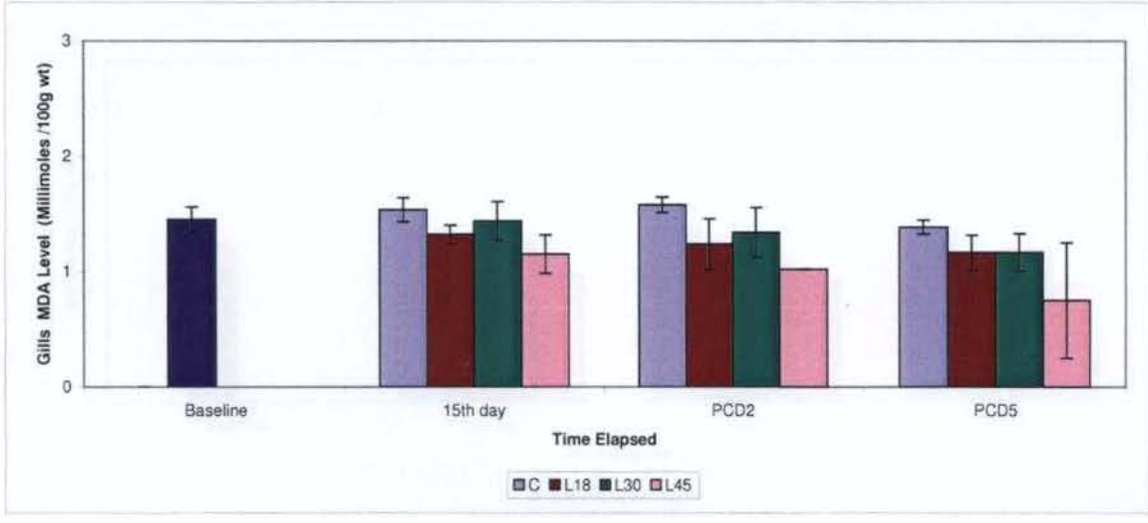


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	1.407 ± 0.012	1.472 ± 0.057 ^{Ba}	1.540 ± 0.063 ^{Ba}	1.355 ± 0.050 ^{Ba}
L18	1.407 ± 0.012	1.286 ± 0.189 ^{Bb}	1.113 ± 0.118 ^{Aa}	1.045 ± 0.048 ^{Aa}
L39	1.407 ± 0.012	1.116 ± 0.174 ^{Ba}	1.353 ± 0.100 ^{Aa}	1.137 ± 0.080 ^{Ba}
L45	1.407 ± 0.012	1.021 ± 0.014 ^{Ab}	0.879 ± 0.387 ^{Aab}	0.203 ± 0.085 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.22: Malondialdehyde content (Mean±SD) in Muscle of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

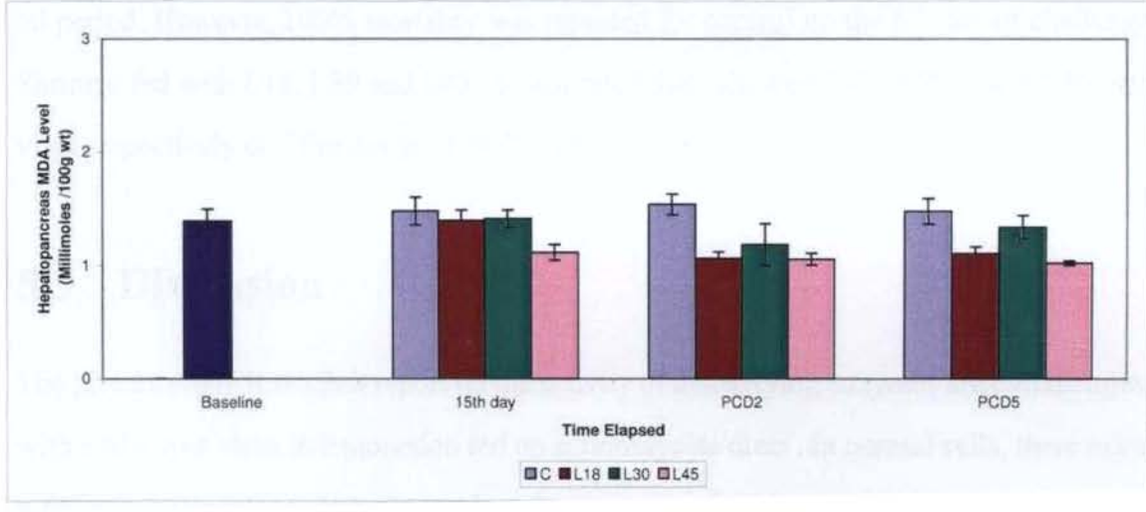


PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	1.453 ± 0.010	1.537 ± 0.105 ^{Abab}	1.579 ± 0.068 ^{Bb}	1.387 ± 0.062 ^{Aa}
L18	1.453 ± 0.010	1.322 ± 0.080 ^{Aba}	1.239 ± 0.218 ^{ABa}	1.166 ± 0.152 ^{Aa}
L39	1.453 ± 0.010	1.438 ± 0.168 ^{Ba}	1.341 ± 0.217 ^{ABa}	1.170 ± 0.163 ^{Aa}
L45	1.453 ± 0.010	1.151 ± 0.167 ^{Aa}	1.021 ± 0.002 ^{Aa}	0.751 ± 0.500 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.23: Malondialdehyde content (Mean±SD) in Gills of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge



PCD - Post challenge day

Feeds	Baseline	15th day	PCD2	PCD5
Control	1.390 ± 0.043	1.482 ± 0.122 ^{Ba}	1.540 ± 0.091 ^{Ba}	1.479 ± 0.112 ^{Ba}
L18	1.390 ± 0.043	1.397 ± 0.093 ^{Bb}	1.065 ± 0.056 ^{Aa}	1.106 ± 0.059 ^{Aa}
L39	1.390 ± 0.043	1.413 ± 0.079 ^{Ba}	1.185 ± 0.184 ^{Aa}	1.339 ± 0.104 ^{Ba}
L45	1.390 ± 0.043	1.116 ± 0.069 ^{Ab}	1.057 ± 0.051 ^{Aab}	1.021 ± 0.020 ^{Aa}

Capital superscript indicates group variance with respect to Treatment
Small superscript indicates group variance with respect to Time
Values with same superscript or subscript does not vary significantly ($p<0.05$)

Figure 5.24: Malondialdehyde content (Mean±SD) in Hepatopancreas of *P.monodon* fed with different actinomycete diets prior to and after WSSV challenge

5.4.7 Effect of Actinomycete Incorporated Diet on the Susceptibility of *Penaeus monodon* to White Spot Virus

The effect of actinomycete incorporated diet on the susceptibility of *P.monodon* to white spot virus was tested. Mortality was not reported during the fourteen days of the experimental period. However, 100% mortality was reported for control on the 6^{th} day of challenge. Shrimps fed with L18, L39 and L45 incorporated diets showed 20%, 50% and 62.5% survival respectively on 7thh day post challenge (*Fig.4.8*).

5.5 Discussion

The present study is the first report on the activity of detoxifying enzymes after challenging with white spot virus in P.monodon fed on actinomycete diets . In normal cells, there exists a delicate balance between pro-oxidant forces and antioxidant defenses that is known as redox balance. When the antioxidant defenses are overwhelmed by pro-oxidants, oxidative stress occurs. Statistical analysis of the data showed that there are significant variations in the nonenzymic (total reduced glutathione) and enzymic antioxidants (catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase) and lipid peroxidation product (malondialdehyde content) under WSSV infection in actinomycete fed groups and control.

The current study has shown that the activity of lipid peroxidation was significantly higher in controls when compared to actinomycete fed diet groups and this indicates that they are under high oxidative stress. This might be due to increased production and accumulation of reactive oxygen species. All marine organisms contain high levels of polyunsaturated fatty acids that are substrates for lipid peroxidation (*Liu et al.*, 1997). Increased lipid peroxidation can lead to the production of malondialdehyde that enhances

the formation of free radicals from polyunsaturated fatty acids in cell membranes (*Chih et al.*, 2003). Increase of lipid per oxidation due to oxidative stress caused by influenza virus infection in the liver tissues and serum in patients with chronic hepatitis have been reported (*Suematsu et al.*, 1977).

The current study has shown that WSSV-infection in *Penaeus monodon* caused a significant increase in the activity of lipid peroxidation in all tissues and a substantial increase in the activity of antioxidants (total reduced glutathione, catalase, glutathione peroxidase, glutathione-S-transferase) on post challenge day two in controls followed by a decline in the activity of lipid peroxidation and activity of antioxidant enzymes on post challenge day five. The reduction in catalase (CAT) activity following WSSV challenge has been previously reported by *Mohankumar and Ramasamy* (2006) & *Mathew et al.* (2007) . The present study also demonstrated a similar decrease in the CAT activity of WSSV infected tissues in controls on post challenge day 5. *Mathew et al.* (2007) has reported an increase in CAT activity during the initial period of WSSV infection as observed in controls in our study attempting to counteract or neutralize the harmful free radicals generated due to WSSV infection. This may be due to progressive increase in damage to cells of ectodermal and mesodermal tissues. The lower activity of catalase in infected animals may be due to inactivation of these enzymes by the oxidative stress generated singlet oxygen.

Superoxide dismutase (SOD) is one of the main antioxidant enzymes generated in response to oxidative stress. In the present study, the activity of SOD was significantly lowered in haemolymph, hepatopancreas, gills and muscles of WSSV infected controls. The abundantly available singlet oxygen and hydroxyl radicals in the immediate environment may be responsible for the declined activity in SOD observed in the post challenge

days 2 and 5 of the present study. These results are in agreements with the findings of *Lin* (1998) & *Chang et al.* (2003) who have found SOD to decrease in WSSV infected *P.monodon* than that of the uninfected controls. Superoxide dismutase has been reported to contain arginine and histidine residues at its active site (*Mallinowski and Fridovich*, 1979). Free radicals attack these highly reactive amino acids resulting in chemical modification of the protein structure and loss of enzyme activity. This may be the cause of low SOD activity post challenge.

Glutathione peroxidase catalyses the reduction of hydroperoxides, with the conversion of reduced glutathione to glutathione disulfide (GSSH) (*Meister and Anderson*, 1983). It has been reported that GPX is inactivated with increased levels of hydrogen peroxide, which in turn is due to the lower activity of catalase (*Searle and Wilson*, 1980). Since GSH act as the substrate for GPX, the decreased availability of GSH in the tissues of WSSV infected animals might have resulted in a decline in the activity of GPX. *Mathew et al.* (2007) noted a significant reduction in the activities of glutathione-dependant antioxidant enzymes in WSSV infected shrimp compared to control animals. Decline in the activity of GPX makes cellular and subcellular membranes more sensitive to oxidative damage. Besides acting as a hydrogen donor in the glutathione peroxidation reaction, reduced glutathione has a direct antioxidant function by reaction with superoxide, hydroxyl radical and singlet oxygen, leading to the formation of oxidized glutathione and other disulfides (*Kosower and Kosower*, 1978). In tissues, glutathione is maintained predominantly in the reduced form, where it acts as a major source of reducing power and function in the reduction of oxidized tissue components.

Glutathione S-transferase is another important enzyme known to catalyse antioxidant metabolism of thiol compounds, and this in turn protects cells from electrophiles, free

radicals induced damage and oxidative stress (*Dixon et al.*, 1998). Significant reduction in the activities of GPX and GST on post challenge day 5 in controls might lead to the formation of O_2^- and H_2O_2 , which in turn form hydroxyl radical (OH^-) and bring about a number of reactions harmful to cell membranes (*Mathew et al.*, 2007). A decrease in reduced glutathione content may decrease the activities of antioxidant enzymes and hence aggravate the effects of oxidative stress (*Garg et al.*, 1996).

Free radical scavenging enzymes are the first line of defense against oxidative stress. An equilibrium between these enzymes is important for the effective removal of oxygen stress in intracellular organelles (*Andrew and Mathew*, 1989). In shrimps fed with actinomycete incorporated diets antioxidant activity was comparatively less both before and after challenge. However, the survival rate of actinomycete incorporated diet fed animals was more when compared to control . Control fed shrimps showed 100 % mortality at the end of experimental period. Shrimps fed on L18, L39 and L45 incorporated diets showed 20%, 50 % and 62.5% survival respectively on 7^{th} day post challenge. This may be due to the fact that the experimental diet could help in scavenging the free radicals post infection. The present study shows that the experimental diets could effectively bring down the requirement of defensive antioxidant enzymes in various tissues indicating that they can act as an antioxidant by scavenging free radicals produced during infection and thus it could inhibit lipid peroxidation. This also stresses upon the fact that actinomycete fed diets could enhance the immune profile of shrimps against WSSV and also could protect the animals from the deleterious consequences of free radicals.

The factors which contribute to the antioxidant property of actinomycetes are to be studied. The role of dietary antioxidants and their potential benefits in health and disease have attracted great attention (*Kehrer and Smith*, 1994). The use of synthetic antioxidants

has decreased due to their suspected activity of promoters of carcinogensis (*Namiki*, 1990). There is a current worldwide interest in finding new and safe antioxidants from natural sources to prevent oxidative deterioration of food and to minimize oxidative damage to living cells (*Pratt*, 1992). Therefore, screening and selection of actinomycetes with high antioxidant property with a view to enhance disease resistance in shrimps offer tremendous scope. Since the crustaceans are lacking a well-defined acquired immunity against viral infection, the dietary supplementation of synthetic and natural antioxidants possessing health promoting and antimicrobial properties may be an effective alternative and promising disease preventing option for amelioration of WSSV infection in *Penaeus monodon*.

CHAPTER 6

Summary and Conclusion

Aquaculture represents one of the fastest growing food-producing sectors in the world with penaeid shrimp aquaculture being the most important among them. The most common penaeid shrimp species currently being cultured in the world is *Penaeus monodon* and it contributes more than 50% of the world production of penaeid shrimps. The enormous stocking density of animals and their coprophagous behaviour imposed by an intensive culture have triggered the onset of disease outbreaks, which are often severe and sometimes lead to the loss of a complete stock. Moreover, in the last decade there are strong indications that different viral infections have become endemic in some parts of the world. Infectious diseases constitute a main barrier to the development and continuation of shrimp aquaculture in terms of quality, quantity, regularity and continuity. Bacteria, particularly *Vibrio* spp. and viruses especially white spot syndrome viruses are considered as the main cause of mortality of shrimps in Asian countries.

Correct diagnosis, including knowledge of the life cycle and ecology of the pathogens, is

obviously a critical step in any control programme. Chemotherapy, preferably combined with preventive measures, is widely applied in the control of many infectious diseases in aquaculture. However, this type of chemical control should be considered as a last resort because of growing concern for food quality, accumulation of such substances in the environment and increase in the spread of antibiotic-resistant pathogenic strains.

In shrimp culture, new and more often difficult pathogens frequently emerge to replace the solved pathogen problem of yesterday. Therefore, preventive measures should be given priority in the control of diseases. Prophylactics include probiotics, immunostimulants and bioremediators with proper husbandry and management practices. Immunostimulants, like β-glucans, which induce and built up protection against a wide range of diseases, become increasingly important in aquaculture. Nowadays, disease management using proactive control measures involving probiotics or bioremediators are being developed as a package of practice for sustainable shrimp farming .

Among the mulititude of diverse organisms in the marine environment, marine microorganisms stand out as excellent source of many useful bioactive compounds. Of all the marine microbes, the actinomycetes merit special consideration in view of the proven biosynthetic capabilities . The actinomycetes stand out as a unique group of prokaryotic organisms in two respects; the diversity of their morphology and their metabolic products. Against this background, the present study was undertaken to study the potential of marine actinomycetes as a source of antiviral drugs and as probionts in shrimp aquaculture.
The salient findings of the study are as follows.

- A total of 77 marine actinomycetes could be isolated during the study from the sediment samples collected from West coast of India.

- Marine actinomycetes were found to exhibit anti-WSSV property. Incorporation of concentrated whole broth into diet was found to have better effect in terms of survival of the animal on challenge with WSSV . Seven isolates (Seven isolates L10 *Streptomyces sp.*, L27 *Brevibacterium linens*, L33 *Streptomyces flavidofuscus*, L35 *Nocardia nova*, L45 *Streptomyces grieus*, L56 *Streptomyces sp.*, B451 *Streptomyces fradiae*) which showed more than 40 % survival rate were selected for further study.

- The antiviral principle (against WSSV) of actinomycetes L27, L35, L45, L56 and B451 could be extracted by passing the crude culture supernatant through Amberlite (XAD-16) column and eluting with methanol.

- Five actinomycetes (L27, L45, L56 and B451) were selected as potent source of anti-WSSV molecules for application in shrimp culture systems.

- Inhibition 2 pathogenic vibrios was observed in thirty two out of the 99 isolates. The inhibitory activity of actinomycetes (99 Nos) against prawn pathogens was found to be less. Maximum inhibition was exhibited by L39 (81.81%) followed by L45 (72.7%) and L18 (45.45%). However the percentage of pathogens being inhibited by the various actinomycetes was in the range 3-17% . *V.splendidus* was the most susceptible pathogen which was found to be inhibited by 17% of the actinomycete isolates.

- All actinomycete isolates (99 Nos) possessed proteolytic activity , lipase activity was shown by 96 % followed by amylase (95 %) , chitinase (36 %) & cellulase (9.8 %) activity. Culture Nos L17, L18,L25, L39, L45, L56 and B451 were capable of producing all the five enzymes.

- The actinomycetes (L18 (*Prauseria hordei*), L39 (*Nocardia alba*), L45 *(Streptomyces griseus*) selected for probiotic application, were proved to be non pathogenic to shrimps both *in vitro* and i*n vivo*.

- The study on the colonisation of actinomycetes (L18,L39 and L45) in the gut revealed that they were not able to colonise the intestine.

- In the co-culture experiments with *Vibrio harveyi* and selected actinomycetes (L18, L39 and L45) a gradual decline in *Vibrio harveyi* was observed .

- Application of actinomycetes (L18,L39 and L45) to *Penaeus monodon* post larvae rearing tanks also revealed that there was a reduction in *Vibrio harveyi* count while the total bacterial count was more or less steady . The actinomycetes enhanced the survival and growth rate of the post larvae and therefore could be applied as probiotics in shrimp aquaculture systems for improving product yield.

- Immune profile was found to be high in *Penaeus monodon* maintained on actinomycete incorporated diets (L18 (*Prauseria hordei*), L39 (*Nocardia alba*), L45 *(Streptomyces griseus*)) compared to control. Haematological parameters were found to be maximum on post challenge day 2.

- Marine actinomycete fed groups showed maximum survival when challenged with WSSV. L18 (*Prauseria hordei*), L39 (*Nocardia alba*), L45 *(Streptomyces griseus*) incorporated diets showed 20 %, 50 % and 62.5% survival respectively. This study showed that the selected actinomycetes (probionts) act as immunostimulants.

- The antioxidant profile in *Penaeus monodon* fed with actinomycete incorporated diets (L18 (*Prauseria hordei*), L39 (*Nocardia alba*), L45 *(Streptomyces griseus*)) showed that the activity was less before and after challenge when compared to control. Marine actinomycete fed group showed maximum survival rate indicating that

they can act as an antioxidant by scavenging free radicals produced during infection by WSSV.

The present study revealed the importance of marine actinomycetes as a potent source of bio active secondary metabolites. The selected isolates were capable of protecting *Peaneus monodon* against WSSV infection. They also proved to be inhibitory to vibrios and is a rich pool of hydrolytic enzymes. Their capacity to proliferate in saline environments and their property of non-pathogenicity to prawns makes them good candidates to be applied as probionts in penaeid shrimp aquaculture. They also enhanced the immune status of shrimps challenged with WSSV and act as a good source of antioxidants. Exploitation of the potential for the prophylactic and therapeutic measures in aquatic animal health management would be highly rewarding. This work is a preliminary study targeting marine actinomycetes as a source of antiviral compounds and as probionts in *Penaeus monodon* culture systems. More work is needed to understand the nature and mode of action of the bioactive compound, the various aspects of immune and antioxidant responses under challenge and when exposed to proactive treatments, and the dose and frequency of application of such compounds under rearing conditions .

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