A MARINE ISOLATE *CANDIDA SAKE* AS SOURCE OF IMMUNOSTIMULANTS TO *FENNEROPENAEUS INDICUS*

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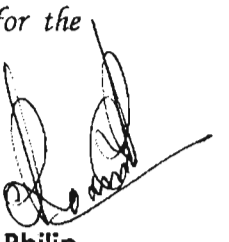
.....to my beloved uncle Late Sri. T.P. Kunhiraman

Certificate

This is to certify that the thesis entitled **"A MARINE ISOLATE CANDIDA SAKE AS SOURCE OF IMMUNOSTIMULANTS TO FENNEROPENAEUS INDICUS"** *is an authentic record of research work carried out by Mr. Sajeevan T.P under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part thereof has been presented before for the award of any degree, diploma or associateship in any university.*



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Declaration

I hereby do declare that the thesis entitled **" A MARINE ISOLATE *CANDIDA SAKE* AS SOURCE OF IMMUNOSTIMULANTS TO *FENNEROPENAEUS INDICUS*"** *is a genuine record of research work done by me under the supervision of Dr. Rosamma Philip, Senior Lecturer, School of Marine Sciences, Cochin University of Sciences and Technology, Cochin – 682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title of any university or institution.*

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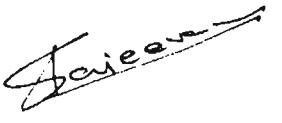

(Sajeevan T.P)

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Sajeevan T. P.

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Chapter 1

General Introduction

1.1. Introduction

Aquaculture has developed rapidly over the last three decades to become an important activity worldwide. The United Nations (UN) estimates that, by 2037 the world's population would exceed 10 billion. This rapid growth in humankind will bring with it severe difficulties not only in terms of shelter, education and health care, but also in maintaining current levels of protein intake. The Food and Agricultural Organization (FAO) of the UN acknowledge that global fishery output must be increased by at least 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 world's 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.

1.2.Crustacean aquaculture

Crustacean aquaculture is considered as a high value activity and tends to have higher monetary value and annual world production is over 8 million metric tonnes (FAO, 2000). Of this figure over half is made up of shrimps and prawns and the proportion of this production coming from farms has increased rapidly since the 1980s. In 2000, more than 85% of the cultured shrimp production was still realised by farmers in the eastern hemisphere, with Thailand as the main farming country, followed by China, Indonesia and India (Rosenberry, 2001). 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, P. chinensis, P. merguiensis, Fenneropenaeus indicus* and *Litopenaeus vannamei.* Much of the world shrimp production still comes from extensive culture. However, research on biology and ecology of penaeid shrimp significantly contributed to its aquaculture development and, related to that, its intensification.

1. 3. Disease in aquaculture

Out break of diseases is being increasingly recognized as a significant constraint on aquaculture production. The most significant diseases of cultured penaeid shrimps have had viral or bacterial aetiologies, but a few have fungal and protozoan agents as their cause.

Many shrimp farms around the world have been badly hit by epidemics of White Spot Syndrome Virus (WSSV) since it emerged first in Asia around 1993 (Lightner, 1996). WSSV infects a wide spectrum of hosts, including shrimps (penaeid and non-penaeid), crabs and aquatic insect larvae (Lightner, 1996; Flegel, 1997). Some species are susceptible enough to become diseased and some are not so highly susceptible so as to succumb to the disease, but the latter are important as carriers, able to spread the pathogen (Wang *et al.*, 1999). The disease is characterized by the appearance of white spots on the carapace and reddish discolouration of the body. WSSV is an enveloped ovoid- virus with a rod–shaped nucleo-capsid with flat ends and having 300kb double stranded DNA as genetic material (Wang *et al.*, 1995; Yang *et al.*, 1997). The economic loss due to this single virus was tremendous and in Asia alone the estimated loss was around US$ 4 to 6 billion (Lightner, 2003). It is quite obvious 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 available for shrimp disease research during the period 1996-2010 (Lundin, 1996).

1.4. Disease control in shrimp aquaculture

Rapid dissemination of viral disease in shrimp aquaculture invites concern over its effective control, rapid diagnosis and treatments. 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 the 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 viral diseases. In this context 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 have to be taken care of.

1.5. Use of antibiotic and other chemotherapeutics in aquaculture

The application of antibiotics or other chemicals to culture ponds is expensive and undesirable as it risks contamination of both the environmental and the final product (Capone *et al.*, 1996). A disquieting observation has been the gradual evolution of drug resistance in many bacteria. The use of almost every antimicrobial agents leads, sooner or later, to the selection of resistant strains from previously sensitive bacterial populations. Emergence of a resistant strain at a farm site renders particular antimicrobials useless, and the resistance can easily spread until it is the norm for that species. Antimicrobials do not cause the genetic and biochemical changes that make a bacterium resistant, but they select strains carrying the genetic information that confers resistance (Munn, 2004). The more an antibiotic is used, greater the selection pressure for resistance to evolve.

For most crustacean species, culture still depends on the use of seed produced in hatcheries, mainly from sexually matured females caught from the wild. So selective breeding programmes and the use of genetically modified strains are still having a long way for providing an ethically acceptable and commercially viable means of reducing the problem posed by epidemics. Therefore there has been a growing interest in finding ways to protect stock prophylatically in a manner conceptually equivalent to the use of vaccines, now routine for humans, agricultural livestock and more

recently farmed fish (Smith *et al.*, 2003). Disease prevention in aquaculture by prophylactic use of chemicals emphasise procedures that prevent infections even if pathogens are present in the environment.

1.6. Crustacean immune system

Invertebrates, especially arthropods, are evolutionarily successful groups that have representation in various environments and ecological conditions and survived without the development of antibody-based immunity, relying instead upon relatively non-specific defence mechanisms. Shrimps possess immune system that, although quite complex, is substantially different from that of vertebrates. There is no specific immunity (no true antibodies and substantially less lymphocyte heterogeneity), though few aspects of specific immunity (inducibility) appear to be present in some cases. Shrimps possess both humoral and cellular immune responses, although they are less specialized than vertebrate immune responses. The innate immunity characterized by a diverse array of humoral factors that originate and/or reside in haemocytes and released during the immune response.

1.6.1. Immune system of shrimp.

The immune system of crustaceans is primarily related to their blood or haemolymph and to its circulating cells or haemocytes. 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 (Bauchau, 1981, 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).

1.6.1.1. Haemocytes

Haemocytes play an important role in cellular responses, including clotting, non-self recognition, phagocytosis, melanisation, encapsulation, cytotoxicity

and cell-to-cell communication. 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 Graves, 1985) and are capable of phagocytosis (Smith and Soderhall, 1983). The percentage population of hyaline cells varies 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% (Gargioni and Barracco, 1998).

The semigranular cells, which contain small granules and display some phagocytic activity, are specialized in particle encapsulation (Persson *et al.*, 1987). Semigranular cells can respond to microbial polysaccharides such as lipopolysaccharides and β-1,3-glucan by degranulation process (Johansson and Soderhall, 1985).

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 (Barracco *et al.*, 1991).

1.6.1.2. Haematopoiesis

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).

1.6.2. Cellular immune responses

1.6.2.1. Phagocytosis

The ability to ingest and kill microorganisms is a key component in the host defence. Phagocytosis is the most common of the cellular defence reactions and together with humoral components constitute 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, a five to seven times higher degree of phagocytosis was observed than untreated control monolayers (Smith and Soderhall, 1983). But the factors, which act as an opsonin in crustacean haemolymph is yet to be isolated.

1.6.2.2.Nodule formation

When the body cavity is invaded by a large number of microorganisms, nodule formation or cell clumping occurs in several invertebrates, including crustaceans. These microorganisms entrapped in several layers of haemocytes, get melanised 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.

1.6.2.3.Encapsulation

In addition to nodule formation and phagocytosis, invertebrate blood cells are capable of immobilizing parasites, that are too large to be ingested by a single blood cell by surrounding them with multicellular sheaths. Considerable confusion exists regarding the types of blood cells involved in encapsulation. Also very little is known about the initiation process of an encapsulation reaction. In crustaceans the only cells to react to foreign

molecules like β-1,3-glucan from fungi or lipopolysaccharides (LPS) from bacteria are the semigranular cells. This cell is also the first one to react to foreign particles and to encapsulate any invading pathogens. Some opsonin factors present in the haemolymph can also mediate the encapsulation process.

1.6.3. Humoral immunity

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

1.6.3.1. Lectins

Lectins have been regarded as potential molecules involved in immune recognition and phagocytosis of microorganisms through opsonisation. They are non-enzyme proteins or glycoproteins without catalytic activity that binds to specific carbohydrates expressed on different cell surfaces. These type of carbohydrate binding proteins, which recognize surface structures common for different pathogens, represent a primitive immune response and called pattern recognition proteins (PRPs). 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 a purified lectin called monodin. Vargas-Albores *et al.* (1993)

reported the ability of purified lectin to react with different marine species of *Vibrio*.

1.6.3.2. The proPhenoloxidase system (proPO System)

The best-studied enzymatic system of crustaceans is phenoloxidase cascade (Sritunyalucksana and Soderhall, 2000). This enzyme is a part of complex system of proteinases, pattern recognition proteins and proteinase inhibitors constituting the so called prophenoloxidase (proPO) activating system. 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 (Soderhall *et al.*, 1990).

The proPO is an inactive zymogen stored in the granular haemocytes, which degranulate and release the inactive enzyme into haemolymph. According to the amino acid sequence, proPO belongs to a family of copper containing proteins including haemocyanin and tyrosinases. The activation of proPO is by a proteolytic cleavage mediated by serine protease (proPO activating enzyme, ppA) which itself is seen in an inactive form in the haemolymph. Microbial polysaccharides, like LPS or β-1,3-glucan can mediate the activation of 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 polymerised into melanin (Cerenius and Soderhall, 2004). Melanisation is involved in the process of tanning of cuticle during the post-molt period, in

wound healing and in defense 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 (Johansson and Soderhall, 1989). 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 (Johansson *et al.*, 1995).

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 have 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). A schematic overview of the important factors in the crustacean defence system is given in Fig 1.1.

1.6.3.3. Antimicrobial peptides

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

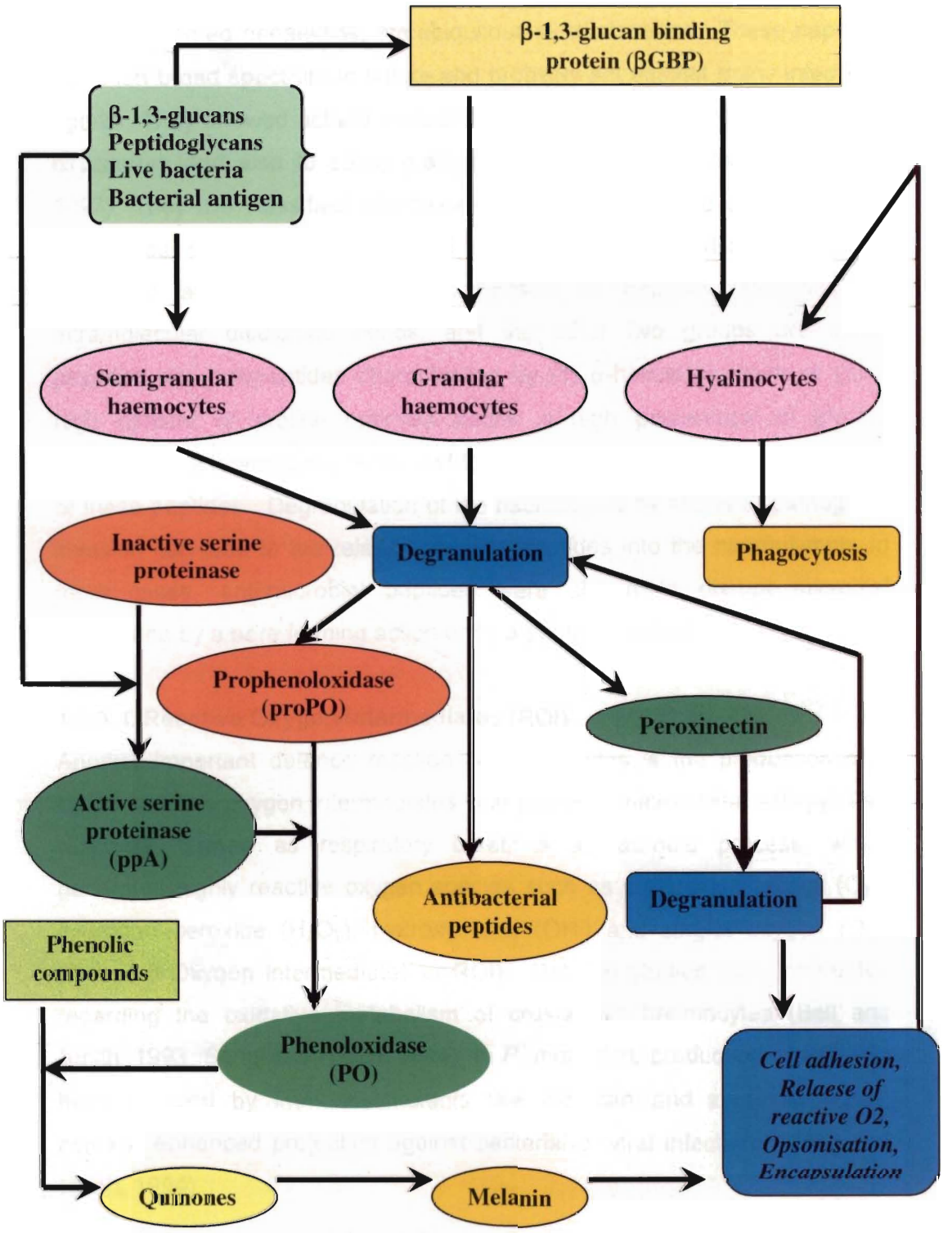


Fig 1.1 Flow diagram showing the crustacean defence system (Redrawn from Smith *et al.*, 2003)

peptides have been isolated and characterized from *P. vannamei* (Destoumieux *et al.*, 1997 and 2000) and recent studies show that these peptides, named penaeidins, 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 (Destoumieux *et al.*, 1997). 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.

1.6.3.4. Reactive Oxygen Intermediates (ROI)

Another important defence reaction of haemocytes is the production of a series reactive oxygen intermediates with powerful microbicidal activity. This response termed as 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_2^1) (Reactive Oxygen Intermediates or ROI). Detailed studies were conducted regarding the oxidative metabolism of crustacean haemocytes (Bell and Smith.,1993; Song and Hsieh, 1994). 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).

1.6.4. The concept of vaccination in shrimps

Vaccination, a strategy developed for generating immunity to the lethal small pox virus, is based on the memory capacity of adaptive immune system. One of the most important attributes of an adaptive immune response is the establishment of a state of 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).

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. 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.

Shrimps possess non-specific immune system, which is substantially different from that of vertebrates. 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 (Teunissen *et.al.*, 1998) 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.* (1992) 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.

1.7. Immunostimulants in aquaculture

Immunostimulants are chemical compounds that activate the immune system of animals and render them more resistant to infections by viruses, bacteria, fungi, and parasites. It has been known for many years that cell wall fragments of microorganisms render animals more resistant to microbial infections (Kiser *et al.*, 1956). The ability of the immune system to respond to microbial surface components is the result of an evolutionary process whereby animals have developed mechanisms to detect common and highly conservative chemical structures of potential pathogenic microorganisms and to use those structures as "alarm signals" to switch on the defence against infection. The immune system will therefore respond to an immunostimulant as if challenged by a pathogenic microbe. Administration of an immuno-stimulant prior to an infection may protect the animal against an infection which otherwise would have become severe or lethal.

Immunostimulants have been obtained from diverse natural sources and a large number have been synthesised chemically with the natural products as structural models, wherein the main natural source of immunostimulants is microbial cell wall. The active principles of immunostimulatory cell wall preparations are various muramylpeptide fragments, lipopolysaccharides (LPS), lipopeptides, acyloligopeptides (Azuma, 1987). The immuno-

stimulants present in the cell walls of mushrooms and yeast are mainly β glucans.

1.7.1.Yeast cell wall glucan as immunostimulant

β-1,3-glucans appear to be the most promising of all immunostimulants so far experimented in fish and shrimp. Glucan belongs to the class of drugs known as Biological Response Modifiers (BRMs). β-Glucans are polyglucose molecules linked through β-1,3 bonds in a long chain and with β-1,6 branches consisting of single glucose molecule or chains of glucose molecules (Fig.1.2). Such glucans can exist in various structural forms and may be in the form of water-soluble oligomers, water soluble or insoluble macromolecules or particulates.

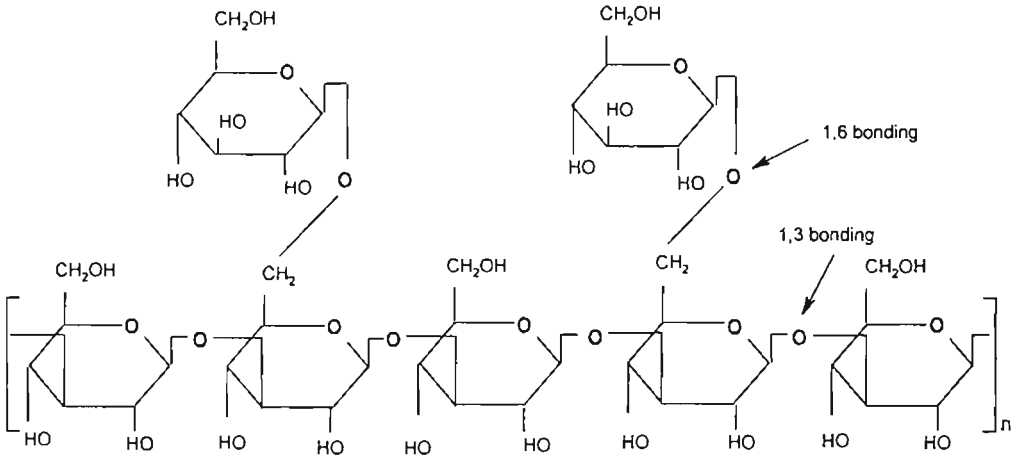


Fig1.2 Structure of β- 1,3-glucan with 1-6 branching

There are well-defined receptors for β-1,3-glucans on the macrophages of warm-blooded animals, fish and on shrimp haemocytes. The β-1,3-glucan receptor on macrophages is highly specific in their action and can "recognise" a β-1,3-glucan chain with more than 3 to 5 glucose units.

Glucan is, from an evolutionary point of view, the most widely and most commonly observed macrophage activator in nature. β-1,3-glucan has been

proven to both stimulate and activate macrophage cells, which can overcome the negative effects of immunosuppression. Activation of macrophages, results in increased non-specific phagocytic activity, killing pathogens more efficiently and thereby preventing disease outbreaks.

The phenoloxidase system is an important element in the disease resistance of crustaceans. It is, however, of crucial biological significance that this latent defence apparatus is able to identify a real infection and not be switched on by signals other than those unique to pathogens. Crustaceans use LPS and the β-1,3-glucan structure as specific signals to activate the prophenoloxidase system. The haemolymph of crustaceans contain proteins that specifically bind to β-1,3-glucans. When this protein has reacted with β-1,3- glucan, it can bind to a specific receptor on the haemocytes and induce degranulation and release of the prophenoloxidase, leading to the proPO cascade and formation of melanin (Cerenius and Soderhall, 2004). Various authors reported that glucan derived from yeast cell wall act as an immunostimulant in penaeid shrimps (Sung *et al.*,1994; Song *et al.*, 1997; Chang *et al.*, 1999, 2000, 2003).

1.7.2.Bacterial cell wall products as immunostimulants

1.7.2.1.Lipopolysaccharides (LPS)

The bacterial cell wall carbohydrates including Lipopolysaccharides (LPS) and peptidoglycan are the two other most common immunostimulants in aquaculture practice. Gram-negative bacteria consist of a complex LPS structure that is anchored to the underlying peptidoglycan (Fig.1.3). Animals have evolved mechanisms to detect the presence of LPS, and this molecular structure has diverse nonspecific action on the immune system, activating both macrophages and lymphocytes (Burrell, 1990).

A low dose of LPS enhances the disease resistance and act as a prophylactic agent (Noworthy, 1983), but their high toxicity to warm blooded animals may limit their use in practice (Bone, 1991). But LPS is less toxic to

fish and shrimps and has been shown to be active as an immunostimulatory complex that increases the disease resistance of fish and shrimps (Jorgensen, 1994; Song and Sung, 1990).

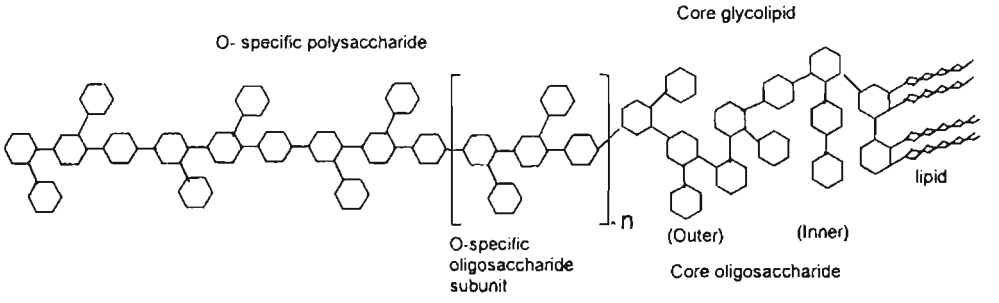


Fig. 1.3. Structure of lipopolysaccharides (LPS) of Gram-negative bacteria

1.7.2.2.Peptidoglycan

Peptidoglycan is a cell wall component of many bacteria, though it is found in greater amount in Gram-positive bacteria (Fig 1.4). Matsuo and Miyazono (1993) have reported that oral administration of peptidoglycans from *Bifidobacterium thermophilium,* protected juvenile rainbow trout challenged with *Vibrio anguillarum.* Boonyaratpalin *et al.* (1995) reported the effects of PG from *Brevibacterium lactofermentum* on the growth, survival, immune response and tolerance to stress in *P. monodon.* Itami *et al.* (1998) noted that peptidoglycan from *Bifidobacterium thermophilum* protected shrimps against White Spot Syndrome Virus (WSSV).

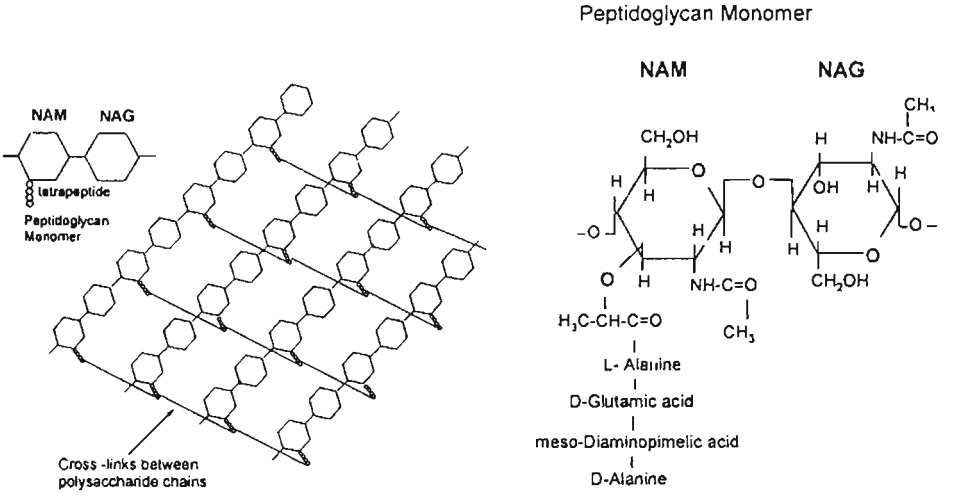


Fig.1.4 Structure of peptidoglycan (PG) of bacteria

Apart from these, various other cell wall preparations of bacteria namely, BCG, Freunds (Complete) Adjuvant, curdlan, and other cellular glycans from mycelial fungi and plants have been in use as immunostimulants with limited success (Sakai, 1999).

1.7.3. Rationale of immunostimulants in shrimp aquaculture

It is an inevitable part of intensive aquaculture to stress the animals by containment, transport, handling, sorting, periodic low oxygen or high ammonia etc and as a result create a physiological condition characterized by a suppressed immune system (Raa, 1996). The development of disease particularly in shrimp aquaculture, resulted not only from an intensification of production, based on zootechnological progress, but also from ecological and environmental disturbances, pollution and nutritional imbalance (Kautsky *et al.*, 2000).

Avoidance of pathogen is not practically sound where a large-scale conventional open culture system exists in a country like India. Nowadays closed culture system or recirculating culture systems are being practiced in many countries, where no or limited water exchange takes place. In shrimp farming system, horizontal transmission of the virus occurs *via* oral ingestion and the waterborne route (Corsin *et al.*, 2001) whereas, in hatcheries vertical transmission has been identified to be the major route (Mushiake *et al.*, 1999). Therefore disease control in aquaculture should focus first on preventive measures related to water quality, technology and husbandry thus significantly eliminating disease promoting factors. Disease risk may be reduced further by the use of improved feeds, higher disease resistance through selective breeding and by the use of immunostimulants, probiotics and vaccines. But in shrimp aquaculture, vaccination is not a relevant option, either because the pathogenic agent is unknown and therefore beyond the scope of a classical approach to vaccine development or because of impaired performance and mortality as a result of environmental stress, which elicit infection by opportunistic pathogens in the environment.

Immunostimulants may reduce the risk of disease under such circumstances. Since there is no obvious biological basis for making vaccines for crustaceans; the use of immunostimulants to increase their disease resistance may be a rewarding option (Raa, 1996). Currently many commercial products are available in shrimp aquaculture sector under the label of immunostimulants and are extensively used by the shrimp farmers. But the nature of these compounds, the dose required for eliciting immunostimulation at optimum level, duration of protection conferred, route of application etc. are still uncertain. These lacunae in the scientific knowledge pertaining to such practices necessitate a comprehensive study on products before they are launched into the market. Present work is aimed at the identification and utilisation of marine yeast as a source of immunostimulant to penaeid prawn culture system. Indian white prawn *Fenneropenaeus indicus* is selected as model organisms to study the immunostimulatory effect of the yeast/yeast products. In this context present study was undertaken with the following objectives

1. Screening of marine yeasts to identify potent strains showing immunostimulant and growth enhancing property in *Fenneropenaeus indicus*.
2. Optimisation of yeast biomass concentration in feed for effective protection against experimental infection with White Spot Syndrome Virus.
3. Optimisation of culture conditions of the selected marine yeast for biomass production.
4. Extraction and partial H-NMR structural characterization of (1→3)-β-D-glucan from selected yeasts
5. Efficacy of (1→3)-β-D-glucan from marine yeast as an immunostimulant to Indian white prawn *Fenneropenaeus indicus*. Optimisation of the dose, frequency and mode of glucan administration to *Fenneropenaeus indicus*.
6. Assessing the immunological profile of *Fenneropenaeus indicus* on administration of whole cell marine yeast/cell wall glucan

First chapter present a general introduction of the topic. The results of the present study are presented in eight chapters. Screening of selected marine yeast for growth enhancing and immunostimulants property in *Fenneropenaeus indicus* is presented in chapter 2. Third chapter deals with the optimisation of culture conditions of selected marine yeast for biomass production. Immunological profile of *Fenneropenaeus indicus* on oral administration of marine yeast is presented in chapter 4. The fifth chapter deals with extraction and partial H-NMR characterisation of cell wall (1→3)-β-D-glucan from selected yeasts. The H- NMR spectra obtained for marine yeast glucan was compared with that of a cell wall glucan extracted from baker's yeast *Saccharomyces cerevisiae*. In chapter 6, the efficacy of cell wall glucan from marine yeast as an immunostimulant to Indian white prawn *Fenneropenaeus indicus* is presented. The dose, frequency and mode application are also presented this chapter. In chapter 7 the dose and frequency of glucan to be administered was confirmed by studying the immunological profile of *Fenneropenaeus indicus*. Chapter 8 describes a comparative study of whole cell marine yeast, it's glucan and a glucan obtained from baker's yeast as an immunostimulant to *Fenneropenaeus indicus*. This is followed by summary, list of references and appendices.

Chapter 2

Screening of marine yeasts for immunostimulant and growth enhancing property in *Fenneropenaeus indicus*

2.1. Introduction

The discovery of marine yeasts dates back to 1894, when Fisher isolated red and white yeasts from the Atlantic Ocean and identified them as *Torula* sp. and *Mycoderma* sp. respectively. The materials collected by Fisher during his cruise in the Atlantic indicated the presence of yeasts not only in the coastal areas, but also far out in the open areas of the ocean. The frequency of the occurrence of the yeasts was higher at higher latitudes. Bhat and Kachwalla (1955) isolated more than 80 cultures of yeast from off Bombay. Kohlmeyer and Kohlmeyer (1979) reported 177 species of yeast from marine environment of which only a few representatives of the genera *Candida, Rhodotorula, Torulopsis, Kluyveromyces, Metschnikowia, Cryptococcus* etc. were truly obligate marine forms.

Recently there has been considerable progress in the isolation and identification of marine yeasts. The isolation frequency of yeasts falls with increasing depth, and yeasts of the order *Ascomycetes* (e.g. *Candida, Debaryomyces, Kluyveromyces, Pichia,* and *Saccharomyces*) are common in the shallow waters, whilst yeasts belonging to Basidiomycetes are most common in deep waters (e.g. *Rhodotorula* has been isolated from a depth of 11,000 m (Munn, 2004). The wide distribution of yeasts in the marine environment not only in the surface layers but also at greater depths, leads to the conclusion that the peculiar conditions of sea do not preclude the possibility of existence of yeast species. Among marine heterotrophic microorganisms developing on the meat-peptone media, yeasts are less abundant than bacteria. Apparently the distribution of yeasts in marine environment is related to the presence of easily assimilable organic substances. Kriss (1963) was of the opinion that the majority of the yeasts in sea were not accidental forms, but species adapted to life under marine conditions. They undoubtedly take part in the process of transforming the inorganic and organic matter and play a definite role in the food relationship of the inhabitants of the sea and oceans.

2.1.1.Nutritional value of yeast

Yeast is a rich source of vitamins particularly vitamin-B complex (Evans, 1985; New, 1987) which are stable and readily available to the animals (NRC, 1983). The vitamins are in conjugated form and there occurs less chance of leaching out into water. The protein content of yeast varies from 45-65% depending on the strain.

Yeasts act as both a protein and an energy source. About 20% of the nitrogen in the yeast occurs as nucleic acid nitrogen especially in the form of RNA (Rumsey *et al.*, 1991). The methionine content is low when compared to chicken egg protein, which is the seriously limiting amino acid in yeast (New, 1987). The minerals are embedded in the yeast cell and are available in an assimilable form. In yeast the sodium content is low but does not need to be supplemented in diets of sea and brackish water cultured animals (Beck *et al.*, 1979).

2.1.2.Use of microbes in aquaculture

The use of antibiotics and chemical therapeutics in prawn farms caused the spread of antibiotic resistance in pathogens causing much concern and scientific community initiated efforts to find alternate strategies for the control of pathogens. Some chemotherapeutics used in aquaculture can result in environmental imbalance (Kautsky *et al.*, 2000) and may have detrimental side effects not only on the treated animals but also on those who consume them (Karunasagar *et al.*, 1994). Disease risk may be reduced further by the use of improved feeds, higher disease resistant strains through selective breeding and by the use of immunostimulants, probiotics and vaccines.

Application of probiotics in aquaculture was envisaged in the early 1970s, whereby the gut micro flora of shrimps could serve as food despite keeping them healthy (Hood and Meyers, 1973). This approach is currently investigated in aquaculture where the beneficial role of non-pathogenic bacteria is exploited to protect larvae and its adults (Gibson *et al.*, 1998;

Verschuere *et al.*, 2000). The use of probiotic bacteria and immunostimulants are two rewarding options developed as preventive methods against diseases (Fuller, 1992). Probiotics may improve digestive activity by the synthesis of vitamins, cofactors or improve enzyme activity (Fuller, 1989, Gatesoupe, 1999).

Bacteria that have been used successfully as probiotic belong to the genera *Vibrio* (Griffith 1995; Garriques and Arevalo, 1995) and *Bacillus* (Moriarty, 1998; Rengpipat *et al.*, 1998). Several mechanisms have been suggested as mode of action for probiotic bacteria. The competitive exclusion mechanism, based on the substitution of the pathogen by the beneficial population has been considered to be relevant by many authors (Fuller, 1989; Moriarty, 1998; Gatesoupe, 1999). There are several ways by which probiotic bacteria can induce bacterial antagonism by producing antimicrobial agents, such as antibiotics, antimicrobial peptides or siderophore substances (Sugita *et al.*, 1998). Certain microorganisms which show probiotic potential are also found to exhibit immunomodulation in crustaceans (Rengpipat *et al.*, 2000; Guillian *et al.*, 2003; Alavandi *et al.*, 2004).

Several studies have suggested replacing the fishmeal component of aquaculture feeds with low cost feed ingredients like soymeal or single cell proteins (SCP) (Tacon and Jackson, 1985; Kiessling and Askbrandt, 1993, Manju and Dhevendaran, 1997). As a protein source, single cell proteins (SCP) of yeast or bacterial origin appears especially attractive because the protein content and amino acid composition of these organisms compare well with that of fishmeal (Spinelli *et al.*, 1979). While considering the economics of shrimp aquaculture practice, the cost of feed accounts 80% of the total expenditure. The essential and expensive component of feed is protein, especially the fishmeal, which is in short supply. Hence, effective replacement of these high cost ingredients with low cost commodity without compromising the nutritional quality of feed is certainly a sensible objective. As a fact many workers tried alternative protein source such as soybean

meal (Akiyama, 1990; Lim and Dominy, 1990; Piedad-Pascual *et al.*, 1990; Tidwel, *et al.*, 1993; Jones *et al.*, 1996; Muzinic *et. al.*, 2004), yeast, bacteria and algal proteins (Matty and Smith 1978; Mahnken *et al.*, 1980; Tamaru *et al.*, 1993; Coutteau *et al.*, 1993; Sanderson and Jolly, 1994; Teles and Gonzalves, 2001; Novoa *et al.*, 2002; Li and Gatlin III, 2003) and results proved to be encouraging. Among these, yeasts have been the most commonly used SCP within aquafeeds (Tacon, 1994).

2.1.3. Utilisation of yeast in aquaculture

The advantages of microbial protein are high productivity, a high proportion of cell mass as proteins, a good profile of desirable amino acids, good performance in feeding livestock and no toxic or carcinogenic compounds. Besides these, the yeasts have the advantage of large size, low nucleic acid content, long history of use as food and ability to grow at low pH (Mitchel, 1974).

Because of their high protein content and relatively low production cost, yeasts have been considered as an algal substitute for several species of filter feeders e.g. rotifers (Fukusho, 1980; Al-Khars *et al.*, 1986) *Artemia* (Johnson, 1980), molluscs (Urban and Langdon, 1984; Coutteau *et al.*, 1990, 1993, 1994a, b; Nell *et al.*, 1996) etc.

Baker's yeast, marine yeasts and lipid-enriched yeast diets are now routinely used as sole diet or in combination with the algae *Chlorella* for rearing the rotifer *B. plicatilis*. In addition considerable progress has been made in the replacement of live algae in the larval rearing of commercially important shrimp species. Mock *et al.* (1980) reported that replacing algae with active dry baker's yeast as feed for blue shrimp *P. stylirostris* larvae gave better results. Use of lactic acid yeast *Kluyvermyces fragilis* at a level of 13% in diet for tiger prawns improved growth performance (Hertrampf and Piedad-Pascual, 2003). Hertrampf and Piedad-Pascual (2003) reported that under field conditions squid meal was replaced successfully by 10 to 15 % lactic

acid yeast in diet for kuruma prawns. Coutteau *et al.* (1994b) reported that in hard clam *Mercenaria mercenaria* replacing 80% of the algae with yeast resulted in increased growth rate of up to 90% of the algal control. Muzinic *et al.* (2004) reported that brewer's grains with yeast could be included in practical diets for red claw crayfish *Cherax quadricarinatus* at a level up to 30% without adverse effects on growth and survival.

Yeasts like, *Candida* sp. and *Saccharomyces cerevisiae,* are believed to have immunostimulatory properties by virtue of their complex carbohydrate components and nucleic acid content (Anderson *et al.,* 1995). So there is a growing awareness in harnessing the health benefits of these microorganisms for both human medicine and animal husbandry. Patra and Mohammed (2003) showed that *Artemia* enriched with a probiotic yeast *Saccharomyces boulardii* exhibited increased resistance to *Vibrio* infection and emphasised the possible use of this technology to deliver probiotic to other aquatic larvae feeding on them. *Saccharomyces boulardii* is well known as human probiotic yeast, isolated from lychee fruits, which is active against *Clostridium dicile* (Buts *et al.*, 1993) and *Vibrio cholera* toxin (Czerucka *et al.*, 1994).

Yeasts have been demonstrated to exhibit immunostimulatory effect when fed to salmon (Robertsen *et al.*, 1990). Dehasque *et al.* (1995) reported that Atlantic salmon fry, fed with a diet containing baker's yeast at 2% showed better survival than control animals when challenged with *V. anguillarum.* Uma *et al.* (1999) reported that immuno-potentiating effect observed in *P. indicus* fed with a commonly available live stock feed "Lacto-sacc" could be due to the presence of yeasts and lactic acid bacteria in the feed given. Vici *et al.* (2000) reported that dried yeast *Acremonium dyosporii* when incorporated into the larval feed of *Macrobrachium rosenbergii* increased the disease resistance against *Vibrio* infection and the larvae showed increased percentage of metamorphosis. Enhancement of vibriosis resistance was reported in *Penaeus vannamei* after feeding with *Phaffia rhodozyma*

incorporated diet (Scholz, *et al.*, 1999). Recently Burgents *et al.*(2004) reported enhanced disease resistance against experimental infection of *Vibrio* in Pacific white shrimp *Litopenaeus vannamei* fed with a *Saccharomyces cerevisiae* incorporated feed.

The immunostimulatory property of yeast cells is attributed to its cell wall content namely β-1,3-glucan (Furkas, 1985). Glucan is a proven immunostimulant both in vertebrates and invertebrates. β-Glucan has been successfully used to enhance resistance in fishes and crustaceans against bacterial and viral infections (Itami *et al.*, 1994; Robertsen *et al.*, 1994; Sung *et al.*, 1994; Song *et al.*, 1997; Chang *et al.*, 1999, 2000, 2003).

The potential of yeast as a food in aquaculture has been proven by their successful application in the rearing of rotifers and some species of penaeid shrimps. However, there are also reports emphasising the limited nutritional value of yeast, which can be attributed to its low digestibility due to its rigid cell wall. The development of techniques to improve the digestibility and the nutritional composition of yeast-based diets resulted in a product with great potential as a substitute for unicellular algae (Coutteau *et al.*, 1990). According to Albentosa *et al.* (1989) manipulated yeast diet can substitute up to 80% of the algal diet in the culture of juveniles of Manila clam *Tapes philippinarum* without significant decrease in growth rate. Disruption or homogenisation of the yeast cell wall markedly increased the apparent digestibility of the yeast protein in rainbow trout (Rumsey, 1988).

Marine yeasts have been proved as safe and have been showing a beneficial impact in biotechnological processes. Gama *et al.* (2001) reported that yeast strains isolated from marine environment showed strong adhesion potential to human cell lines of Hela S3 and KATO III and suggested a possible application of these yeast probiotic supplements in humans.

Utilisation of marine yeast in aquaculture as probiotic, immunostimulant or growth promoter is very limited. Larvae of kuruma prawn *P. japonicus* and tiger prawn *P. monodon* were fed with marine isolate of *Saccharomyces cerevisiae* to obtain high survival rates particularly at zoea stages (Furukawa *et al.*, 1973; Aujero *et al.*, 1985). Kamel and Kawano (1986) studied the utilisation of a marine yeast *Candida* as a feed ingredient for zooplankton *Brachionus plicatilis* larvae of *Penaeus semisulcatus* and *Metapenaeus affinis*. Nell *et al.* (1996) reported that algae from the feed of Sydney rock oyster, *Saccostrea commercialis* could be substituted with marine isolates of *Debaryomyces hansenii* and *Dipodascus capitatus*.

Reports on enhanced survival and immunostimulation by purified β-glucan are more common than that of yeast (Itami *et al.*, 1994). However, utilisation of marine yeasts as a source of immunostimulant in aquaculture is not reported earlier.

The present chapter deals with the screening of marine yeast isolates for their immunostimulant and growth enhancing properties in *Fenneropenaeus indicus*.

2.2. Materials and methods

2.2.1. Primary screening of yeasts for utilisation as a feed supplement and immunostimulant

2.2.1.1 Yeast strains

Seven marine yeast strains isolated from coastal waters off Cochin and maintained in the microbiology laboratory of School of Marine Sciences were used for the study. These seven strains were characterised and identified at IMTECH, Chandigarh (Table 2.1) as *Debaryomyces hansenii* S8, *Debaryomyces hansenii* S87, *Debaryomyces hansenii* S100, *Candida sake* S165, *Debaryomyces hansenii* S169, *Candida tropicalis* S186 and *Torulospora delbruecki* (S303) (Table 2.2). Biomass of the strains were produced by culturing on Malt Extract Agar media (Malt Extract- 20 gm,

Table 2.1. Taxonomical characteristics of the selected strains of yeast (as obtained from IMTECH Chandigarh, India)

Sl.No.	Name of the Test	S8	S87	S100	S165	S169	S186	S303
1	D-Glucose fermentation	+	+	+	+	+	+	+
2	D-Galactose fermentation	-	-	-	+	-	?	-
3	Maltose fermentation	-	-	-	+	-	+	-
4	Me alpha-D-glucoside fermentation	-	-	-	-	-	-	-
5	Sucrose fermentation	+	-	+	+	+	+	-
6	Alpha, alpha-Trehalose fermentation	-	-	-	-	-	?	-
7	Melibiose fermentation	-	+	-	-	-	-	-
8	Lactose fermentation	-	-	-	-	-	-	-
9	Cellobiose fermentation	-	-	-	-	-	-	-
10	Melezitose fermentation	-	-	-	-	?	-	-
11	Raffinose fermentation	-	-	-	-	-	-	-
12	Inulin fermentation	-	-	-	-	-	-	-
13	Starch fermentation	-	-	-	-	-	-	-
14	D-Galactose growth	+	+	+	+	+	+	+
15	L-Sorbose growth	+	-	?	-	+	-	+
16	D-Glucosamine growth	-	-	-	-	-	-	-
17	D-Ribose growth	?	?	?	?	+	-	-
18	D-Xylose growth	+	+	+	+	+	+	+
19	L-Arabinose growth	+	+	+	-	+	-	+
20	D-Arabinose growth	+	+	+	-	+	-	-
21	L-Rhamnose growth	+	+	+	-	+	-	-
22	Sucrose growth	+	+	+	+	+	+	+
23	Maltose growth	+	+	+	+	+	+	+
24	Alpha, alpha-Trehalose growth	+	+	+	+	+	+	+
25	Me alpha-D-glucoside growth	+	+	+	+	+	+	-
26	Cellobiose growth	+	+	+	+	+	+	-
27	Salicin growth	+	+	+	?	+	?	-
28	Arbutin growth	+	+	+	-	+	+	-
29	Melibiose growth	-	+	+	-	-	-	-
30	Lactose growth	-	-	-	-	-	-	-
31	Raffinose growth	+	+	+	-	+	?	?
32	Melezitose growth	+	+	+	+	+	+	+
33	Inulin growth	+	+	+	-	+	-	-
34	Starch growth	-	-	-	-	-	-	-
35	Glycerol growth	+	+	+	+	+	+	+
36	Erythritol growth	-	-	-	-	-	-	-
37	Ribitol growth	+	+	+	+	+	+	+
38	Xylitol growth	+	+	+	-	+	-	-
39	L-Arabinitol growth	+	+	+	-	+	-	-
40	D-Glucitol growth	+	+	+	+	+	+	+
41	D-Mannitol growth	+	+	+	+	+	+	+

	Galactitol growth	-	+	+	-	-	-	-
	Myo-inositol growth	-	-	-	-	-	-	-
	D-Glucono-1,5-lactone growth	-	-	-	+	-	+	+
	2-Keto-D-gluconate growth	+	+	+	+	+	+	+
	5-Keto-D-gluconate growth	-	-	-	-	-	+	-
	D-Gluconate growth	-	-	-	+	-	+	-
	D-Glucuronate growth	-	-	-	-	-	-	-
	DL-Lactate growth	-	-	-	?	-	-	-
	Succinate growth	+	?	+	-	+	?	?
	Citrate growth	+	-	?	-	+	-	?
	Methanol growth	-	-	-	-	-	-	-
	Ethanol growth	+	+	+	+	+	+	+
	Growth at 25 deg C	+	+	+	+	+	+	+
	Growth at 30 deg C	+	+	+	+	+	+	+
56	Growth at 35 deg C	?	?	?	?	?	?	?
57	Growth at 37 deg C	+	+	+	+	+	+	+
	Growth at 42 deg C	?	-	+	-	+	+	?
59	0.01% Cycloheximide growth	-	-	-	-	-	-	-
60	0.1% Cycloheximide growth	-	-	-	-	-	-	-
61	50% D-Glucose growth	+	?	+	-	?	-	?
62	60% D-Glucose growth	?	-	-	-	-	-	-
63	Stach formation	?	?	?	?	?	?	?
64	Acetic acid production	-	-	-	-	-	-	-
65	Urea hydrolysis	-	-	-	-	-	-	-
66	Diazonium Blue B reaction	-	-	-	-	-	-	-
67	Pink colonies	-	-	-	-	-	-	-
68	Budding cells	+	+	+	-	-	+	+
69	Polar budding	?	?	?	?	?	?	?
70	Splitting cells	-	-	-	-	-	-	-
71	Filamentous	+	+	-	+	+	+	-
72	Pseudohyphae	+	+	-	-	-	-	-
73	Septate hyphae	-	-	-	+	+	+	-
74	Arthroconidia	-	-	-	-	-	-	-
75	Ballistoconidia	-	-	-	-	-	-	-
76	Symmetric ballistoconidia	-	-	-	-	-	-	-
77	Ascospores	-	-	-	-	?	?	-
78	Ascospores, round, conical, reniform	-	-	-	-	?	?	?
79	Ascospores cap, hat,saturn,walnut-shaped	-	-	-	-	?	?	?
80	Ascospores needle-shaped or whip like	-	-	-	-	?	?	?

Mycological peptone- 5 gm, Agar- 20 gm, 30 ppt Seawater-1L, pH 6.2). Lawn culture on malt extract agar plates were harvested at log phase using sterile seawater at 30ppt. From this suspension, cells were separated by centrifugation at 7000 rpm for 15 min. in a refrigerated centrifuge (Remi C-30, Mumbai) and stored at 4°C until use.

2.2.1.2. Experimental diet

Experimental feeds were prepared by incorporating the wet whole cell yeast biomass in the feed ingredients (Table 2.3). Powdered ingredients except yeast biomass were mixed well into dough with 100 ml distilled water and steamed for 10 min. in an autoclave. When the temperature lowered to 45-50°C the yeast biomass was added, mixed well and pelletised using a laboratory model hand pelletiser having a 1 mm die. Pellets were dried in an oven at 50°C for 18hrs and were fragmented into pieces of 4-5mm size. Seven different feeds were prepared incorporating the biomass of seven strains of yeasts and a control diet without yeast biomass. The stability of feeds was examined visually by immersing the pellets in seawater for 8hrs. Feeds were stored in airtight polythene bags at -20°C in a freezer until use.

2. 2.1.3. Animals used

Fenneropenaeus indicus post larvae (PL20), of the size range 20 to 25mg, which are PCR negative for White Spot Syndrome Virus (WSSV), were brought from a shrimp hatchery located at Kannamali, Cochin and was acclimatised to laboratory conditions. The PCR test for white spot syndrome virus was done at Centre for Fish Disease Diagnosis and Management, School of Environmental studies, Cochin University of Science and Technology. These larvae were maintained on control diets for a period of one week.

2. 2.1.4. Experimental design

Twenty-five animals were stocked in Fibre Reinforced Plastic (FRP) tanks of 30L capacity containing 20L seawater (Fig.2.1). Feeding experiments were

Table 2.2 List of marine yeasts used for screening experiment

Species	Strain No
Debaryomyces hansenii	S8
Debaryomyces hansenii	S87
Debaryomyces hansenii	S100
Candida sake	S165
Debaryomyces hansenii	S169
Candida tropicalis	S186
Torulospora delbrueckii	S303

Table 2.3 Composition of experimental feed

Ingredients	Control Feed (g)	Experimental Feed (g)
Fish meal powder	28	28
Prawn shell powder	20	20
Rice bran	10	10
Soya flour	10	10
Groundnut oil cake	8	8
Agar	2	2
Refined wheat flour	20	0
Yeast biomass	0	20
Mineral supplement & Vitamin premix*	2	2

*mineral supplement and vitamin premix

Mineral mix mg kg^{-1} diet

K_2HPO_4 ,11.69; $Ca_3(PO_4)_2$, 15.91;$MgSO_4.7H_2O$, 17.78; and $NaH_2PO_4.2H_2O$, 4.62.

Vitamin mg kg^{-1} diet

p-amino benzoic acid,150.8; biotin, 6.3; inositol, 6320.0; niacin, 632.0; Ca pantotenate, 948.0; pyridoxin HCl, 189.6; riboflavin, 126.4; thiamin HCl, 63.2; menadione, 63.4; α-tocopherol, 316.0;β-kerotene' 151.7; calciferol, 19.0; cyanocobalamin, folic acid ,12.6; choline chloride ,9480.0

Table 2.4 Rearing conditions and water quality

Parameters	Experiment
Stocking Density (per tank)	25 Post Larvae (Primary screening)
	10 Juveniles (Secondary screening)
Tank Capacity	30 L
Feeding level	15 -20 % body wt
Feeding frequency	Twice daily
Feeding period	21 days
Water temperature	24-27°C
pH	7.5 – 8
Salinity (ppt)	24-26 ppt
NH_3	0.01 - 0.02 mg/L
NO_3	Nil
NO_2	0.00 - 0.01mg/L
Dissolved oxygen	6 -7 mg/L

Fig.2.1: Bioassay system used for feeding experiment with *F. indicus* post larvae for testing immunostimulant property of marine yeasts and beta-1, 3-glucan.

done in triplicates for each yeast incorporated diet along with the control for a period of 21 days. Before the feeding experiment, total biomass in each tank was determined after removing water from the body surface of the animals with a blotting paper and weighing the animals on a precision balance (accuracy 0.001g). The average body weight of individual post larva was around 0.022g. 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 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 (Table 2.4). Continuous aeration was provided from 1 HP compressor. Health status of the larvae was monitored regularly and dead larvae, if any, were removed. After 21 days of feeding the final weight of shrimps in each tank was determined and absolute growth was recorded.

2.2.1.5. Challenging with white spot syndrome virus.

After 21 days of feeding experiment the animals were challenged with White Spot Syndrome virus (WSSV) *via* oral administration of white spot virus infected prawn flesh after a period of 12hrs of starvation. Infection with WSSV was confirmed by examining the carapace of dead larvae for white spots. Post challenge survival was recorded for a period of seven days.

2.2.2. Screening of marine yeasts for pathogenicity

Marine yeasts were tested for pathogenicity by observing haemolysis on prawn blood agar as per Chang *et al.* (2000).

2.2.2.1. Collection of haemolymph

Adult *Fenneropenaeus indicus* 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).

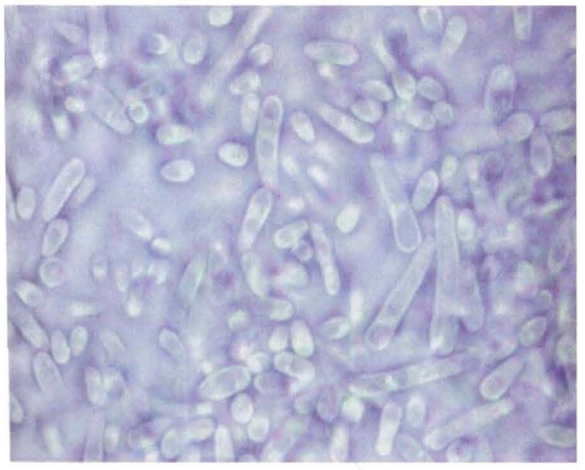
2.2.2.2. Haemolysis assay

One ml haemolymph was mixed with 130µl 3% (w/v) rose bengal stain prepared in shrimp anticoagulant solution (3%W/V) in order to stain the haemocytes. Malt extract agar 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 15ml 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. Yeast strains were streak inoculated on to this prawn blood agar plate along with a standard reference haemolytic strain of *Vibrio* sp, (MBCS 6), isolated from a diseased prawn (isolated and characterised by Centre for Fish Disease Diagnosis and Management, 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.

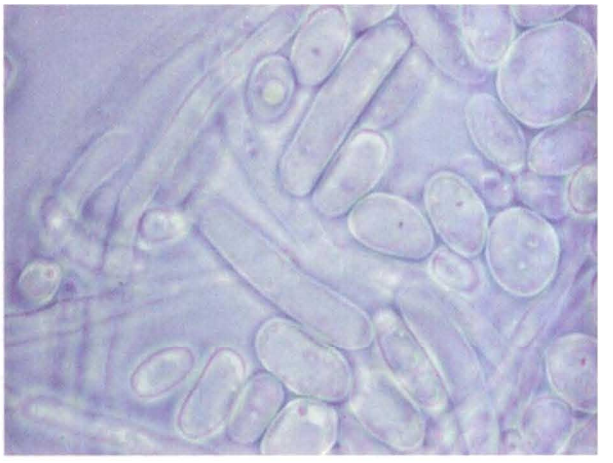
2.2.3.Secondary screening of marine yeasts

2.2.3.1. Yeast strains

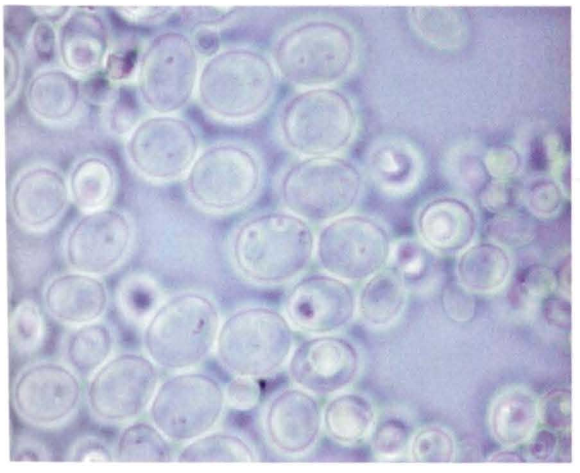
Based on the primary screening, four strains of yeasts were selected for secondary screening. They were *Debaryomyces hansenii* S8, *Candida sake* S165, *Debaryomyces hansenii* S169 and *Candida tropicalis* S186 (Fig 2.2). Experimental feeds were prepared as per section 2.2.1.2.



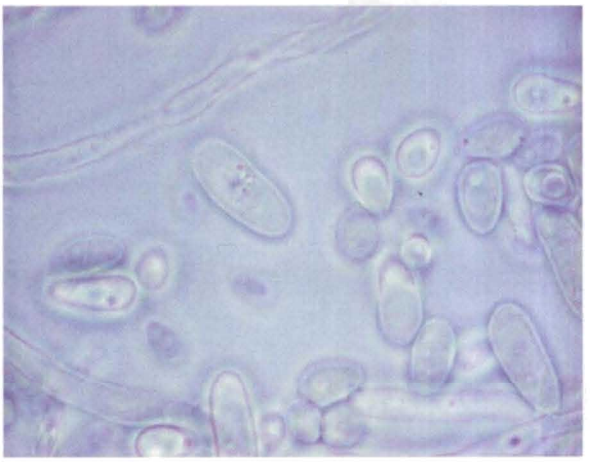
Debaryomyces hansenii **S8**



Candida sake **S165**



Debaryomyces hansenii **S169**



Candida tropicalis **S186**

Fig.2.2: Light Microscopic photograph of selected marine yeasts (400x)

2.2.3.2. Animals used

Fenneropenaeus indicus juveniles of the size range 3.5 to 4 g were brought from a prawn farm located at Kumbalangi, Cochin, acclimatised to laboratory conditions and maintained on control diet for one week.

2.2.3.3. Experimental design

FRP tanks of 30L capacity were used for the study. Ten animals each were stocked in the tanks. The experiment was done as mentioned in section 2.2.1.4. After 21 days of feeding absolute growth was recorded and animals were challenged with white spot virus orally. Post challenge survival was recorded for seven days.

2.2.4. Optimisation of yeast biomass concentration in the diet and treatment for improving digestibility of yeast cells.

2.2.4.1.Yeast strain

Based on the secondary screening experiment (Section 2.2.3) yeast strain *Candida sake* S165 was found to give better growth enhancement and immunostimulatory property and hence selected for further study.

2.2.4.2. Animals used

A homogenous population of *Fenneropenaeus indicus* post larvae (PL25), PCR–negative for WSSV, were brought from a shrimp hatchery located at Kannamali, Cochin and was acclimatised to laboratory conditions. The stock was maintained on control diet for a period of one week

2.2.4.3. Experimental diet

The objectives of the study were to find out the optimal concentration of yeast required in feed, and the improvement of the digestibility of the yeast which confer an effective protection against WSSV challenge and support maximum growth. For this, three categories of experimental feeds were prepared by incorporating graded concentrations of yeast *C. sake* S165 (i.e.,1, 5, 10, 15, 20%) into the standard diet (Section 2.2.1.2). The

concentration of refined wheat flour in feed was adjusted depending on the concentration of yeast biomass.

Treatment of yeast biomass

Yeast biomass of *Candida sake* S165 to be incorporated in the diet was prepared in three ways.

a. *Yeast cells: untreated*

Lawn culture of yeast was prepared, harvested using sterile seawater and centrifuged at 7000 rpm for 15 min to separate the biomass (as given in 2.2.1.1). This yeast biomass was stored in the freezer and was incorporated at different concentrations in the diet.

b. *Yeast cells: Autoclaved*

Yeast biomass was prepared as given in section 2.2.1.1 and autoclaved at 121°C for 15 min. This autoclaved yeast biomass was incorporated at different concentrations in diet.

c. *Yeast cells: 2- Mercapto- ethanol treated*

Yeast biomass was prepared as given in section 2.2.1.1. Chemical treatment was performed to remove the outer mannan coating of yeast as per the method described by Coutteau *et al.* (1990). Briefly 10 g wet weight of yeast biomass was suspended in 100ml Tris buffer (0.1 M, pH 7.6), 2% (V/V) of 2-mercapto-ethanol was added to this and kept for incubation at room temperature for 30 min. The cells were collected after centrifugation at 7000 rpm for 10 min. and extensively washed with sterile seawater. The cells were collected again and were incorporated at different concentrations in diet. All the experimental diets were stored at 4^0C until use.

2.2.4.4. Feeding experiment

The feeding experiment was carried out with post larvae of *F. indicus* (PCR–negative for WSSV) for 21 days and the maintenance was done as per

Section 2.2.1.3. At the end of feeding experiment for 21 days, absolute growth was recorded. All the animals were challenged with WSSV (orally) and the post challenge survival was recorded for 7 days.

2.3. Statistical Analysis

Data generated from the above feeding experiments were analysed using one-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means by using SPSS 10.0 for windows. Whereas in the case of experiment done in section 2.2.4. two way ANOVA was done. Significant differences were indicated at $p<0.05$.

2.4. Results

2.4.1 Primary screening of yeasts for utilisation as a feed supplement and immunostimulant

It was noted that the feed S165 with yeast *Candida sake*, supported maximum growth (0.039g) followed by S8 (0.037g), S186 (0.030g) and S87 (0.028g) (Fig. 2.3). The control feed showed a production of 0.01g. Rest of the experimental feeds showed almost similar performance as that of the control feed.

Post challenge survival at the end of 7thday of WSSV challenge was maximum for S165, *Candida sake* (18.35%) followed by S186, *Candida tropicalis* (14.3%), S169 *Debaryomyces hansenii*, (10.38%), and S8 *Debaryomyces hansenii*, (10.01%) (Fig.2.4). All these four yeast diets differ from the control feed significantly ($p<0.05$) and were segregated for the secondary screening.

2.4.2. Screening of marine yeasts for pathogenicity.

From the prawn blood agar haemolysis test, all seven strains of yeasts were shown to be non-haemolytic whereas, the pathogenic *Vibrio* sp. MBCS6, exhibited haemolysis giving a distinct clearance zone visible around the colony (Fig 2.5)

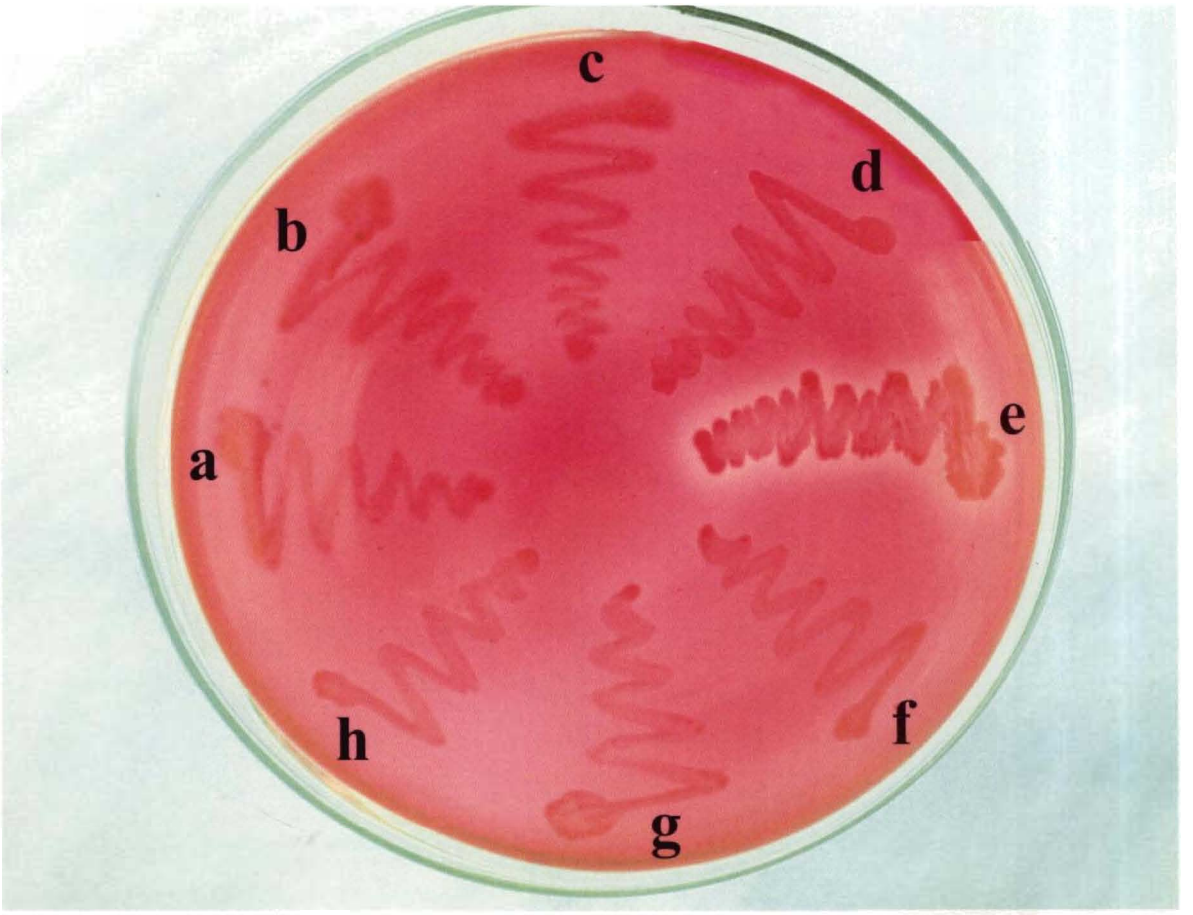


Fig.2.5: Haemolytic assay of marine yeasts using prawn blood agar.

a. ***Debaryomyces hansenii*** **S 8**
b. ***Debaryomyces hansenii*** **S 87**
c. ***Debaryomyces hansenii*** **S 100**
d. ***Candida sake*** **S 165**
e. ***Vibrio*** **sp. MBCS 6**
f. ***Debaryomyces hansenii*** **S 169**
g. ***Candida tropicalis*** **S 186**
h. **Torulospora delbrueckii S 303**

2.4.3 Secondary screening

Out of the four strains screened, the yeast *Candida sake* S165 incorporated diet supported maximum growth (0.15g) followed by *Debaryomyces hansenii* S8, (0.14g), *Candida tropicalis* S186, (0.12g) and *Debaryomyces hansenii* S169, (0.11g) (Fig 2.6). The control showed a production of 0.1152g. Post challenge survival against WSSV infection was found to be maximum with S169 (15.81%) followed by S165 (13.65%) (Fig 2.7). whereas, S 8 and S 186 showed 7.16 and 8.37% survival respectively. Even though the strain S169 showed better post challenge survival the absolute growth with this strain was less or even below the control feed. The control showed a survival of 7.6% at the end of 7^{th} day post challenge.

2.4.4 Optimisation of yeast biomass concentration in feed

Based on the absolute growth and post challenge survival data in secondary screening, yeast *Candida sake* S165, was selected for further study. Among the three categories of feeds *viz*, whole cell untreated yeast, autoclaved yeast and mercapto-ethanol treated yeast, mercapto-ethanol treated group supported better growth when compared to other two categories of feeds (Fig. 2.8). Among feeds prepared with untreated whole cell yeast, 20% yeast incorporated feed supported maximum growth and a gradual increase in growth could be observed in prawn maintained on 1% to 20% yeast diets. No significant difference in the performance could be observed between 10, 15 and 20% yeast biomass incorporated feeds.

Autoclaved and mercapto-ethanol treated yeast diets supported maximum growth at 10% inclusion level in the diet and showed a suppression in growth when yeast biomass inclusion level was above 10% in the diet. Among the autoclaved yeast diets, no significant difference could be observed between 1, 5, 10 and 15% yeast. However among the mercapto-ethanol treated diets the performance of 10 and 15% yeast diets were found to be significantly

different from other feeds. From this study 10% yeast diet was found to be optimal for application in penaeid culture systems.

Post challenge survival data also reflected the same pattern where the maximum survival was recorded with feed group incorporated with mercapto-ethanol treated yeast (Fig. 2.9a-d). Here also the 10% yeast feed showed better survival (40.94%) against WSSV at the end of 7^{th} day of post infection. In autoclaved yeast fed category, the feed with10% yeast showed a survival of 38.17% on the 7^{th} day of post challenge, whereas in whole cell live yeast fed group it was 33.68% for 10% yeast feed. When the concentration of yeast in feed was above 10% a decreasing trend in post challenge survival was noticed.

2.5. Discussion.

Aquaculture has developed rapidly over the last three decades and has become an important activity world wide, providing millions of job opportunities, billions of revenue and augmentation of world food supply with high quality protein. As in any other animal husbandry management, disease outbreak especially of viral origin has become the most important bottleneck in shrimp aquaculture industry.

The major strategies for controlling the diseases in aquaculture are application of vaccines, chemotherapeutics, probiotics and immuno-stimulants. Chemotherapeutics like antibiotics are very effective in controlling disease, but incidence of emergence of drug–resistant bacteria became a major problem in aquaculture ponds (Aoki, 1992). Moreover, antibiotic residues in prawn and other fishery products invited the attention of seafood importing countries like USA, European Union, and Japan and strict guidelines were imposed to the usage of such antibiotics in culture practice. As a matter of fact Govt. of India imposed ban on the use of certain antibiotics or even fixed a minimum permitted level for such antibiotics in aquaculture use.

Vaccination is the most reliable method to control diseases. Vaccination against a single antigen seldom protects the animals from other pathogens. However, incidence of red mouth disease and furunculosis of salmon could be reduced by proper vaccination. The same cannot be expected to work in shrimps against white spot virus or vibrios infection, since success story of vaccination rely on the peculiar property, memory, of the immune system of vertebrates. It is very evident that there exists an anticipatory (memory) and non-anticipatory immune response in vertebrates, but only non-anticipatory immune responses were observed in invertebrates (Klein, 1997). So vaccination could not be a relevant option in shrimp aquaculture to protect them against infection, instead the use of immunostimulants and probiotics may reduce the incidence of disease outbreaks.

The probiotic treatments may be considered as methods of biological control, the so-called "biocontrol" that termed the limitation or elimination of pests by the introduction of adverse organisms, like parasites or specific pathogens (Gatesoupe, 1999), whereas, immunostimulants are substances of microbial origin, which enhance the defence status of the host organism to certain extent thereby reducing the risk of infection. These methods attempt to stimulate the immune system and are therefore more suited for immuno-prophylaxis rather than post-infection therapy.

Immunostimulants, which received maximum attention, are glucan of yeast cell wall, peptidoglycan and lipopolysaccharides of certain bacteria. Yeast cell wall architecture is peculiar in nature having 30% alkali-insoluble glucan, 30% mannan, 10-15% protein, 8-9% lipid and 1-2% chitin and the cell wall represent nearly 15% of the dry weight of the cell (Duffus *et al*, 1982). So yeast is generally regarded as a good source of immunoactive glucan and extraction of glucan have been carried out for many studies. In aquaculture also exploitation of this immunostimulant property of yeast or yeast-derived

glucan is of recent interest. Smith *et al.*(2003) recently reviewed the application and effectiveness of immunostimulants in penaeid prawns.

Only few attempts were made to utilise live yeast as immunostimulants rather than purified cell wall content (Scholz, *et al.*, 1999; Burgents *et al.*, 2004). Most of the shrimp feeding experiments were performed with either baker's yeast or brewer's yeast, which are, quite alien to a marine or brackish water environment. The penaeid shrimp culture practice is being restricted to such brackish or seawater conditions having a salinity of about 15 ppt to 35 ppt. In this high salinity, the fresh water forms like *Saccharomyces cerevisiae* may find sea water as hyperosmotic and cause cell rupture due to osmotic shock which further leads to pollution of culture waters (Kawano and Ohsawa, 1971). One practical solution to this problem is to identify a salt resistant, halophilic form, which can be used to substitute the common freshwater yeast as a feed ingredient in prawn culture systems. The halophilic property of marine yeasts would be an advantage in this context and can be used in brackish/marine culture systems without any osmolarity problems.

Report on the utilisation of marine isolates of yeasts, as a source of immunostimulants to shrimp aquaculture is very limited. So in the present study marine yeasts were utilised for screening their immunostimulant and growth enhancing property in penaeid prawn. In the preliminary screening with seven marine isolates of yeast for disease resistance and growth enhancement in *Fenneropenaeus indicus*, four strains showed promising result in terms of growth enhancement and post challenge survival against WSSV infection. The strains were identified as *Debaryomyces hansenii* S8, *Candida sake* S165, *Debaryomyces hansenii* S169 and *Candida tropicalis* S186. Among these four isolates, *Candida sake* S165 showed better growth enhancement property and post challenge survival (0.0385g and 18.18 % respectively), which was significantly different from all other diets including the control diet. The post challenge survival on 7^{th} day challenge showed

that S186, S169 and S8 were comparatively better than the other three yeast strains *viz.* S100, S87 and S303. The trend in the production was also similar but S87 showed better value than S169. However, the post challenge survival was poor (7.8%) for this strain. So in the secondary screening S169 was selected along with other three strains which showed better result *viz.* S8, S165 and S186. The poor performance of the strains like S100 and S303 could be due to the adverse effect of some components of the diet, which would have circumpassed the beneficial effects imparted by the nutritive value of yeast. Gentles and Touche (1969) and Madri *et al.* (1966) reported that all marine yeasts were not suitable for mariculture since some may secrete substances, which were toxic to animals.

The result of the secondary screening showed that post challenge survival was best for S169 (15.82%) followed by S165 (13.65%), but the production for S169 was not promising (0.10g) and was ranked 4^{th} among the four strains tested. However, the production for S165 was the highest. Since growth and survival are the two important parameters used to test the effectiveness of a feed, S165 was found to be a better candidate for immunostimulation. The growth enhancing property of yeast could be ascribed to their nutritive values, where yeasts are generally considered as good sources of protein, nucleic acids, vitamins, polysaccharides and other micronutrients.

The difference in the post challenge survival with different yeast strains could be attributed to the difference in the structure of glucan, where structure of the glucan also exert role in their effectiveness as immunostimulant as reported by Yanaki *et al.* (1983). Scholz *et al.* (1999) had the opinion that the higher immunity index of the shrimp fed with yeast *Phaffia rhodozyma* compared to animals fed with *S. cerevisiae* might have been due to the difference in the presence of carotenoid pigments in the yeast. Burgents *et al.* (2004) reported that the positive influence of dried *S. cerevisiae* yeast

feed on the disease resistance of *Litopenaeus vannamei* could be due to the presence of its glucan content.

Nutritional value of any diet depends on its degree of digestibility. Yeast cells are known to have a complex and thick cell wall and thereby the poor digestibility is an important constraint in the use of these organisms as food source for aquaculture species. Coutteau *et al.* (1990) reported that the digestibility of yeast *Saccharomyces cerevisiae* could be increased either by autoclaving or by chemical treatment. These authors have the opinion that chemical treatment using 2-mercapto-ethanol will effectively remove the outer mannan layer from the yeast cell surface and will facilitate easy access for the digestive enzymes.

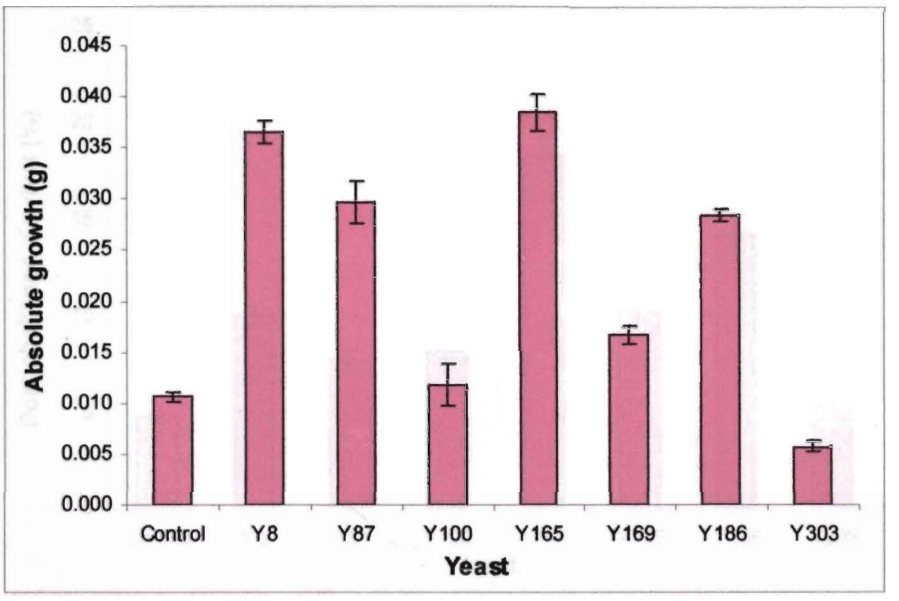
In the present study *Candida sake,* at about 10% gives better post challenge survival and the survival showed a decreasing tendency at higher concentration (15% and 20%). The autoclaved and chemically treated yeast also showed better survival at 10% concentration, which shows that autoclaving/chemical treatment might have enhanced the digestibility of yeast. Feeds prepared with autoclaved and mercapto- ethanol treated yeasts showed better performance both in terms of post challenge survival and absolute growth due to increased digestibility. However, the autoclaving might have resulted in loss of some heat labile nutrients from yeast especially vitamins, which may be the reason for reduced performance when compared to the chemically treated yeast feed. Coutteau *et al.* (1990) reported that lower growth of *Artemia* obtained with the autoclaved yeast in comparison to the thiol treated yeast could be due to the extreme conditions of heat treatment, which probably caused lysis of yeast cell during the culture period. The decrease in post challenge survival in all three feed groups at higher concentration of yeast could be due to the overdose of yeast immunostimulants, probably glucan, which may result in a condition called "immune fatigue" in animals. The reports of many workers that immunostimulants are not dose-dependent and high doses may not enhance

or may suppress the immune responses (Sakai, 1999) is agreement with the present finding. Also the decline in production with increase in yeast ratio in feed could be explained in the light of above finding where the animals put extra energy to bring back the immune system from stress condition to a normal level. Coutteau *et al.* (1993) reported a negative effect of higher concentrations of yeast in feed on growth of Manila clam. Similar result was reported by Rumsey *et al.* (1991a) who observed a feed intake depression in rainbow trout due to reduced acceptability of diets including more than 25% of brewer's yeast.

The protective effect of yeast *Candida sake* on shrimps could be attributed to the β-glucan component of the cell wall of yeast and also to the production of carotenoid pigments, nucleotides and polyamine synthesis. Carotenoid pigments have a positive effect on the physiology and health status of animal and polyamines help in colonising the probiotic microbial community inside the gut epithelia. Sorenson *et al.* (1998) demonstrated that the beta glucan from *Candida sake* increases the phagocytosis and super oxide anion production in rat alveolar macrophage. In the present study it is evidenced that high concentration of yeast in feed have adverse effect on survival as an immunostimulant. So checking the dose–response relationship of the compound in the target organisms using bioassay studies has great significance in the successful usage of any immunostimulant in aquaculture field

To be considered as an immunostimulant or probiotic the putative microbial strain should be proved to be non-pathogenic to the target species. Here all the seven marine yeasts used for screening were shown to be non-haemolytic on prawn blood agar plates and were considered to be non-pathogenic to penaeid shrimps whereas, the pathogenic *Vibrio* sp. (MBCS6) showed haemolytic property as evident by the clear zone around the colony. Under a light microscope this area showed the remains of lysed haemocytes whereas it is absent around yeast colonies (non pathogenic) where intact

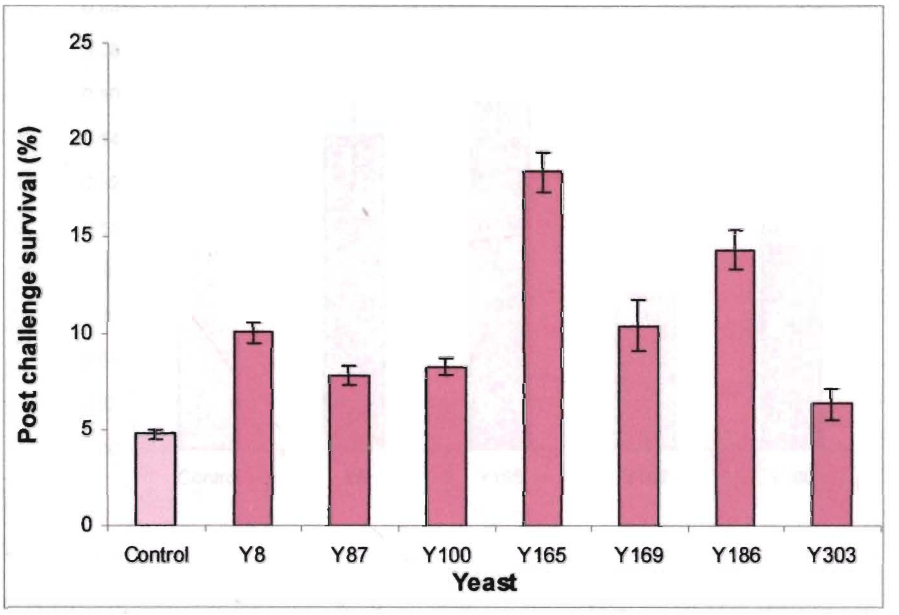
haemocytes could be observed. So it is concluded that marine yeast used for this study are safe for aquaculture application and could be used even as live feed supplement for prawns. Moreover the feeding of these strains for 21 days did not cause any apparent change, both behavioural and morphological, in the shrimp larvae emphasising their non-pathogenic nature.



Yeast Feed	Absolute Growth (g) *
Control	0.011 ± 0.001^{b}
Y8	0.037 ± 0.001^{f}
Y87	$0.03 \pm .002^{e}$
Y100	0.012 ± 0.002^{c}
Y165	0.039 ± 0.002^{f}
Y169	0.017 ± 0.001^{d}
Y186	0.028 ± 0.001^{d}
Y303	0.006 ± 0.001^{a}

*Values with same superscript dose not vary significantly ($P<0.05$)

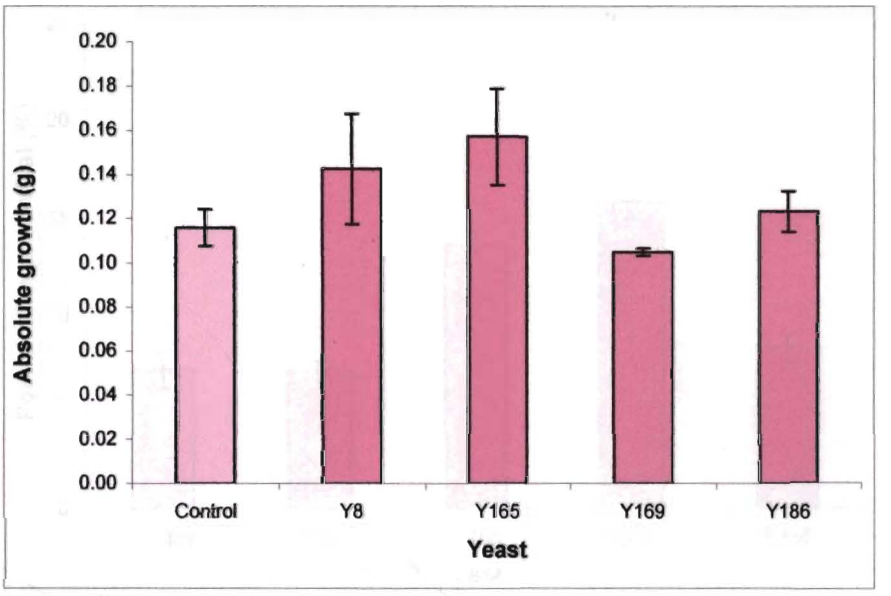
Fig 2.3 Absolute growth (production) of *F. indicus* post larvae fed with seven different marine yeast incorporated diets.



Yeast Feed	Post challenge survival (%)*
Control	4.77 ± 0.23^{a}
Y8	10.01 ± 0.5^{d}
Y87	7.81 ± 0.45^{c}
Y100	8.24 ± 0.41^{c}
Y165	18.35 ± 1.02^{f}
Y169	10.38 ± 1.33^{d}
Y186	14.3 ± 1.05^{e}
Y303	6.31 ± 0.86^{b}

* Values with same superscript dose not vary significantly ($P<0.05$)

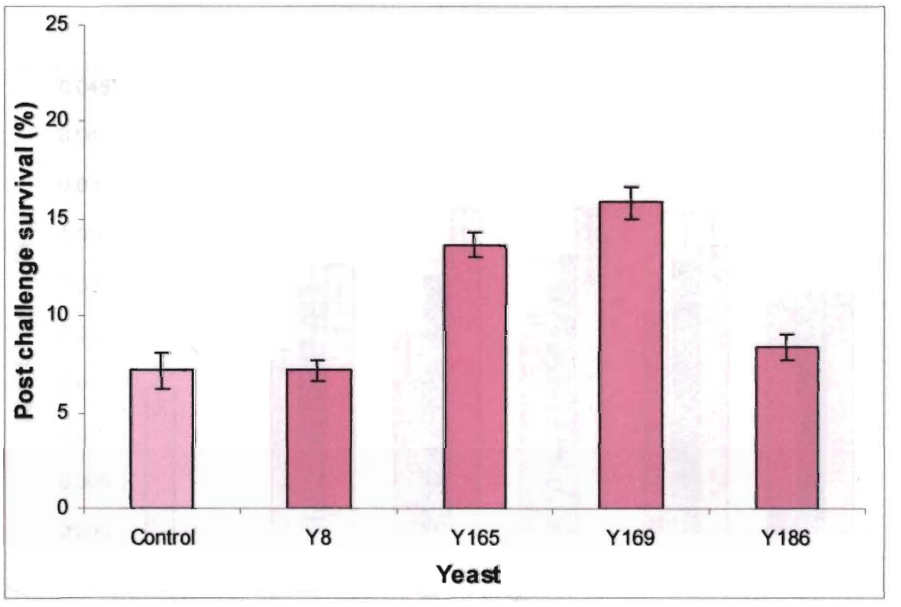
Fig 2.4 Post challenge survival in *F. indicus* post larvae when fed seven different yeast incorporated diets and challenged with WSSV.



Feed	Absolute growth* (g)
Control	0.116 ± 0.008^{ab}
Y8	0.142 ± 0.025^{bc}
Y165	0.157 ± 0.022^{c}
Y169	0.105 ± 0.002^{a}
Y186	0.123 ± 0.009^{ab}

* Values with same superscript dose not vary significantly ($P<0.05$)

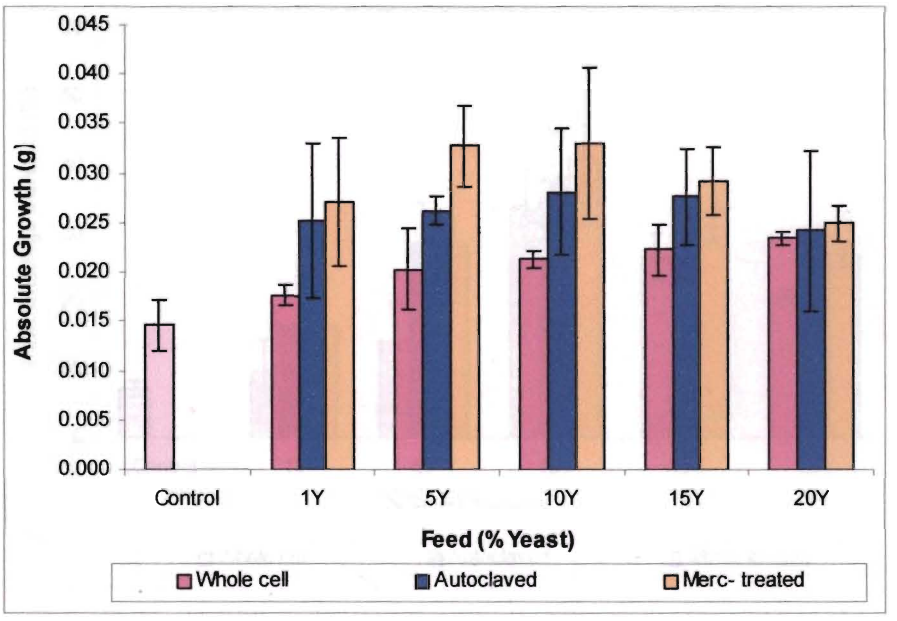
Fig 2.6 Absolute growth (production) of *F. indicus* juveniles when fed four selected yeast incorporated diets.



Feed	Post challenge survival (%)*
Control	7.16 ± 0.92[a]
Y8	7.16 ± 0.52[a]
Y165	13.65 ± 0.62[b]
Y169	15.82 ± 0.83[c]
Y186	8.37 ±.0.7[a]

* Values with same superscript dose not vary significantly (P<0.05)

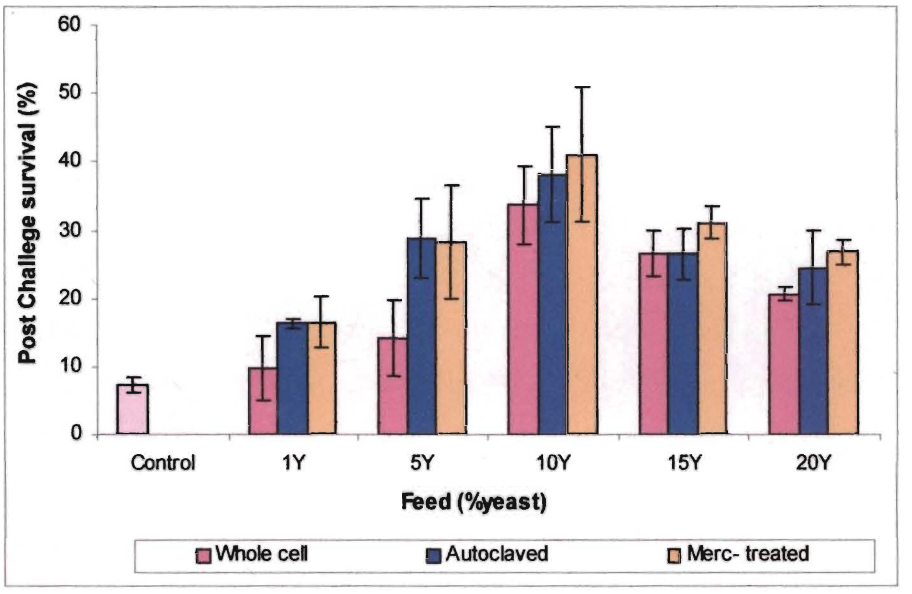
Fig.2.7 Post challenge survival in *F. indicus* juveniles when fed four selected yeast incorporated and challenged with WSSV.



Yeast feeds	Absolute growth (g)*		
	Whole cell	Autoclaved	Merc- treated
Control	0.0147 ± 0.003*[a]	0.0147 ± 0.003**[a]	0.0147 ± 0.003**[a]
1Y	0.0176 ± 0.001*[b]	0.0252 ± 0.008**[b]	0.0270 ± 0.006**[b]
5Y	0.0202 ± 0.004* [b]	0.0262 ± 0.002** [b]	0.0327 ± 0.004** [b]
10Y	0.0213 ± 0.001* [b]	0.0281 ± 0.006**[b]	0.0330 ± 0.008** [b]
15Y	0.0223 ± 0.003* [b]	0.0276 ± 0.005**[b]	0.0292 ± 0.003** [b]
20Y	0.0234 ± 0.001* [b]	0.0242 ± 0.008**[b]	0.0249 ± 0.002**[b]

* Values with same superscript dose not vary significantly (P<0.05)

Fig 2.8 Absolute growth of *F. indicus* fed with yeast (whole cell, autoclaved and mercapto-ethanol treated) incorporated feeds at different concentrations.



Yeast feeds	Post challenge survival (%)*		
	Whole cell	Autoclaved	Merc- treated
Control	7.24 ± 1.04^{*a}	7.24 ± 1.04^{**a}	7.24 ± 1.04^{**a}
1Y	9.65 ± 4.69^{*b}	16.23 ± .74^{**b}	16.39 ± 3.76** ^{b}
5Y	14.04 ± 5.52* ^{c}	28.77 ± 5.82^{**c}	28.17 ± 8.22** ^{c}
10Y	33.68 ± 5.67* ^{d}	38.17 ± 6.96^{**d}	40.94 ± 9.80** ^{d}
15Y	26.53 ± 3.26* ^{c}	26.43 ± 3.83** ^{c}	31.05 ± 2.39** ^{c}
20Y	20.58 ± 1* ^{c}	24.38 ± 5.35** ^{c}	26.74 ± 1.79** ^{c}

*Values with same superscript dose not vary significantly (P<0.05)

Fig. 2.9 Post challenge survival in *F. indicus* fed with yeast (whole cell, autoclaved and mercapto-ethanol treated) incorporated feeds at different concentrations.

Chapter 3

Optimisation of the culture conditions of *Candida sake* for biomass production

3.1.Introduction

All organisms need a variety of elements as nutrients like carbon, nitrogen, phosphorous trace elements etc. for growth. In nature these nutrients are dispersed among large number of compounds, which are either inorganic or organic forms. Carbon is one of the most important elements required for microbial growth. Heterotrophs require preformed organic compounds like carbohydrates, amino acids, peptides and organic acids. All wild type yeasts utilize glucose, mannose, fructose, sucrose etc as carbon source. Yeasts generally utilize ammonium salt as sole source of nitrogen (Jones *et al.*, 1981). Diammonuim phosphate is utilized more preferentially than ammonium chloride (Spencer *et al.*, 1997). Some strains of yeast utilize urea as source of nitrogen apart from ammonium ions. Other elements like hydrogen, oxygen, sulphur and phosphorus also seem to be essential for microbial growth.

Thus a good culture medium should contain all these essential nutrients needed for the growth at optimal level. Not only the optimal nutritional requirements but also the physico–chemical parameters like temperature, salinity and pH should be taken care of while designing a culture medium for mass cultivation of a microorganism. Cost reduction by utilizing less expensive substrates seems to be an economically viable option. In this chapter attempt was made to utilize cane sugar molasses as the sole carbon source. Other nutritional requirements and physicochemical parameters were optimised by considering one-factor-at-a time for marine yeast *Candida sake* S165.

3.2. Materials and methods

3.2.1.Preparation of inoculum

Yeast inoculum was prepared by harvesting young culture (2 days old growth) of yeast *Candida sake* S165 into sterile seawater of salinity 15ppt.

Optical density of the culture suspension was measured at 540 nm and adjusted to 0.1 OD using sterile seawater. 10 µl of this suspension was used as inoculum in 10 ml culture medium for the following optimisation processes.

3.2.2. Optimisation of salinity

Molasses based culture medium of nine different salinities like 0, 5, 10, 15, 20, 25, 30, 35 and 40 ppt. were prepared and sterilized. 10 µl inoculum was added to each medium and incubated for 48hrs at room temperature and growth was measured turbidometrically using Hitachi 2001 UV series spectrophotometer at 540nm.

3.2.3.Optimisation of pH

To find out the pH optima, molasses based seawater (15ppt) medium with pH of 3, 3.5, 4, 4.5, 5, 5.5, 6, 7and 8 were prepared. The pH of the medium was adjusted either by using 0.1N NaOH or 0.1N HCl solution. 10 µl each of the cell suspension was added to the medium (10 ml) and after 48 hrs of incubation, growth was measured at 540nm.

3.2.4.Optimisation of temperature

10 µl inoculum of *Candida sake* was added to 10ml molasses based seawater (15ppt) medium and incubated at different temperatures like 20, 25, 27,30, 35, 40 and 45°C and growth was measured at 540nm after 48 hrs of incubation.

3.2.5.Optimisation of carbon source

Sugar cane molasses, the main by-product from the sugar industry, can be used after proper dilution as a cheap carbon source for fermentation process as it is rich in sugar, mostly in the form of sucrose, fructose and glucose. Sugar cane molasses, obtained from a private distillery located at Cherthala, was diluted with distilled water (100gm in 200ml distilled water) and used as stock solution, the total sugar of which was determined by Anthrone method

(Roe, 1955). Molasses medium with varying concentrations of total sugar viz. 0.5, 1, 2, 3, 4 and 5 mg/ml were prepared using the stock solution. After inoculation with 10µl yeast suspension, incubation was done at room temperature (28 ± 2°C) for 48 hrs and the growth was measured at 540nm.

3.2.6.Optimisation of nitrogen source

Three different compounds KNO_3, Urea and $(NH_4)_2SO_4$ were tested as a source of nitrogen for yeast. Molasses based seawater (15ppt) medium with five different concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5%, of each of these three compounds were prepared and inoculated with 10 µl yeast suspension of 0.1 OD. After incubation at room temperature (28 ± 2°C) for 48 hrs, growth was measured at 540nm.

3.2.7.Optimisation of phosphorus source

As source of phosphorus KH_2PO_4 was used at different concentrations like 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5% in molasses based seawater (15ppt) medium and 10 µl yeast inoculum was added to 10 ml culture medium. Cultures were incubated at room temperature (28 ± 2°C) for 48 hrs and growth was measured at 540nm.

3.2.8.Optimisation of magnesium

$MgSO_4.7H_2O$ was used as the source of magnesium in which different concentrations, 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05% were tested to find out the optimum concentration required for growth. 10 ml molasses based seawater (15ppt) medium were prepared and inoculation of 10 µl yeast suspension of 0.1 OD was done. After incubation at room temperature (28 ± 2°C) for 48 hrs growth was measured at 540nm

3.2.9.Optimisation of calcium

Different concentrations of $CaCO_3$ like 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5% were tested to find the optimal concentration required for growth of *C. sake*. After inoculation with 10µl yeast suspension in molasses based seawater (15ppt)

medium, incubation was done at room temperature (28±2°C) for 48 hrs and the growth was measured at 540nm.

3.3. Results

Growth was found to be maximum at 15 ppt salinity. However, *C. sake* exhibited good growth rate at 0 to 35 ppt. (Fig.3.1). *Candida sake* was found to grow at a wide range of pH from 3 to 8 without showing much difference in growth. The optimum growth was found to be at pH 4.5 (Fig 3.2). Growth was found to be maximum at 30°C and it remained more or less the same between the room temperature (28±2°C) to 35°C. (Fig.3.3). Beyond this range considerable reduction in growth could be observed.

Molasses concentration was expressed as total sugars (mg/ml medium). A concentration of 2mg/ml was found to be optimal for the growth of *Candida sake* (Fig. 3.4). However no significant change in growth could be observed when the concentration was between 0.5 and 3 mg/ml. Concentration above 3 mg/ml resulted in reduced growth.

Ammonium sulphate was found to be the most preferred nitrogen source. Even though growth was found to be maximum with 0.3% $(NH_4)_2SO_4$, no significant variation in growth could be observed at a concentration range of 0.1 to 0.5% $(NH_4)_2\ SO_4$ (Fig.3.5). With urea as nitrogen source at 0.1 and 0.2% the growth was found to be almost equal to that of $(NH_4)_2SO_4$ medium. However, higher concentrations of urea resulted in drastic reduction in growth. Growth was uniformly lesser at all concentrations of KNO_3 (0.1 to 0.5%) compared to the other two nitrogen sources.

Growth was found to be maximum with 0.3% K_2HPO_4 in the medium (Fig 3.6). A considerable increase in growth could be observed when K_2HPO_4 concentration in the medium was increased from 0.5 to 0.3% and a gradual reduction was noticeable beyond this level.

Growth was found to be almost at the same level with 0.02 to 0.05% $MgSO_4$ concentrations in the medium, the maximum being at 0.05% (Fig 3.7). At 0.2% $CaCO_3$ concentration the growth of *C. sake* was found to be maximum (Fig 3.8). A gradual reduction in growth could be observed with increased concentration of $CaCO_3$ in the medium.

3.4. Discussion

The indigenous nature of marine yeasts is not yet clear, whether they are true inhabitants of the sea or merely transient forms introduced from freshwater or terrestrial habitats. *Candida sake* S165 used in the present study was found to be growing optimally at 15 ppt and comparatively good growth could be observed at 0 to 35 ppt salinity without showing much variation. This shows the ability of the particular yeast strain to survive both in fresh water and seawater conditions. Kriss (1963) reported that the majority of yeasts in the sea are not accidental forms, but species adapted to the life under marine conditions. The present study also supports this observation.

Other physical parameters for growth like temperature and pH show that *C. sake* S 165 prefer 30°C and pH 4.5. Yeasts generally prefer an acidic pH for growth, which would be advantageous to prevent bacterial contamination during mass production, where absolute sterility cannot be ensured. Furlan *et al.* (2001) also reported that lower pH was important for better cell growth of yeast *Kluyveromyces marxianus*. Anas and Singh (2003) reported that yeast *Acremonium dyosporii* preferred pH 4 for higher cell yield. In the present work, temperature preference of *C. sake* was 30°C for optimal growth, which was perfectly suitable for mass cultivation in a tropical country like India were the ambient atmospheric temperature is similar to this optimum value.

The development of an economically viable culture medium is necessary to obtain high quantity of biomass. Carbon substrate has a dual role in

biosynthesis and energy generation, with carbohydrates being the usual carbon source for microbial fermentation process (Stanbury *et al.*, 1995). The most widely available carbohydrate is starch obtained from maize grains, other cereals, potatoes and cassava. However, majority of the published papers deal with supplementation with compounds such as yeast extract, a highly effective, but expensive material, which enhances the biomass production. Costa *et al.* (2002) reported that soluble starch provided good growth yields when yeast extract was used as a nitrogen source for *Pantoea agglomerans.* Therefore, from aquaculture point of view the chance of using such an uneconomical material appears unlikely. Consequently there is a need for more extensive investigation on the utilization of cheap raw materials as supplements for yeast biomass production, in particular, increased attention has to be paid to the possibility of using by-products and waste materials from the food industry. A number of unrefined carbon sources have been utilized in fermentation industry by various workers; cane molasses (Haard, 1988), sugar cane juice (Fontana *et al.*, 1996), corn wet milling co-products (Hayman *et al.*, 1995) and grape juice (Meyer and Du Preez, 1994).

Cane sugar molasses is an abundant by-product of the sugar industry, only partially used in fermentative processes and its disposal still remains a problem (Chiarini *et al.*, 1992). Many workers have reported the utilization of molasses as a source of carbon in fermentation processes (Oderinde *et al.*, 1990; Ergun *et al.*, 1997; Furlan *et al.*, 2001). Aksu and Kutsal (1986) studied the lactic acid production by *Lactobacillus delbrueckii* by using beet molasses as carbon source. Goksungur *et al.* (2002) reported that beet molasses solution supplemented with yeast extract and corn steep liquor was an attractive medium for the production of β-carotene. In the present study *C. sake* grew optimally in medium containing molasses as a carbon source (total sugars 2mg/ml). The higher concentrations of molasses did not show any increase in SCP production, which may be due to the hyper osmotic environment of the medium. Jones *et al.* (1981) had opined that

higher sugar concentration in culture medium probably inhibits fermentation where plasmolysis of yeast cells could occur. Bajaj *et al.* (2003) reported loss of viability of yeast strains in molasses medium due to higher osmotic pressure of medium with reduced water activity and inhibitory substances of molasses.

The nitrogen content of yeast is about 10% of dry weight and as such it represents an important constituent of any growth medium. Yeast generally utilize ammonium salt as sole source of nitrogen and diammonium phosphate is utilized most efficiently and ammonium chloride least whereas some strain can even utilize urea (Spencer *et al.*, 1997). Complex nitrogen sources such as peptone, yeast extract and tryptone, contain nitrogenous fractions like amino acid, peptides, nucleic acids etc. in an undefined proportion. In the present study, $(NH_4)_2SO_4$ at a concentration of 0.3% was found to be optimum for growth and proved to be a better nitrogen source for commercial production of yeast biomass. A similar observation was made by Khan *et al.*(1995) who reported that $(NH_4)_2SO_4$ was a suitable nitrogen source for the production of yeast biomass. Incorporation of urea in media at concentration of 0.1 to 0.2% was found to support good growth and the lesser growth at higher concentrations could be due to its toxic effect.

Yeasts utilize inorganic phosphates for growth. It is taken up as the monovalent anion, $H_2PO_4^-$, and more is taken up as monobasic potassium salt than the dibasic sodium form (Spencer *et al.*, 1997). Present experiment showed that *C. sake* preferred 0.3% KH_2PO_4 in culture medium.

Metal ions such as Mg^{2+}, Ca^{2+} and K^+ directly influence fermentation metabolism in yeast (Gamarallage *et al.*, 1997). Magnesium and potassium are regarded as bulk cations establishing the required ionic environment of the cell, and magnesium transport by yeast has been reported to be dependent on the presence of potassium (Bahadur and Verma, 1959; Borst-Pauwells, 1981). In the present study supplementation of molasses medium

with 0.03% magnesium in the form of $MgSO_4 .7H_2O$ showed optimum growth for *Candida sake*. Similarly the $CaCO_3$ supplementation at a concentration of 0.2% showed optimum growth and higher concentrations induced slight decrease in cell growth. Kotzamanidis *et al.*, (2002) reported a similar observation in the lactic acid production from beet molasses by using *Lactobacillus delbrueckii* where reduced cell growth was observed due to high concentration (7% w/v) of $CaCO_3$. Calcium carbonate controls the pH of the fermentation medium. Tanaka and Omura (1986) reported calcium carbonate as the most common buffering agent used in fermentation experiments. Supplementation of all other micronutrients appears to be not necessary if seawater is used for media preparation, where seawater contains all these compounds and other trace elements.

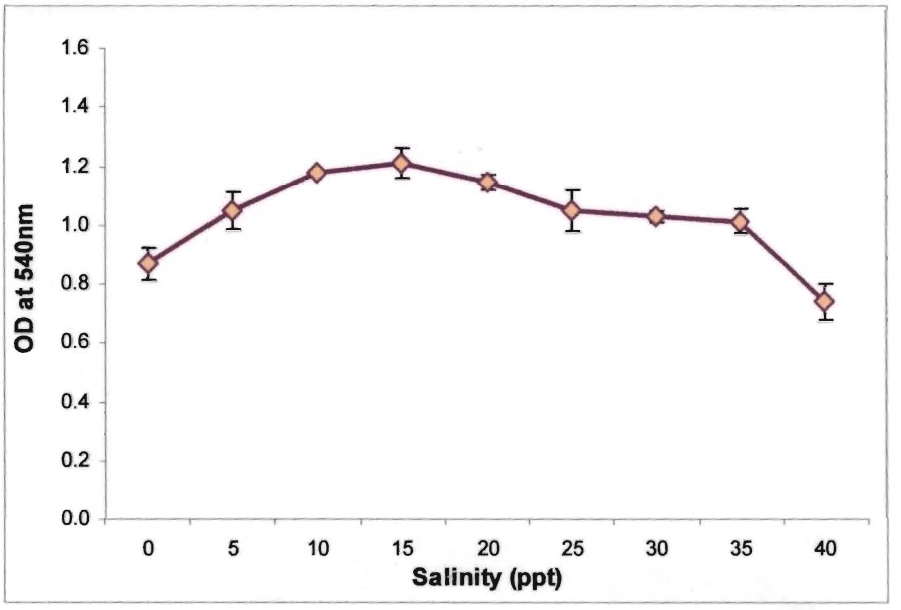


Fig.3.1 Effect of salinity on the growth of *Candida sake*

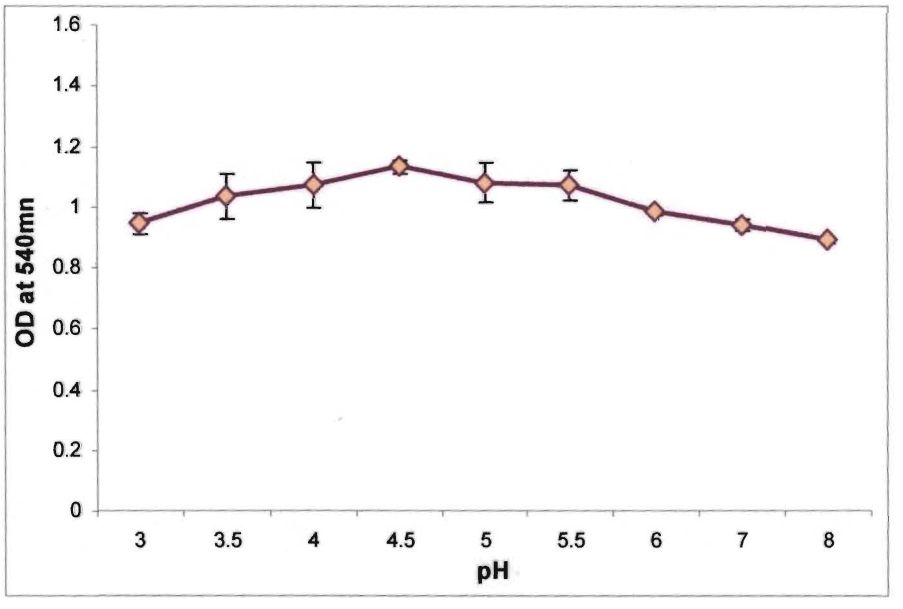


Fig.3.2 Effect of pH on the growth of *Candida sake*

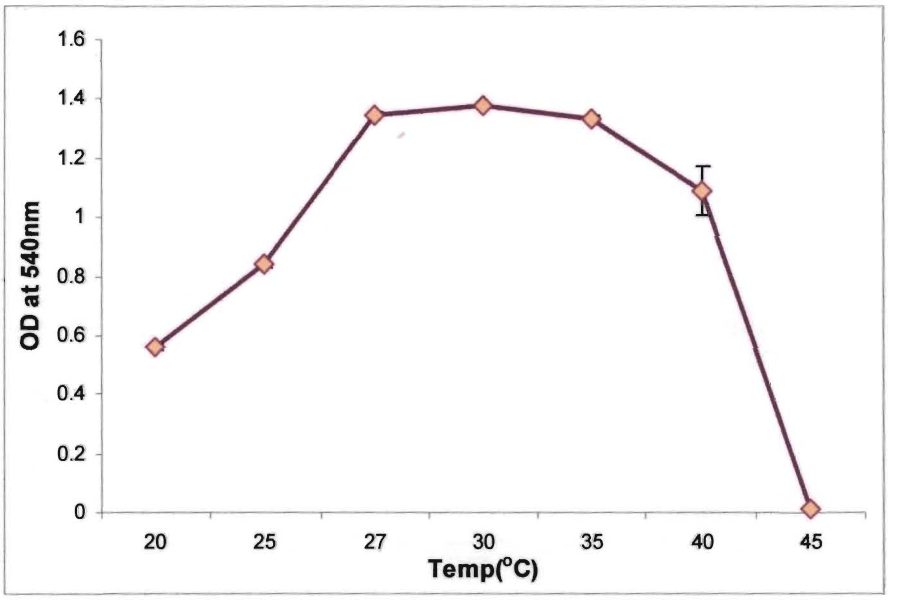


Fig.3.3 Effect of temperature on the growth of *Candida sake*

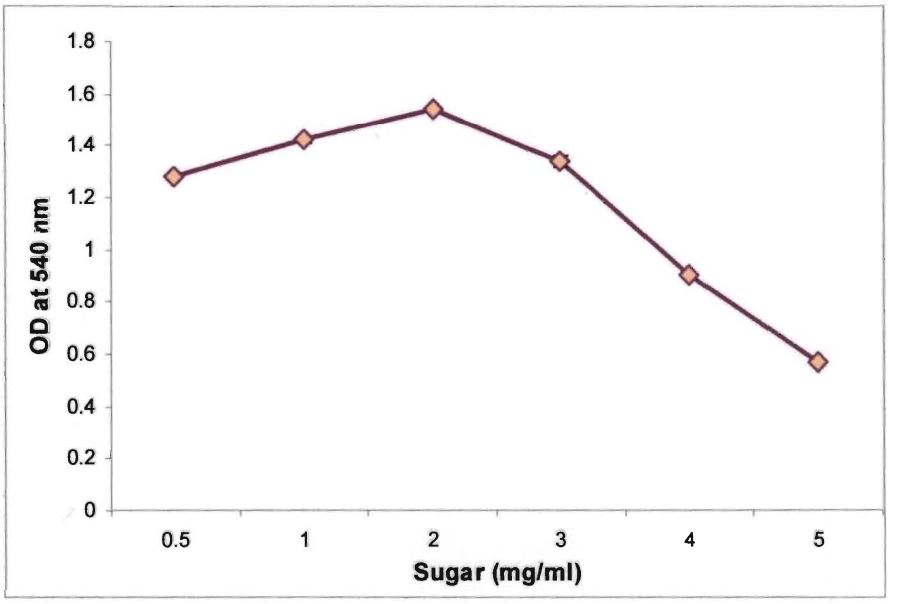


Fig.3.4 Effect of molasses on the growth of *Candida sake*

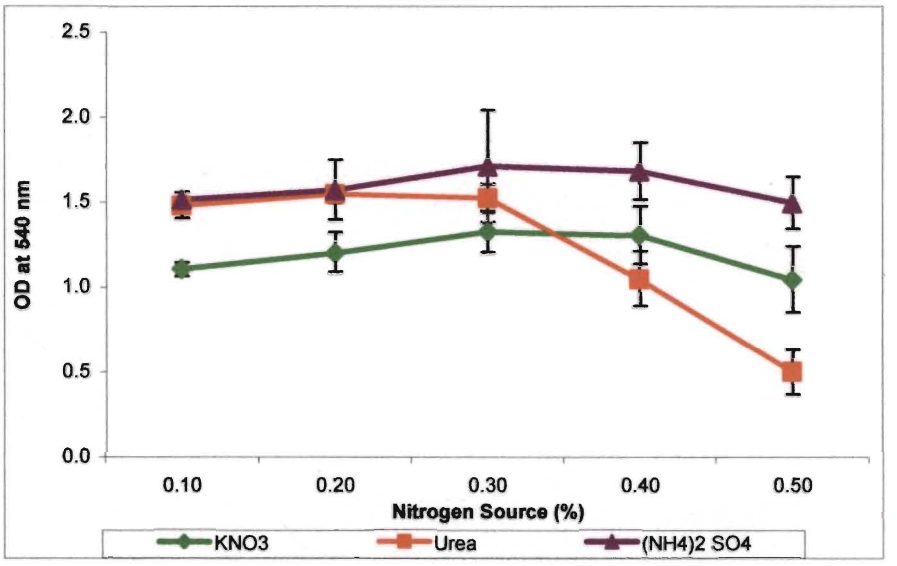


Fig.3.5 Effect of nitrogen sources on the growth of *Candida sake*. Data with same superscript do not varies significantly ($P<0.05$)

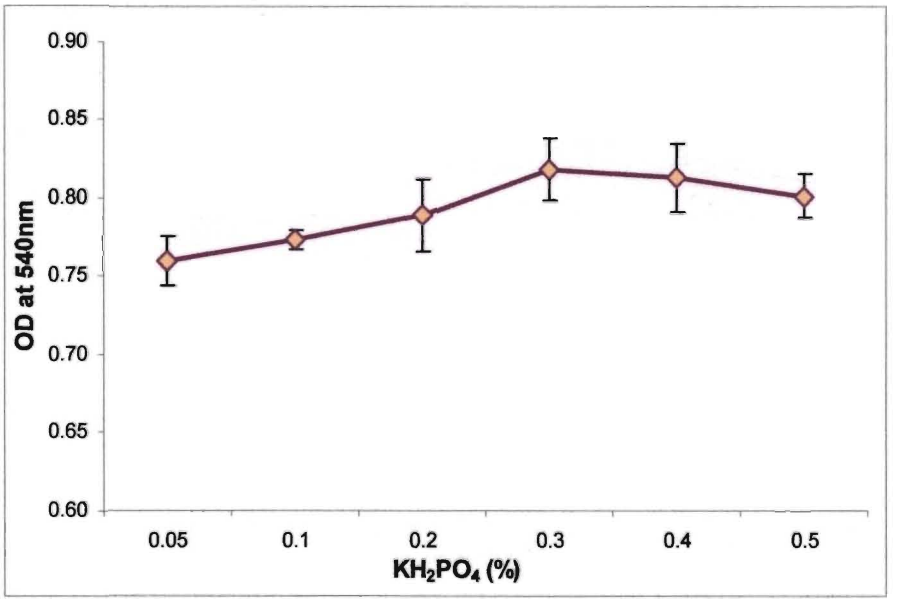


Fig.3.6 Effect of phosphorus on the growth of *Candida sake*

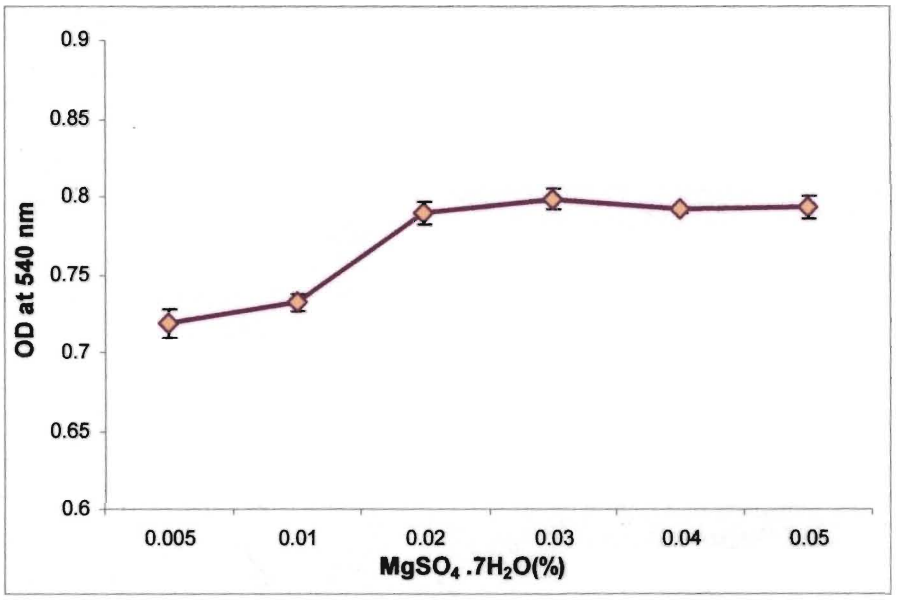


Fig.3.7 Effect of magnesium on the growth of *Candida sake*

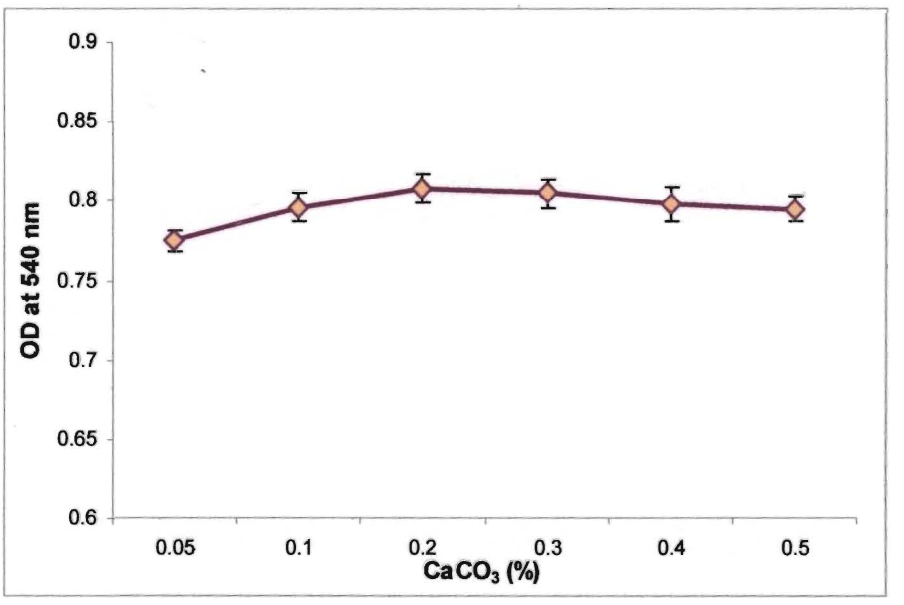


Fig.3.8 Effect of calcium on the growth of *Candida sake*

Chapter 4

Immunological profile of *Fenneropenaeus indicus* on administration of *Candida sake* incorporated diets

4.1. Introduction

One of the constraints in penaeid shrimp culture industry is the frequent outbreak of viral diseases. Among them, white spot syndrome virus (WSSV) causes high mortality in cultured shrimp species including *P. monodon*, *Fenneropenaeus indicus*, *Marsupenaeus japonicus* and *P. semisulcatus* (Lightner, 1996; Lo *et al.*, 1997). In the Indian subcontinent this virus has been causing severe economic loss since early 1995.

β-1, 3 glucan of certain fungi and yeast have been successfully used as immunostimulant to enhance the defence potential of fishes and shellfishes against bacterial or viral infection (Oliver *et al.*, 1986; Robertsen *et al.*, 1990; Sung *et al.*, 1994; Song *et al.*, 1997; Chang *et al.*, 1999, 2000 and 2003). Sung *et al.*, (1994) reported enhanced vibriosis resistance in *P. monodon* post larvae administered with β-glucan. Increased post challenge survival could be observed in glucan fed *P. monodon* when challenged with WSSV (Song *et al.*, 1997). Chang *et al.*(2003) showed that oral administration of β-glucan at an optimal level of 10 g kg^{-1} diet for 20 days effectively enhanced the immune system resulting in an improved survival against WSSV infection in *P. monodon.*

Mock *et al.* (1980) reported that replacing algae with active dry baker's yeast as feed for blue shrimp *P. stylirostris* larvae gave better result. Larvae of kuruma prawn *P. japonicus* and tiger prawns *P. monodon* fed with marine isolate of *Saccharomyces cerevisiae* to obtain high survival rates particularly at zoea stages have been reported (Aujero *et al.*, 1985, Furukawa *et al.*, 1973). Lactic acid yeast *Kluyvermyces fragilis* at a level of 13 % in diet for tiger prawns improved growth performance (cited in Hertrampf and Piedad-Pascual, 2003). Also under field conditions in Japan Squid meal was replaced successfully by 10 to 15 % lactic acid yeast in diet for kuruma prawns (cited in Hertrampf and Piedad-Pascual, 2003).

Scholz *et al.* (1999) compared the efficacy of 5 different yeast supplemented diets in prawns and reported that *Phaffia rhodozyma* incorporated feed gave better performance in terms of bacterial clearance and phenoloxidase value. Recently Burgents *et al.* (2004), reported enhanced disease resistance in pacific white shrimp *Litopenaeus vannamei* against experimental infection of *Vibrio* when fed with a *Saccharomyces cerevisiae* incorporated feed.

In the present study, the immunostimulatory effect of a marine isolate of *Candida sake* was tested in *Fenneropenaeus indicus* against white spot virus infection. Total haemocyte count, phenoloxidase activity and production of superoxide anions (O_2^-) were measured during the experimental period to assess the immune status of the shrimps and the possible role of yeast glucan and nucleotides as immunostimulants have been discussed.

4.2. Materials and methods

4.2.1. Yeast biomass

Candida sake S165 was used for the study. Lawn culture of the yeast was prepared using Malt Extract Agar (Malt Extract, 20 gm; Mycological peptone, 5 gm; Agar 20 gm, 20 ppt Seawater 1 L; pH 6). The yeast biomass was harvested at log phase using sterile seawater (15 ppt) and cells were separated by centrifugation at 7000 x g in a cooling centrifuge (Remi-C-30) for 10 minutes and stored at –20°C until use.

4.2.2. Experimental diet

Three experimental diets were prepared by incorporating yeast biomass at varying concentrations 1%, 10% and 20% to a referral shrimp diet (Table2.3). The referral diet without supplementation of yeast was used as control. All the diets were stored at –20°C until use.

4.2.3. Animals used

A batch of apparently healthy adult *Fenneropenaeus indicus* (mean body weight 15.6±1.5 g) were brought to the laboratory of School of Marine Sciences from a shrimp farm located at Kannamali, Cochin. The shrimps were randomly divided into four groups of 60 shrimps each into aquarium tanks (Fig 4.1) of 500 L capacity and acclimatised to the laboratory conditions for one week.

4.2.4. Feeding experiment

Among the four groups of experimental animals first group received control diet, second group the feed with 1% yeast, third group feed with 10% yeast and the fourth group received feed with 20% yeast. Feeding was done twice daily (8 A.M and 7 P.M) at a rate of 10-15% wet body weight of the shrimp. Physico-chemical parameters of the rearing water were monitored regularly. Salinity, NH_3-N, NO_2-N, NO_3-N and dissolved oxygen were estimated (APHA, 1995) and maintained at optimal level by water exchange. Total culture period was extended up to 28 days and at the end of feeding experiment animals were challenged with white spot syndrome virus (WSSV) *via* orally. Maintaining the animals on same diet, the haematological parameters were assayed.

4.2.5 Assay of immunological parameters

4.2.5.1.Collection of haemolymph

Immunological assays were performed to study the immunostimulatory effect of the yeast diets. Haemolymph was withdrawn aseptically from the rostral sinus of the shrimps and transferred in to a sterile microcentrifuge tube containing measured quantity of cold sterile shrimp anticoagulant solution (0.02M sucrose, 0.01M tri-sodium citrate in 0.01M Tris-HCl, pH 7.6) (Song and Hsieh, 1994). A specially designed sterile capillary tube having a diameter of 0.5mm, pre-rinsed with anticoagulant solution was used for haemolymph collection. Haemolymph was collected from five shrimps of each treatment group and assayed separately. Sampling was done at the

Fig.4.1: Bioassay system used to study the efficacy of marine yeast and beta -1, 3-glucan as immunostimulant to *F. indicus* (adult).

beginning of the feeding experiment (0 day/base line), day 15, day 28 besides post challenge day 1 (PCD1), post challenge day 2 (PCD2) and post challenge day 3 (PCD3). Samples were diluted three-fold with shrimp salt solution (450mM NaCl, 10mM KCl, 10mM EDTA.Na_2, 10mM HEPES, pH 7.3) as described by Vargas-Albores and Ochoa (1992) and analysed individually.

4.2.5.2.Total haemocyte count

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

4.2.5.3.Phenoloxidase (PO) activity

Phenoloxidase activity of haemolymph was measured spectrophotometrically using L-3, 4-dihydroxyphenylalanine (L-DOPA) as substrate according to Soderhall (1981). Briefly 100µl of haemolymph was incubated with 100µl of 1% SDS for three minutes at 25°C. Then 1 ml of L-DOPA was added to the haemolymph. Increase in absorbance at 495 nm was measured at an interval of 30 sec with in a span of 3 min. using a UV-Visible Spectrophotometer (Hitachi. U-2001). L-DOPA with distilled water was used as blank. Enzyme activity was expressed as increase in absorbance per minute per 100µl haemolymph

4.2.5.4.Superoxide anion (NBT reduction) assay

Respiratory burst activity of haemocytes was measured spectro-photometrically as per the method described by Song and Hsieh (1994) with minor modifications. Nitro blue tetrazolium (NBT, SRL Chemicals, India) was used as substrate that gives a blue formazan colour due to its reduction by O_2^- produced during phagocytosis of heamocytes. 100 µl of haemolymph was taken into a microcentrifuge tube precoated with 0.2% poly- L-lysine (Sigma). Poly- L-Lysine coating increases the haemocyte adhesion to the microcentrifuge tube. 100 µl NBT solution (2 mg/ml) prepared in Tris-HCl buffer (pH 7.6) was added to the haemolymph and incubated at room

temperature for 30 min. Tubes were centrifuged at 300 x g for 10 min. in a cooling centrifuge. Discarded the supernatant and stopped the reaction by adding 1ml absolute methanol followed by incubation for 10 min. Spun down the tubes again, discarded the supernatant and left the tubes for air-drying for 30 min. The tubes were washed thrice with 50% methanol and a final washing was done using PBS of pH 7.6. 2M KOH (120 µl) followed by 140 µl dimethylsulphoxide (DMSO, SRL Chemicals) were added to the tubes. Finally 2ml distilled water was added. The optical density at 620nm was recorded using UV-Visible Spectrophotometer (Hitachi. U-2001) against a blank consisting of reagents (KOH and DMSO) and 2 ml distilled water and expressed as NBT activity per 100µl haemolymph.

4.2.6. Statistical analysis

In order to determine significant difference if any, in immunological parameters between the different treatment groups the results were analysed using one way analysis of variance (ANOVA) and Duncan's multiple comparison of the means by using SPSS 10.0 for windows. Significant differences were indicated at $p<0.05$.

4.3. Results

4.3.1.Total haemocyte count (THC)

Total haemocyte count was maximum in prawns fed with 10% yeast diet both during the feeding experiment and on post challenge. An increase in haemocyte count could be noticed during the feeding experiment in all the treatment groups followed by sudden decrease in the count on post challenge. However, a substantial increase could be noticed on day 3 post challenge showing the immune boost up to face the challenge (Fig 4.2 and Table 4.1 of appendix). Diet wise comparison showed that 10% yeast diet supported maximum cell count and this difference in performance was significant on day 3 post challenge. Generally the count proved to be

remarkably low for prawns fed 20% yeast diets except on day 3 post challenge.

4.3. 2. Phenoloxidase activity

Generally shrimps fed with 10% yeast diet showed a PO value significantly higher than that of control and other diets fed groups (Fig 4.3 and Table 4.2 of appendix). During the first day of post challenge the PO value does not show any significant difference, even though the group fed with 10% yeast showed higher values than the others. On the third day of post challenge this difference was significant and 10% yeast fed group showed an overall peak value for PO (1.54 OD at 495 nm). PO value for the group fed with 20% yeast was next to that of 10% yeast diet fed groups.

4.3. 3. NBT reduction assay

NBT reduction was found to be best in prawns fed with 10% yeast diets followed by 20% and 1% yeast fed groups (Fig 4.4 and Table 4.3 of appendix). This difference between various treatment groups was found to be significant on day 2 and day 3 post challenge. A remarkable increase in NBT value could be witnessed on day 3 post challenge, the maximum value (3.705) being recorded by the 10% yeast fed groups. Unlike other immunological parameters the NBT value remained more or less same throughout the experimental period except for the post challenge.

4.4. Discussion

Administration of immunostimulants like glucan, peptidoglycan and lipopoly-saccharides have been found to increase the disease resistance of penaeid shrimps against pathogenic microbes (Sung *et al.*, 1994; Itami *et al.*, 1994, 1998; Karunasagar *et al.*, 1996; Song *et al.*, 1997; Newman, 1999; Takahashi *et al.*, 2000; Chang *et al.*, 2000, 2003). Among these immunostimulants, glucans derived from yeast gained much importance as an immunostimulant in crustacean aquaculture. Glucans are the structural components of cell wall of yeast and certain fungi and principally contains

glucose molecules linked by β-1,3 linkages with occasional 1-6 branching. Extraction of glucan from yeast cell wall requires harsh chemical treatments and the method of extraction may affect the immunostimulatory property of glucan. This can be the reason why some authors report the inefficiency of glucan in stimulating the immune response (Scholz *et al.*, 1999). Glucan extraction is a labour intensive process involving both alkali and acid treatments resulting in the removal of valuable nutrients from the yeast cells.

Yeast is generally considered as a good source of protein, nucleic acid, vitamins and polysaccharides. In the present experiment a marine isolate of *Candida sake* was utilised as a feed ingredient at graded concentrations for *F. indicus* to study its immunostimulatory effect. The yeast *C. sake* was found to be nonpathogenic to penaeid shrimps by prawn blood agar haemolysis assay (Section 2.2.2). Of the 3 different concentrations of yeast-incorporated feeds 10% yeast feed showed better immunostimulatory property as evidenced from values of THC, phenoloxidase and NBT. The total haemocyte count (THC) of the treatment group, which received 10% yeast, showed higher values than that of other groups. Tsing (1987) and van de Braak *et al.* (2002) reported that an increase in circulation of young and immature haemocytes might have be an indicator of an intense proliferation of haematopoietic tissue. A slight drop in the THC just after WSSV challenge was noticed in all the test groups including control, but during the subsequent days it regained. So this may be due to the mobilsation /proliferation of haemocytes on recognizing an infection. A probable explanation for the slight decrease in total haemocyte count at the very beginning of infection could be the infiltration of haemocytes, especially semigranular cells, to connective tissues, stomach and gills on WSSV infection as reported by Munoz *et al.* (2002). Chang *et al.* (2003) made a similar observation in the reduction in total circulating haemocytes at the first day of WSSV infection in *P. monodon*. However, it is interesting to note that in the group fed with 20% yeast, THC count was less when compared to group that received 10% yeast and sometimes even lesser than the control.

At the end of 28 days of feeding the PO level of this group was doubled as compared to that of control on same day. During the first two days of post challenge this difference was not significant though, PO showed an elevated level and on third day of post challenge the difference was very significant. Since PO is considered as the key enzyme in shrimp defence the response of this enzyme during infectious period is having paramount importance in host resistance. NBT values also showed a similar pattern where the group fed with 10% yeast displayed a significant difference from all other groups. There occurs a gradual increase in NBT level during post infection periods and very prominent hike in the NBT level on the third day of post challenge. This can be attributed to an increase in phagocytosis resulting in the production of more super oxide anions for checking infection. Throughout the experiment the performance of the group fed with 20% yeast diet was poor when compared to 10% yeast diet fed group. This could be due to a higher dose of yeast-derived immunostimulant administered to the prawns resulting in over activation of immune system leading to a condition called "immune-fatigue".

The immunostimulant property of yeast could be attributed to its glucan content. Scholz *et al.* (1999) reported that *S. cerevisiae* and *Phaffia rhodozyma* incorporated diets showed higher survival in *P. vannamei*. Apart from cell wall glucans the nucleotide contents of the yeast also would have contributed to immunostimulation.

Nucleotides are low molecular weight biological components that play a major role in almost all biological processes like encoding genetic information, mediating energy metabolism, and signal transduction (Carver and Walker, 1995; Aggett *et al.*, 2003) They are generally considered as non-essential nutrients because deficiency signs have not been observed (Carver and Walker, 1995). Although most cell types are capable of synthesising nucleotides from purines and pyramidines, *de novo* synthesis and salvage synthesis of nucleotides are thought to be a costly process in

terms of energy requirements. An exogenous source of nucleotides may optimise the functions of rapidly dividing cells, such as those of immune system, which lack the capacity to synthesise nucleotides and therefore must depend on pre-formed nucleotides (Carver, 1994; Carver and Walker, 1995). Moreover information regarding the synthesis and metabolism of nucleotides in fishes and crustaceans are extremely limited to date (Li and Gatlin III, 2003).

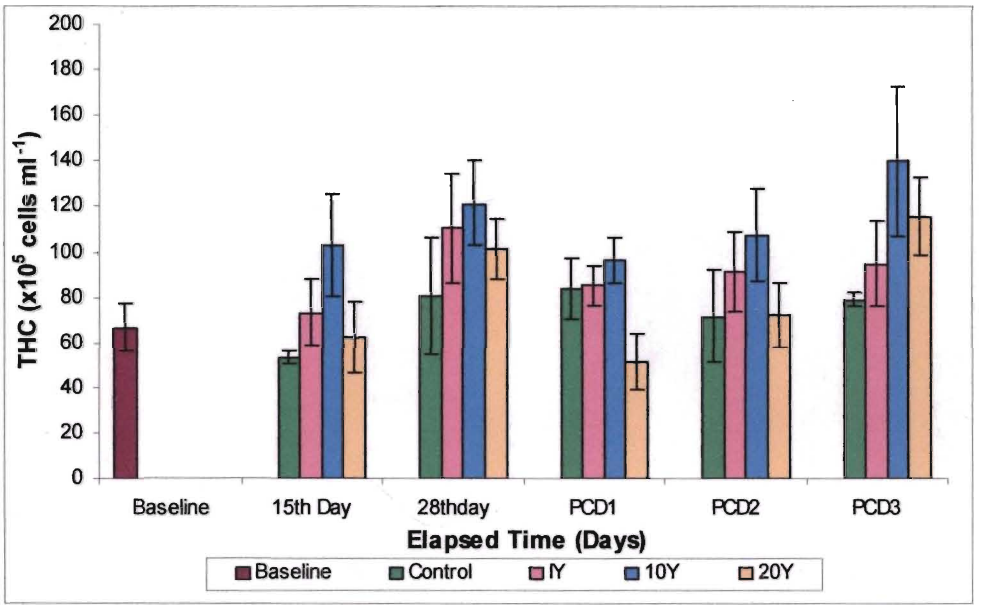
Studies conducted by Burrells *et al.* (2001a&b) showed that the supplementation of standard aquaculture diets with additional dietary nucleotides could improve the health status of salmonids by increasing the resistance of fish to various bacterial, viral and rickettsial infection and a reduction in the severity of ectoparasite infection. Dietary nucleotides were also shown to have a positive effect on stress tolerance, vaccine efficiency, osmoregulatory capacity at seawater transfer and growth rates of salmon (Burrells *et al.*, 2001b). Sakai *et al.* (2001) reported that the nucleotides from brewer's yeast RNA were capable of enhancing the phagocytic and oxidative activities of kidney phagocytic cells, serum lysozyme in common carp as well as resistance to *Aeromonas hydrophila*. Li and Gatlin III (2003) also reported that brewer's yeast *Saccharomyces cerevisiae* positively influenced growth performance and feed efficiency of hybrid striped bass as well as resistance to *Streptococcus iniae* infection and emphasised the possible role of yeast nucleotides in immunostimulation.

Low *et al.* (2003) examined the relative expression of certain immune genes of *Scophthalmus maximus* after feeding with nucleotide supplemented diet and showed that there occurs an increased expression of genes of IgM, interleukin etc. Recently Chuo *et al.* (2005) studied the signal transduction of the proPhenoloxidase activating system of *Macrobrachium rosenbergii* haemocytes and reported that intracellular phenoloxidase activity in haemocyte lysate supernatant (HLS) was increased after treating with CpG oligonucleotides. Here the oligonucleotides function as an immunostimulant,

similar to that of glucan, where it increases the phenoloxidase activity and degranulation of granular haemocytes, but failed to induce proPhenoloxidase synthesis at transcriptional level.

So it is reasonable to believe that the immunostimulatory effect of whole cell yeast *Candida sake* is not only due to its glucan contents, but also due to the nucleotide pool and both can act as immunostimulants in shrimps under stress such as infection with WSSV. In the present experiment *C. sake* at a concentration of 10% in feed was optimal and showed an enhanced immunity in *F. indicus.* Whereas a high concentration of 20% yeast diet showed a comparatively low immune profile of shrimp probably due to over dose of immunostimulants which lead to a reduced immunity. This result corroborate with the finding of Chang *et al.* (2000) who reported that a higher dose of immunostimulant β-glucan reduce the non-specific immunity and disease resistance to pathogens in shrimps.

Most of the shrimp feeding experiments were performed with either baker's yeast or brewer's yeast, which are, quite alien to a marine or brackish water environment. The penaeid shrimp culture practice is being restricted to such brackish or seawater conditions having a salinity of about 20 ppt to 35 ppt. In this context it is reasonable to state that the fresh water forms like *S. cerevisiae* may find sea water as hyperosmotic and cause the cell rupture due to osmotic shock which further leads to pollution of culture waters (Kawano and Ohsawa 1971). One practical solution to this problem is to identify a salt resistant, halophilic form, which can be used to substitute the common freshwater yeast as a feed ingredient in prawn culture systems. The halotolerant property of yeast *C. sake* would be an advantage in this context and can be used in brackish water or seawater aquaculture where it will not cause problem of cell burst and related water quality deterioration.



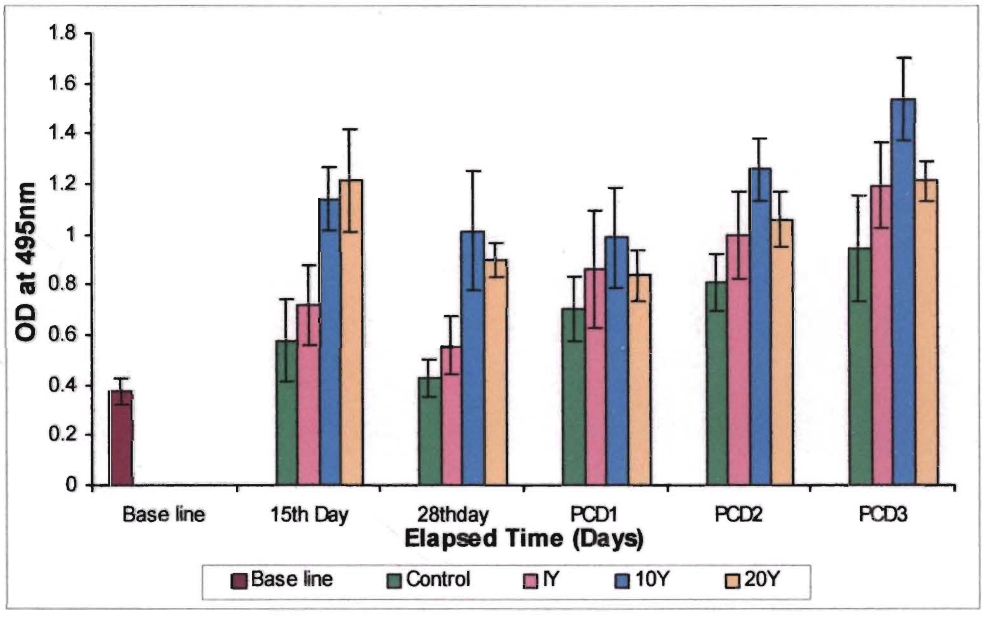
Feeds	THC (x 10^5 cells ml^{-1})*					
	Base line	15th Day	28thday	PCD1**	PCD2	PCD3
Control	66.77±10.09^a	53.25±2.88^a	80.50±25.50^a	83.75±13.52^b	71.67±20.36^a	79.25±2.88^a
IY	66.77±10.09^a	73.42±14.39^a	110.67±24.13^a	85.30± 8.73^b	91.17±17.32^{ab}	94.79±18.69^a
10Y	66.77±10.09^a	102.67±22.25^b	121.33±18.76^a	96.25±9.59^b	107.37±20.20^b	140.00±32.57^b
20Y	66.77±10.09^a	62.08±15.90^a	101.17±12.96^a	51.17±12.41^a	72.17±13.82^a	115.67±16.74^{ab}

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

1Y- 1 % yeast diet; 10Y- 10% yeast diet; 20Y- 20% yeast diet;

PCD1- Post challenge Day 1; PCD2- Post challenge Day 2; PCD3- Post challenge Day 3

Fig. 4.1. Mean (± S.D) THC of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV.



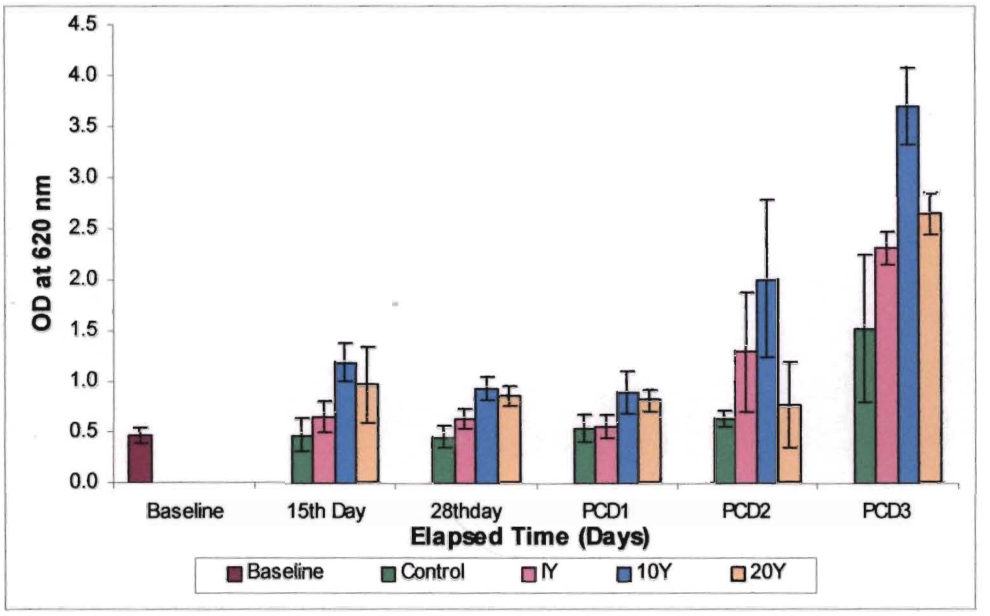
Feeds	Phenoloxidase (increase in OD min^{-1}/100µl haemolymph)					
	Base line	15th Day	28thday	PCD1	PCD2	PCD3
Control	0.376 ± 0.05^{a}	0.575 ± 0.16^{a}	0.427 ± 0.07^{a}	0.703 ± 0.12^{a}	0.810 ± 0.11^{a}	0.942 ± 0.21^{a}
IY	0.376 ± 0.05^{a}	0.722 ± 0.15^{a}	0.558 ± 0.11^{a}	0.863 ± 0.23^{a}	0.998 ± 0.17^{ab}	1.195 ± 0.16^{a}
10Y	0.376 ± 0.05^{a}	1.143 ± 0.12^{b}	1.015 ± 0.23^{b}	0.987 ± 0.19^{a}	1.257 ± 0.12^{b}	1.540 ± 0.16^{b}
20Y	0.376 ± 0.05^{a}	1.215 ± 0.2^{b}	0.902 ± 0.67^{b}	0.837 ± 0.1^{a}	1.060 ± 0.11^{ab}	1.212 ± 0.08^{a}

* Data at the same exposure time with different superscripts are significantly different ($P<0.05$).

1Y- 1 % yeast diet; 10Y- 10% yeast diet; 20Y- 20% yeast diet;

PCD1- Post challenge Day 1; PCD2- Post challenge Day 2; PCD3- Post challenge Day 3

Fig. 4.2. Mean (±S.D) phenoloxidase (PO) value of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV.



Feeds	NBT activity (OD at 620 nm/100µl haemolymph)*					
	Baseline	15th Day	28thday	PCD1	PCD2	PCD3
Control	0.457 ± 0.07[a]	0.470 ± 0.15[a]	0.452 ± 0.10[a]	0.542 ± 0.13[a]	0.630 ± 0.08[a]	1.512 ± 0.72[a]
IY	0.457± 0.07[a]	0.645 ± 0.14[ab]	0.625 ± 0.09[a]	0.562 ± 0.11[a]	1.293 ± 0.58[ab]	2.308 ± 0.15[ab]
10Y	0.457± 0.07[a]	1.182 ± 0.18[bc]	0.920 ± 0.11[b]	0.890 ± 0.20[ab]	2.005 ± 0.77[a]	3.705 ± 0.37[b]
20Y	0.457± 0.07[a]	0.967 ± 0.37[c]	0.852 ± 0.09[b]	0.810 ± 0.10[b]	0.767 ± 0.41[b]	2.648 ± 0.19[c]

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

1Y- 1 % yeast diet; 10Y- 10% yeast diet; 20Y- 20% yeast diet;

PCD1- Post challenge Day 1; PCD2- Post challenge Day 2; PCD3- Post challenge Day 3

Fig. 4.3. Mean (± S.D) NBT value of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV.

Chapter 5

Extraction and partial H-NMR structural characterisation of (1→3)-β-D-glucan from *Candida sake* and *Saccharomyces cerevisiae*

5.1. Introduction

The immunotherapeutic effects of glucan and other biological response modifiers (BRM), largely depend on their structure. The yeast cell wall contains β (1→3) D-glucan, β (1→6) D-glucan, chitin, mannan, proteins and lipids (Duffus *et al.*, 1982). The cell wall polysaccharides are separated in two groups according to their solubility in hot alkali solution. The structural skeleton of the yeast cell wall, i.e., β-1,3-glucan is alkali insoluble. The overall composition of the yeast cell wall usually comprises of glucan (50%, of which 20% is alkali soluble), mannan (30%), protein (10-15%), lipid (8 – 9%) and chitin (1-2%), were the cell wall representing about 15% of the dry weight of the cell (Duffus *et al.*, 1982). But these values may vary with the species and strains of yeast, its conditions of growth and with the methods used for the preparation of the cell walls and their fractionation.

Most commonly employed method for glucan extraction is the alkali-acid hydrolysis method of Hassid (1941) later redefined by Williams *et al.* (1991) according to which more than 97 % pure form of glucan could be obtained (Lowman and Williams, 2001). Further quantification and structural analysis of the water-insoluble glucans is hampered by their relative insolubility. Methylation analysis is considered as an important technique in polysaccharide studies, whereas yeast glucan is notoriously difficult to methylate with the conventional Haworth reagents.

Manners and Patterson (1966) carried out methylation, periodate oxidation and also enzymic degradation studies to conclude that yeast glucan had a branched structure, containing main chain of (1→6) linked β-glucose residue to which were attached linear side chains of (1→3) linked β-glucose residues. Later Bacon and Farmer (1968) proved that yeast glucan was in fact a mixture of a major (1→3) linked β-glucan component and a minor (1→6)-β-glucan component.

More recently structural studies of the yeast glucan have been done with Nuclear Magnetic Resonance (NMR) spectroscopy and detailed structure of various glucans were elucidated (Ensley *et al.*, 1994, Kim *et al.*, 2000, Lowman *et al.*, 2003).

In the present experiment, alkali insoluble cell wall glucan of marine yeast *Candida sake* was extracted and its characterisation was done with proton NMR and compared with a standard glucan prepared by Lowman and Williams (2001). Glucan extracted from *Saccharomyces cerevisiae* (MTCC 36) by following the same protocol was also subjected to NMR analysis. So far there is no report available pertaining to a holistic study involving extraction of biologically active glucan, its structure elucidation and purity confirmation by NMR studies and testing its efficacy as an immunostimulant in penaeid shrimps.

5.2. Materials and methods

5.2.1. Extraction of glucan

Candida sake S165, which showed better immunostimulatory potential in penaeid prawns in the screening experiment, was used for glucan extraction and further study. Along with this a strain of bakers yeast *Saccharomyces cerevisiae* (MTCC36) obtained from IMTECH Chandigarh was also used for glucan extraction. Biomass of both yeast were prepared by lawn culturing on malt extract agar media and was harvested on 6th day of culture. Cells were separated by centrifugation at 7000 rpm for 15 min. in a refrigerated centrifuge and then dried at 80°C for 24 hrs. Glucan from the dried biomasses of yeasts were extracted as per the method of Williams *et al.* (1991). The detailed extraction procedure of particulate glucan is given in Fig 5.1.

5.2.2. H-NMR spectroscopy

Spectral data were collected using a JEOL Model Eclipse+ 600 NMR spectrometer operating at 80°C in 5-mm OD NMR tubes. Glucan isolated

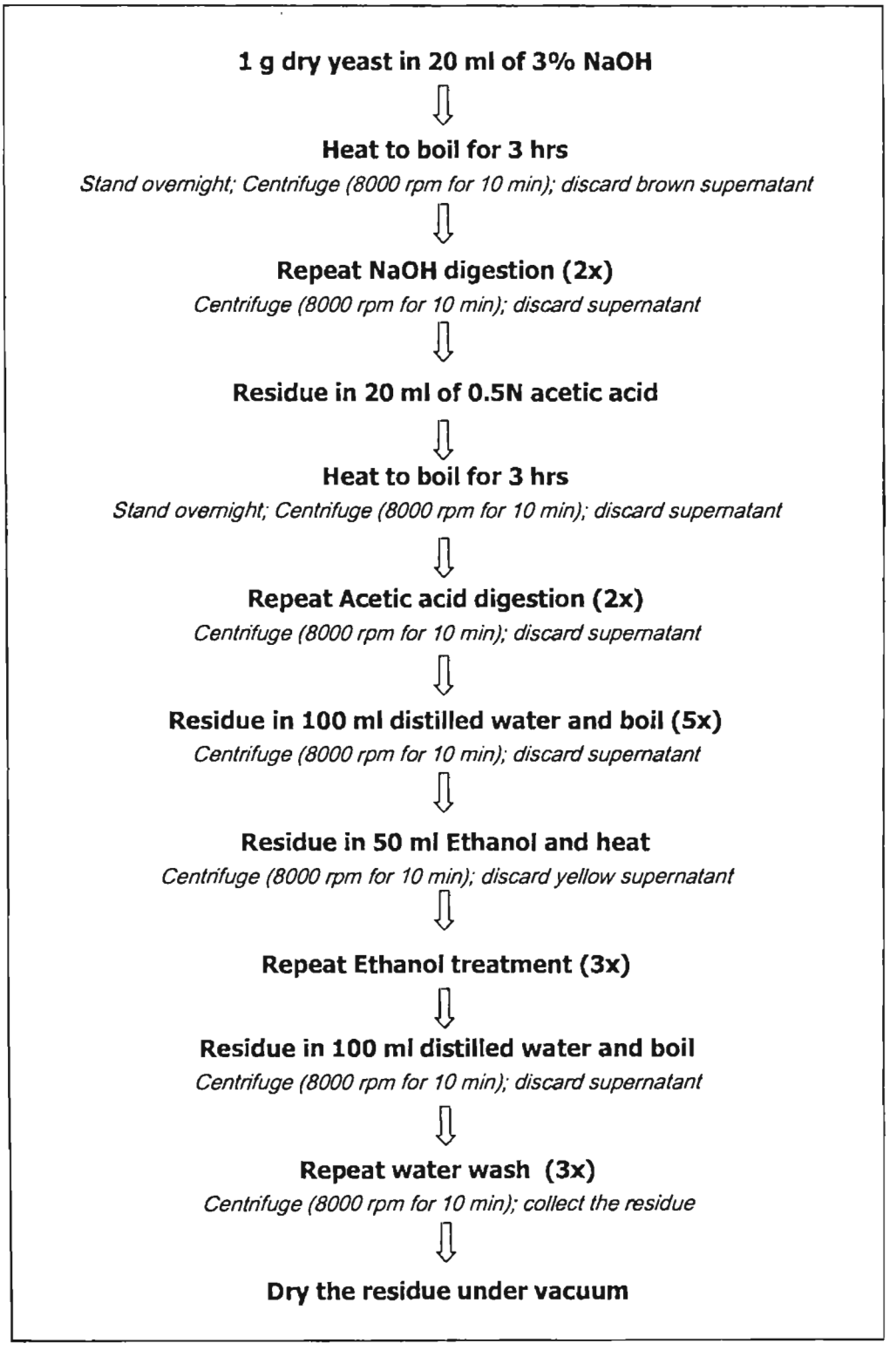


Fig.5.1 Flow chart describing the procedure for extraction of water insoluble β- glucan from yeast cell wall.

from *Candida sake* S165 and *S. cerevisiae* MTCC36 were compared with a standard glucan isolated from *Saccharomyces cerevisiae*, which showed 97% pure soluble glucan (Lowman and Williams, 2001). Briefly 20-25 mg of the glucan was dissolved in 1 mL of dimethyl sulfoxide-d_6 (DMSO-d_6) at 80 °C. A few drops of deuterated trifluoroacetic acid (TFA-d, 99.8% deuterated, Cambridge Isotope Laboratories) were added to the solution to shift the water resonance downfield (Ross and Lowe, 2000). Proton chemical shifts were referenced to the residual DMSO-d_6 proton resonance at 2.50 ppm. NMR spectral collection and processing parameters were the following: 25 ppm spectral width centered at 7.5 ppm, 32768 data points, 1024 scans, 15 sec relaxation delay, 2.18 sec acquisition time, and exponential apodisation.

5.3. Result

5.3.1.Extraction of glucan

The extraction of glucan from *Candida sake* S165 gave a yield of 12.3% particulate glucan whereas in the case of *Saccharomyces cerevisiae*, MTCC36, it was only 9.13% (Table 5.1).

Strain	Production % (mean ± S.D)
Candida sake S165	12.31 ± 0.35
Saccharomyces cerevisiae MTCC36	9.13 ± 0.39

Table 5. 1 The percentage yield of glucan extracted by alkali extraction

5.3.2. NMR spectra

Fig 5.2 to 5.4 shows the proton NMR spectra of the carbohydrate region of water insoluble particulate glucan isolated from *Candida sake* S165 and *Saccharomyces cerevisiae* MTCC36 in comparison with that of standard glucan. Figure 5.2 shows the spectra full scale in both dimensions–X and Y. Figure 5.3 is an X-axis expansion of the carbohydrate region from 5.6 to 2.8 ppm.

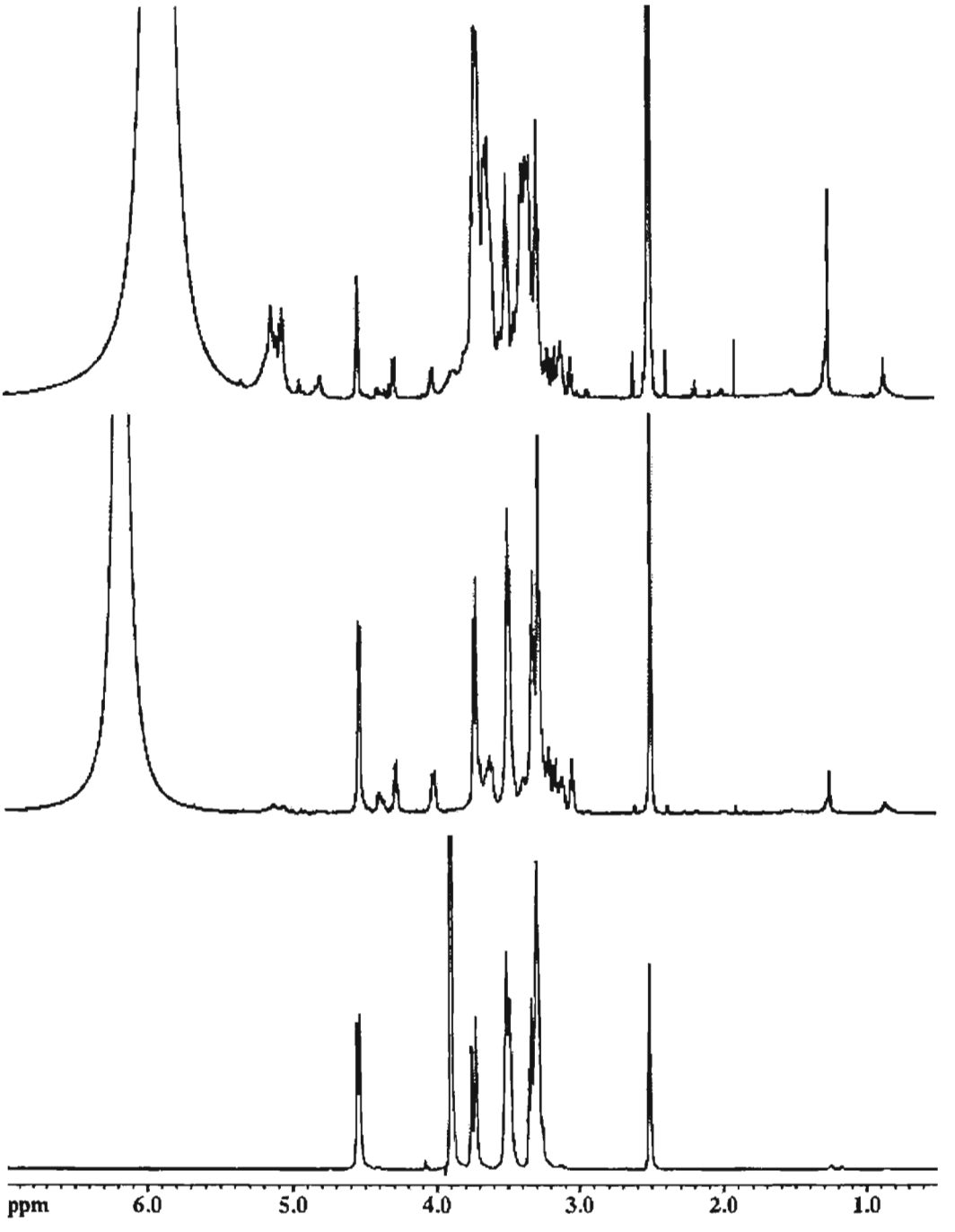


Figure 5.2. ^{1}H NMR spectrum of the glucan isolated from *Candida sake* S165 (top) and *Saccharomyces cerevisiae* MTCC36 (middle) with the standard glucan of 97% purity (bottom)

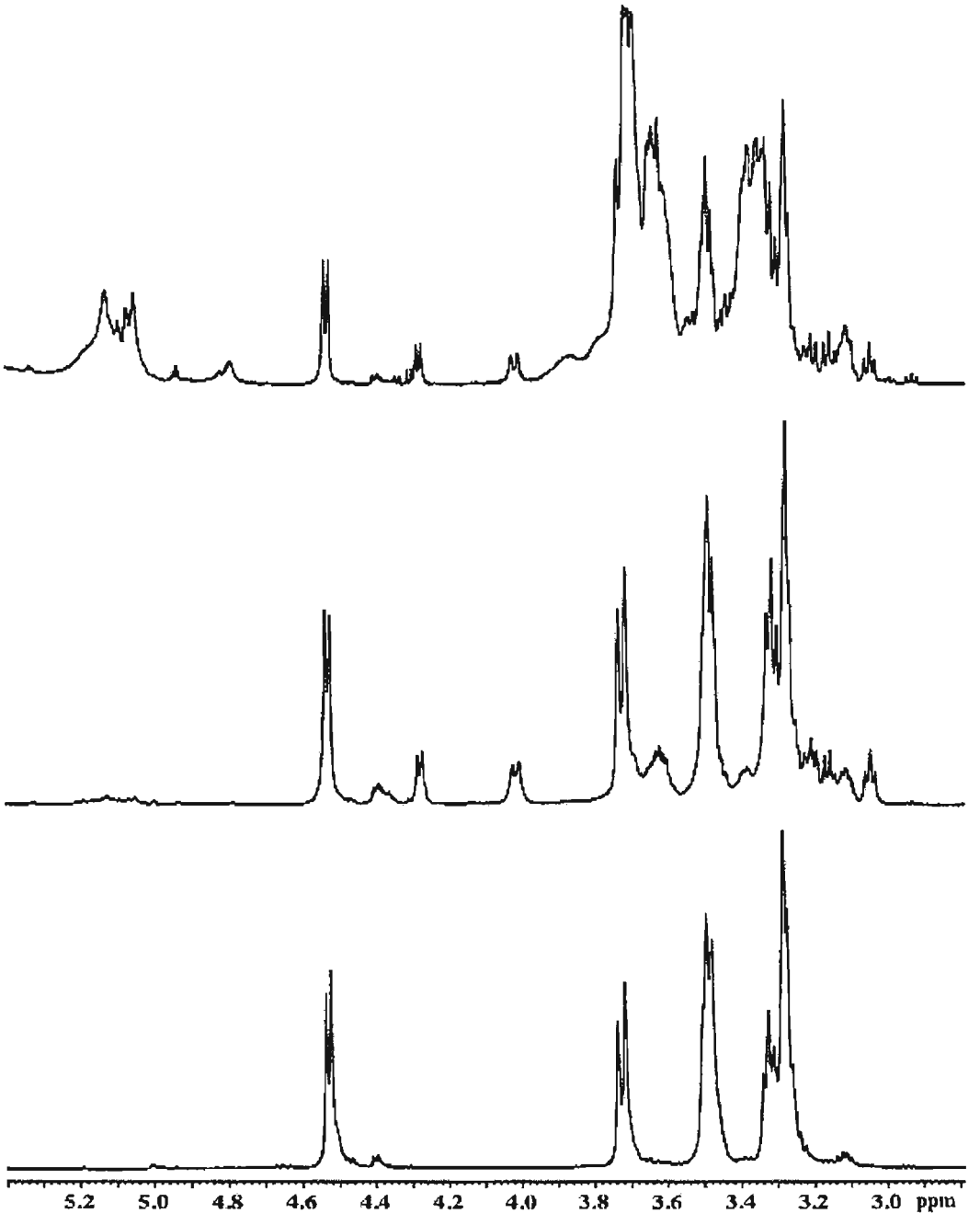


Figure 5.3. ^{1}H NMR spectrum of the glucan isolated from *Candida sake* S165 (top) and *Saccharomyces cerevisiae* MTCC36 (middle) with the standard glucan of 97% purity (bottom) showing X-axis expansion of the carbohydrate region

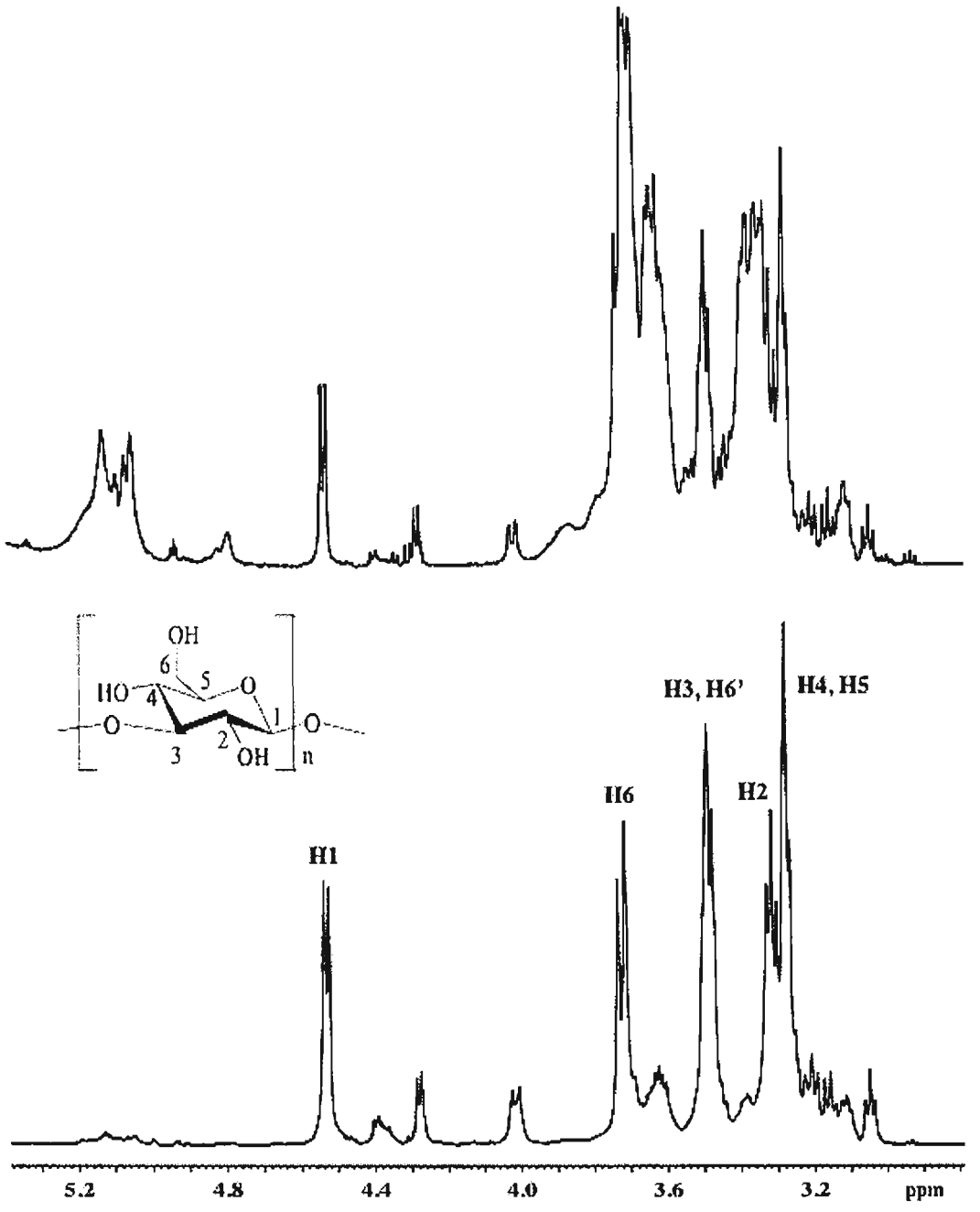


Figure 5.4. Anomeric proton region of NMR spectrum of glucan from *C. sake* S165 (top) and glucan from *S. cerevisiae* MTCC36 (bottom). Resonance labels are described in the text

The top one in the Fig 5. 2 and 5.3 represents the glucan of *C. sake* S165, middle one that of *S. cerevisiae* MTCC36 and the bottom line represent spectrum of standard glucan used for comparison. Figure 5.4 is an expansion of the anomeric proton and H6 proton spectral region (expanded from 5.1 to 3.9 ppm) of glucan of *C. sake* S165 (top) and *S. cerevisiae* MTCC36 (bottom).

Fig 5.4 is a comparison of the proton NMR spectra of glucan isolates from *C. sake* (Top) and *S. cerevisiae* (Bottom), showing the carbohydrate spectral region. The list below details the chemical shift assignments for the resonances observed for samples in Figure 5.4.

5.0 ppm:	Anomeric proton of the alpha anomer at the reducing terminus (RT) of the glucan.
4.94 ppm:	Anomeric proton of glucose.
4.52 ppm:	Anomeric protons of the backbone chain anhydroglucose units.
4.47 ppm:	Possibly resonances from the anomeric proton of the glucosyl unit second from the RT, also referred to as the SRT repeat unit.
4.33 to 4.44 ppm:	Resonances for the anomeric proton of the beta anomer of the glucan and anomeric protons from the non-reducing terminus (NRT)
4.31 ppm:	Doublet resonance from the beta anomer of glucose.
4.28 and 4.02 ppm:	Anomeric proton and one of the H6 protons, respectively, involved in the (1-6)-linked glucosyl side chain

The large resonance at about 6.0 ppm visible in spectra results from the exchangeable proton of TFA-D. Other resonances outside the carbohydrate region include the following: residual protons of DMSO-d_6 and its C-13 satellite resonances (2.50, 2.6, and 2.4 ppm, respectively), acetic acid and acetyl substitution (1.9 and 2.0, respectively) and lipid (1.5, 1.25 and 0.85 ppm). Resonances between 5.01 and 5.25 ppm suggest the presence of mannan in the preparation. The triplet resonance (2.94 ppm) and doublet resonances (4.31 and 4.94 ppm) result from glucose. Based on the above data a schematic representation of the structural details characterised for these glucan isolates is given in Fig. 5.5

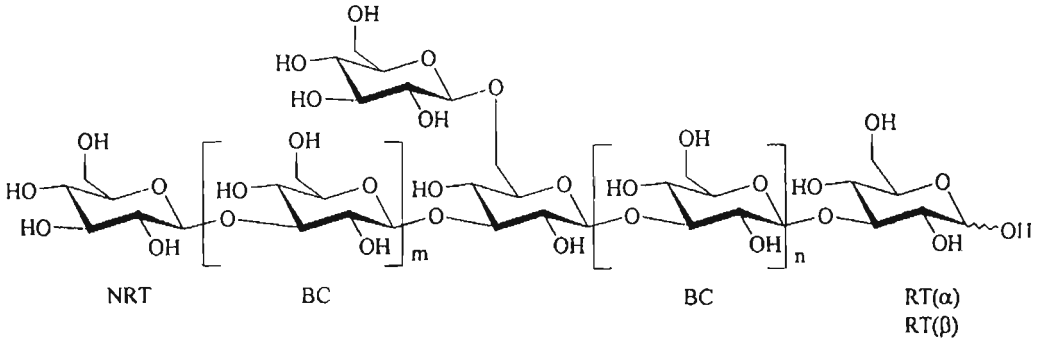


Figure 5.5. Schematic representation of the structural details characterised for glucan isolates.

5.4. Discussion

5.4.1. Glucan extraction and NMR analysis

Extensive structural studies using proton nuclear magnetic resonance spectroscopy (NMR) have been conducted on glucans isolated from the unicellular yeast, or blastospore, form of the saprophytic fungus *Saccharomyces cerevisiae* (Ensley *et al.*, 1994; Kim *et al.*, 2000; Lowman and Williams, 2001) as well as blastospores and hyphae of the polymorphic pathogenic fungus *Candida albicans* (Yu *et al.*, 1993; Kruppa *et al.*, 2003; Lowman *et al.*, 2003). Deciphering the structural features of these carbohydrates is an essential pre-requisite for understanding how fungal glucans are recognized by the innate immune system. In this study proton

NMR spectral analysis was done to characterise structural details of water-insoluble particulate glucans isolated from *C. sake* S165 and *S. cerevisiae* MTCC36 and the result was compared with that of a standard water soluble glucan extracted from *S. cerevisiae* by Lowman and Williams (2001).

Figure 5.2 - 5.4 shows the proton NMR spectra of the carbohydrate region of the water-insoluble particulate glucans isolated from *C. sake* S165 (top) and *S. cerevisiae* MTCC36 (middle) in comparison with a stranded water-soluble glucan from *S. cerevisiae* (bottom). The results indicate appreciably good glucan content in both *C. sake* S165 and *S. cerevisiae* MTCC36. The Fig 5.3 is a spectrum of *C. sake* and *S. cerevisiae* MTCC36 alone. Clearly, from Figure 5.3 the major component of these isolates is identified as (1-6)-branched (1-3)-β-D-glucan as reported by Ohno *et al.* (1999). Resonances between 5.01 and 5.25 ppm suggest the presence of mannan in the preparation. The glucan preparation from *C. sake* S165 alone showed the presence of mannan, which was almost absent in *S. cerevisiae* MTCC36 preparation. The triplet resonance (2.94 ppm) and doublet resonances (4.31 and 4.94 ppm) result from glucose. The sloping baseline in the downfield portion of the top spectrum results from the close proximity of the exchangeable proton resonance from TFA-d.

Structural characterisation of these glucan isolates benefits from a detailed examination of spectral regions containing the anomeric protons and the protons from H6. A schematic presentation of the structure of a typical branched glucan is presented in Figure 5.6 along with labels for various protons identified below. These proton labels follow the suggested nomenclature of Kim *et al.* (2000) for most of the anomeric protons. The proton-numbering scheme used for anhydroglucose repeat units (AGRUs) is also shown in Figure 4.6.

Several structural features, evident in these spectra and shown structurally in Figure 5.6, include the following: α -anomeric proton on the reducing

terminus AGRU, RT(α) (5.00 ppm); anomeric proton of the AGRUs along the polymer backbone, BC (4.52 ppm); resonances from the anomeric proton of the AGRU second from the RT, SRT (4.47 ppm); resonances from the β - anomeric proton on the RT AGRU, RT(β), anomeric proton from the non-reducing terminus AGRU, NRT, anomeric proton and one of the H6 protons involved in the (1-6)-linked side chain (4.28 and 4.02 ppm, respectively).. The other non-equivalent 6-position methylene proton resonates near 3.63 ppm. Resonance assignments shown here are based upon previous work from Kim *et al.* (2000), Ensley *et al.* (1994), Lowman *et al.* (2003), Kruppa *et al.* (2003) and Monteiro *et al.* (2000).

For these insoluble particulate glucan isolates, the AGRUs on the surface of the particulate are most likely solvated by DMSO, which results in sufficient mobility for them to be observable by NMR. Protons inside the particulate do not have similar mobility since they are not solvated and are therefore not as observable. The side chain glucosyl group is evident here as along with a few other anomeric protons in disproportionate amount since they are associated with AGRUs on the surface of the insoluble particulate glucan in DMSO. Based on these results, the structure of these glucan isolates is best described as a linear (1-3)-linked glucan with (1-6)-linked side chains.

Chapter 6

Cell wall (1→3)-β-D-glucan of *Candida sake* as immunostimulant to *Fenneropenaeus indicus*

6.1. Introduction

Polysaccharides from a variety of sources have been found to enhance the immune system of animals. Pharmacologically they are classified as biological response modifiers (BRMs). The most active appears to be (1→3)-β-D- glucans, also referred to as (1→3) (1→6)-β-D-glucans (Bhon and Be Miller, 1995). These naturally occurring (1→3)-β-D-glucan polymers can be isolated from bacterial and fungal cell walls (Ensley *et al.*, 1994, Christensen *et al.*, 2001) and a mixed linkage (1→3, 1→4)- β-D-glucan polymer can be isolated from plant and cereal grain extracts (Williams *et.al.*,1996). There is a growing awareness of the potential health benefits of glucans, which has increased their importance in the 'nutraceutical' industry (Lowman and Williams, 2001).

Di Luzio (1983) reviewed the role of glucan as a broad-spectrum enhancer of host defence mechanisms against microbial infections and neoplasia and as a unique adjuvant in diverse vaccines. Chihara *et al.* (1987) and Kraus and Franz (1991) have reported that (1→3)-β-D-glucans are effective against allogenic, syngenic and even autochthonous tumours. Various studies have shown that (1→3)-β-D-glucan polymers will increase the function of macrophages (Williams and Di Luzio, 1980), neutrophils (Williams *et al.*, 1988) and other cells of immune system. Observations pertaining to the action of glucan on immune system components have led to the novel concept about glucan as 'immunostimulant' in biomedical applications. The molecular mechanism by which glucan exerts its action on immune cells have been studied and the presence of glucan specific receptors on such cells have been postulated (Williams, 1997).

Glucan polymers can exist as a single polymer strand with a helical conformation (single helix) or as stable complex of three polymer strands forming a triple helix (Williams *et al.*, 1991) and the triple helix appears to be

the preferred form (Ensley *et al.*, 1994). It is reported that the immunostimulatory effect of glucan depends on their structure and size. Yanaki *et al.* (1983) reported that antitumor activity of schizopyllan, a glucan extracted from *Schizopyllum commune*, is related to its triple helical conformation. It is suggested that the activity of these polysaccharides also depends on their size, with high molecular weight (100,000 -200,0000) fraction being most active (Faber *et al.*, 1984). β-Glucan with an average MW of < 200,000 and no ordered structure show great antitumor activity (Blaschek *et al.*, 1992).

6.1.1.Glucans in aquaculture

β-Glucans have been successfully used as immunostimulants to enhance the defence resistance of fishes and crustaceans against bacterial or viral infection (Oliver *et al.*, 1986; Robertsen *et al.*, 1990; Sung *et al.*, 1994; Song *et al.*, 1997; Chang *et al.*, 1999, 2000, 2003). β-Glucan obtained from *Saccharomyces cerevisiae* enhanced the vibriosis resistance in post larvae of *P. monodon* (Sung *et al.*, 1994). Using a mixture of 1,3 and 1,6 glucan extracted from cell wall of *S. cerevisiae,* Song *et al.* (1997) reported an enhanced disease resistance of *P. monodon* towards experimental infection of white spot virus. Chang *et al.* (2000) observed that feeding *P. monodon* with glucan derived from *Schizophyllum commune* at a concentration of 2 g/kg in diet showed an increase in phagocytic index and superoxide anion production by haemocytes. Apart from glucan, cell wall components like peptidoglycan and lipopolysaccharides of certain bacteria also showed considerable immunostimulation in shrimps. Itami *et al.* (1998) reported the enhancement of disease resistance in kuruma shrimp, *P. japonicus*, fed with peptidoglycan derived from *Bifidobacterium thermophilum* against penaeid rod-shaped DNA virus (PRDV). Takahashi *et al.* (2000) reported that lipopolysaccharides (LPS) extracted from *Pantoea agglomerans* fed to kuruma shrimp *P. japonicus* at varying concentrations of 20, 40 and 100μg / kg shrimp body weight/day showed a better phagocytic index and phenoloxidase activity for the group of shrimps which received 20 μg / kg shrimp body weight/day for 7 days. Song and Hsieh (1994) reported that

zymosan, a protein-carbohydrate (mannan and glucose) complex extracted from *S. cerevisiae* and β-glucan extracted from *S. cerevisiae* increased the overall defence system of tiger shrimp *P. monodon in vitro*.

In this present work the efficacy and utilisation of glucan, extracted from marine yeast *Candida sake*, as an immunostimulant to Indian white prawn *Fenneropenaeus indicus* was estimated. Dose, frequency and mode of glucan application were also studied.

6.2. Materials and methods

6.2.1. Oral administration of glucan and optimisation of dose in feed for *F. indicus*

6.2.1.1.Preparation of glucan incorporated feed.

Glucan extracted from *C. sake* as per section 5.2.1 was homogenised and coated on a standard diet (Table 2.3) using a commercial binder "BINDEX" (Matrix Biosciences Ltd, Hyderabad). Five different types of feeds were prepared with varying glucan concentrations like 0.05, 0.1, 0.2, 0.3 and 0.4%. Feed without glucan was used as control.

6.2.1.2.Animals used

Fenneropenaeus indicus post larvae (PL20), of the size range 25 to 30mg, which are PCR negative for white spot syndrome virus, were brought from a shrimp hatchery located at Kannamali, Cochin and was acclimatized to laboratory conditions for one week.

6.2.1.3.Experimental design.

Twenty five animals were stocked in 20L seawater taken in Fibre Reinforced Plastic (FRP) tanks of 30L capacity (Fig2.1). Feeding experiments were done in triplicate with experimental feeds along with control diet for a period of 21 days. At the end of feeding experiment the animals were subjected to WSSV challenge and post challenge survival was recorded for 7 days.

6.2.2. Dip treatment with glucan and optimisation of the concentration

6.2.2.1. Preparation of glucan suspension.

Glucan prepared from *C. sake* as given in section 5.2.1 was subjected to sonication by using an ultrasonicator (SOINC VIBRA, USA). For this, particulate glucan was suspended in distilled water (1% w/v) and sonicated at 20Khz at 100W for 15 min and solutions of different strength (0.1 mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml) were prepared in 15 ppt seawater.

6.2.2.2. Experimental design

Post larvae (PL18) of *F. indicus* PCR–negative for WSSV were used for the study. After acclimatisation to laboratory conditions the animals were dipped in various concentrations of glucan suspensions (500 post larvae per litre glucan suspension) prepared as above for a period of 3hrs. After three hours of dip treatment, larvae were transferred to FRP tanks with aerated seawater of 15 ppt. and were maintained on standard diet. Post larvae dipped in seawater without glucan were considered as control. After 10 days, (post dip 10^{th} day) 60 animals were separated from each treatment groups, transferred 20 each into four FRP tanks and challenged with WSSV orally (via diet). Similarly on post dip 15^{th} and post dip 20^{th} day, the animals were challenged with WSSV orally.

6.2.3.Frequency of administration of glucan

6.2.3.1.Experimental design

Based on glucan dose optimisation study (section 6.2.1) in *F. Indicus,* 0.2 % glucan diet was selected for further work. Post larvae (PL 25) of *F. indicus*, PCR–negative for WSSV were used for the study. The optimal frequency for the administration of glucan was studied for both dip treated and non-dip treated animals. For this the animals were segregated into two groups and first group was subjected to glucan dip treatment in a glucan solution of strength 1mg/ml for a period of 3 hrs (section 6.2.2) and the other group was used without glucan treatment. Two groups were again divided in to six

subgroups and each subgroup was fed with 0.2% glucan incorporated diet at different frequencies (ie. 1) daily, 2) once in two days, 3) once in five days, 4) once in seven days and 5) once in ten days). Control diet without glucan was given on the rest of the days. Sixth group was maintained as control. Four replicates were maintained for each group. Rest of the rearing conditions were similar to that described in section 2.2.1.4. After 40 days of feeding all the animals were challenged with WSSV orally and post challenge survival was recoded till 7days.

6.2.4.Statistical analysis

Data generated from the above feeding experiments were analysed by One-way Analysis of Variance (ANOVA) and mean of the treatment results were compared by using SPSS 10.0 for windows and significant difference were brought out at a level of $p<0.05$

6.3. Result

6.3.1. Oral administration of glucan and optimisation of dose in feed for *F. indicus*

Post challenge survival against WSSV at the end of 7 days was shown to be maximum with 0.2% glucan (54.17±3.62%) incorporated feed and this was found to be significantly different from control group which showed only 4.74 ± 0.99% survival on the same day. This is followed by the feed with 0.3% (34.45 ± 5.65%) and 0.4% (26.06 ±2.74%) glucan. Feed with lesser concentration of glucan like 0.05% and 0.1% exhibited a survival of 16.41 ± 3.84% and 31.59 ± 5.3% respectively. (Fig 6.1 and 6.2).

6.3.2. Dip treatment with glucan and optimisation of the concentration

The study showed that animals subjected to dip treatment with 0.5-1mg/ml glucan exhibited better survival on post challenge with WSSV. The challenge data shows that on dip treatment with glucan the protection offered against WSSV was not long lasting and was only up to 2 weeks. No significant

difference in the survival could be noted among the various treatment groups on the post dip 20th of day challenge.

Post dip 10th day challenge

Post challenge survival for the 10th day challenge group is given in Fig 6.3. Post larvae which were treated with 0.5 mg/ml and 1mg/ml glucan suspension exhibited higher percentage of survival (26.67 ± 5.77%) which was significantly different from control (10%). Animals dipped in 0.1mg/ml glucan showed 16.67 ± 5.77% survival, whereas, lowest survival (6.67±5.77%) was recorded in post larvae subjected to 2mg/ml glucan solution dip treatment.

Post dip 15th day challenge

Survival data of the 15th day challenge group is presented in the Fig 6.4. It is evident from the graph that 0.5 and 1mg/ml glucan treatment showed better survival of 22 ± 4.77 and 24 ± 5.48% respectively. 0.1 mg/ml treated group showed 16 ± 5.48% survival whilst, 2 mg/ml treated group showed only 12 ± 4.47% survival. The control exhibited a survival of only 10% at the end of 7 days post challenge.

Post dip 20th day challenge

No significant difference in the post challenge survival was seen in the case of treatment groups challenged on 20th day of glucan immersion. The four different concentrations of glucan used for immersion failed to protect the animals on the 20th day of glucan treatment (Fig.6.5). The post challenge survival of the control and 0.1mg/ml glucan treated group was 4 ± 5.48%, while that of 0.5mg/ml and 1mg/ml was 6 ± 5.48% only. The 2mg/ml group showed higher survival (8 ± 4.47%), even though not significantly different from others.

6.3.3. Frequency of administration of glucan

The result of the experiment to find out the frequency of administration of glucan feed is presented in fig 6.6. Frequency of administration of glucan (0.2%) in the diet was tested in both dip treated (0.5mg/L) and non-dip treated post larvae. Administration of glucan once in seven days was found to be optimal for both dip treated and the other groups (non-dip). A significant increase in the survival (36.25 ± 4.33% for dip and 35 ± 10 % for non-dip) could be observed in these animals when challenged with WSSV compared to all other groups (daily, once in 2 days, once in 5 days and once in ten days). Both the dip treated and non-dip treated animals showed a similar trend in post challenge survival with the various treatment groups. However the post challenge survival was always found to be high for the dip treated animals compared to the non-dip treated groups. The cumulative mortality also showed similar pattern in both the groups of animals.

6.4. Discussion

In the present study glucan extracted from *Candida sake* was found to confer protection against WSSV challenge in *F. indicus*. Among the five different concentrations of glucan tested the feed with a glucan content of 0.2% showed optimal protection and this was reflected till the day 7 post challenge and was found to be significantly different from the control feed. High concentrations of glucan in the feed (0.3% and 0.4%) showed less protective effect. A similar observation was made by Chang *et al.* (2000) where long term administration of dietary glucan for 40 days at a concentration of 2g kg^{-1} feed resulted in reduced immunity index in *P. monodon*. Robertsen *et al.* (1990) reported that high concentration of glucan gave less protection in salmon compared to low concentrations. Low survival of shrimps at higher concentrations of glucan could be attributed to the overproduction of superoxide anions and free radical generated from the respiratory burst activity of the phagocytic haemocytes, which can cause non-specific host injury. Moreover, higher concentration of glucan causes the degranulation of both granular and semigranular heamocytes resulting in

the release of prophenoloxidase and exhaustion of the immune system. A higher dose of glucan in feed for longer period may exert an immunosuppression and it is more likely that such animals cannot survive infection by potent pathogens and may easily succumb to death due to such infections.

There occurs a very few studies related to the dose/response of immunostimulants in shrimps. Unlike many chemotherapeutics, immunostimulants dose not show a linear dose/effect relationship (Bliznakov and Adler, 1972). In fact they often show a 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 also 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 intra peritoneal injection of immunostimulants enhances the function of leucocytes and protection against pathogens, this method is labour intensive, time consuming and stressful to the animals. While oral administration is non-stressful and allows mass administration regardless of the size of the fish. The fate of orally administered glucan is not clearly understood. The hepatopancrease of shrimps could be the major site of absorption, however, the lymphoid tissue, which lies in close apposition with the former could be a possible route of glucan in to the systemic circulation.

Nikl *et al.* (1993) showed that oral administration of β(1-3)-D-glucan together with bacterial antigen enhanced non-specific resistance against bacterial infection in salmon, where indirect evidence indicates that β(1-3)- D-glucan is absorbed from the gut. Dalmo and Seljelid (1994) showed by means of autoradiography and fluorescence microscopy that laminarin was absorbed

from the posterior intestine after anal administration of the immunomodulator to Atlantic salmon. In an immunohistochemical examination to study the possible route of vibrio antigen given as an immersion for *P. monodon,* Sung and Song (1996) reported that soon after exposure (5min to 6hrs) vibrio antigen could be located at hepatopancreas, lymphoid organs, haematopoietic tissue and haemolymph of the animals.

In the present study the animals were subjected to dip treatment in five different concentrations (0, 0.1, 0.5, 1 and 2 mg ml^{-1}) of glucan. After dip treatment the animals were challenged with WSSV separately on 10^{th} day post dip, 15^{th} day post dip and 20^{th} day post dip for finding out the duration of protection conferred by dip treatment. Hatchery reared post larvae of shrimps are particularly susceptible to microbial infection when stocked in ponds. Moreover transportation of post larvae from hatchery to aquaculture ponds exerts stress. Therefore it is advisable to use immunostimulants to enhance the non-specific defence status of these animals before transportation to ponds. Immersion treatment is the most reliable and effective way of administration of drugs to larvae or fingerlings of aquatic organisms in hatchery.

0.5mg/ml and 1mg/ml glucan showed promising post challenge survival in *F. indicus* on the 7^{th} day of WSSV challenge, which was significantly different from control, and 2mg/ml glucan treated groups. Here also it was evident that the high concentration of glucan, ie, 2mg/ml resulted in lesser post challenge survival. This shows that animals of this group got stressed due to the high concentrations of glucan in dip. In this group considerable death was also observed just after 3 hrs dip treatment, which again shows the adverse effect of high concentration of glucan. A similar observation was made by Sung *et al.* (1994) in *P. monodon* post larvae treated with 2 mg/ml glucan suspension. They also reported that considerable damage was caused to gill tissue due to the high concentration of glucan in suspension.

On the 15th day of post challenge 1 mg/ml treated group showed better survival than 0.5 mg/ml concentration. Here again 2mg/ml glucan treated group showed less survival compared to the control. This slight increase in survival of 1mg/ml group when compared to control may be sign of recovery of the animals from the adverse effect of higher concentration of glucan by a matter of 15 days. Also the result shows that at the 15th day of immersion concentrations like 0.5mg/ml and 1mg/ml was able to protect the animals from infection.

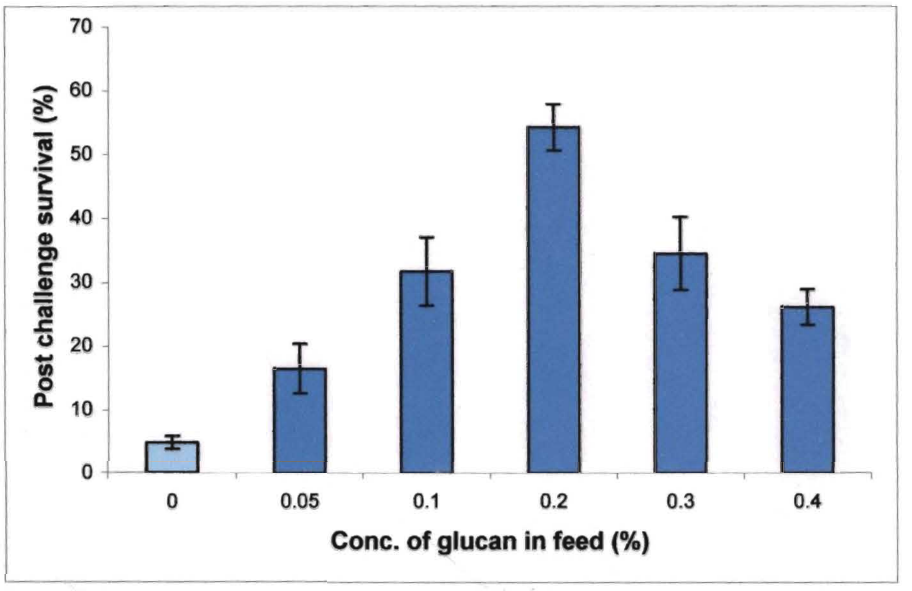
On the 20th day of post dip, the 2mg ml^{-1} treated groups showed slightly better survival against WSSV challenge. However no significant difference could be observed between the four different concentrations of glucan and the survival recorded was meagre for all treatment groups and therefore the observation shows that the protective effect was only short lived, may be up to 15 days or so.

A similar observation was made by Sung *et al.* (1994) in *P. monodon* where glucan protection lasted until 18th day of treatment and completely unobservable on 43rd day of treatment. In the present observation of 15 days short term protective effect of glucan is in line with the total life span of a heamocytes i.e. 15 to 18 days.

Many workers have reported successful immunostimulation via immersion treatment. Baba *et al.*(1993) reported that carp immersed in levamisole solution (10μg/ml for 24 hrs) showed activated phagocytic activities, chemotactic abilities and the production of reactive oxygens in the head kidney phagocytes and enhanced protection against *A. hydrophila.* Anderson *et al.* (1995) showed that rainbow trout immersed in glucan or chitosan showed increased protection against *A. salmonicida* after treatment for 3 days. Jeney and Anderson (1993) reported that rainbow trout bathed in *A. salmonicida* O-antigen in combination with immunostimulants like levamisol, quaternary ammonium chloride etc enhanced phagocytosis by

leucocytes and antibody titer against *A. salmonicida* and showed adjuvant effect with vaccination.

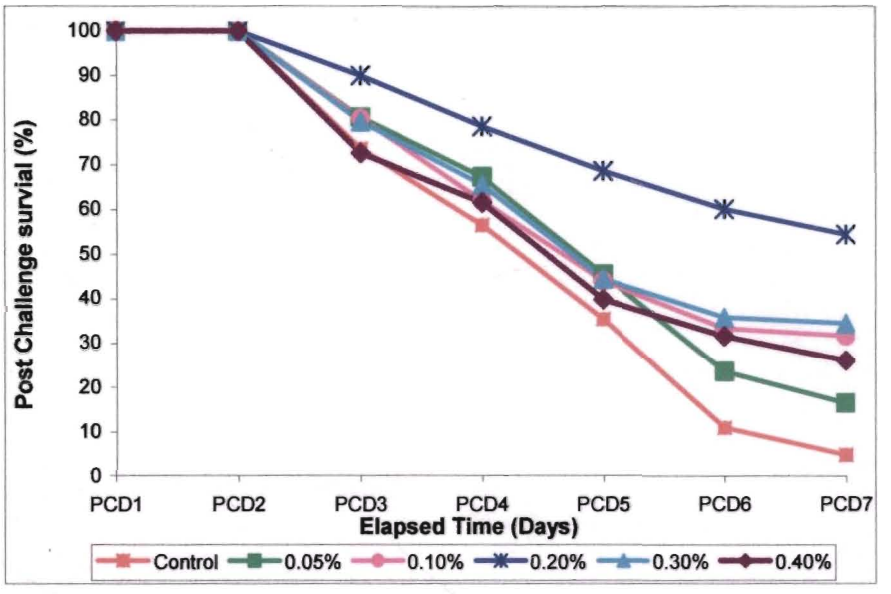
The short lived nature of protection, lack of linear dose/effect relationship and lack of immune memory makes the regimens of immunostimulant application complicated. In the present study post larvae both dip treatment and non-dip treated ones were subjected to the study. In both the cases animals, which received glucan diet once in seven days, showed higher survival when challenged with WSSV. Chang *et al.* (2000) also reported that prolonged use of beta glucan even at optimal dietary levels decreases the immunity of the shrimps and suggested that an intermittent use of immunostimulants would have been a choice for maximizing the effectiveness. In the present experiment the larvae pre-dipped with glucan suspension and fed with glucan showed better survival than the other (non-dip treatment). This shows that dip treatment imparts enhanced protection to post larvae compared to the animals maintained on oral administration of glucan alone. During the larval stages the best method of glucan application is dip treatment and can be done at post larval stage 18 or above, during which they are ready for stocking in ponds. So it is advisable to give a dip treatment to larvae intended for transportation and further stocking. After ten days of stocking feeding with 0.2% glucan incorporated feed can be done at an interval of seven days throughout culture period. The decrease in disease resistance at higher dose of glucan could be attributed to the over stimulation of immune system. In the case of animals fed with glucan diet once in ten days the survival was low. This may be due to the fact that this group received only sub optimal dose of glucan.



Glucan (%)	Post challenge survival (%)*
Control	4.74± 0.99[a]
0.05	16.41± 3.84[b]
0.1	31.59± 5.30[cd]
0.2	54.17± 3.62[e]
0.3	34.45± 5.65[d]
0.4	26.06± 2.74[c]

* Values with same superscript dose not vary significantly (P<0.05)

Fig.6.1 .Post challenge survival on 7th day of WSSV challenge in *F. indicus* post larvae fed with different concentrations of yeast glucan ((0.05, 0.1, 0.2, 0.3, and 0.4%) incorporated diet.

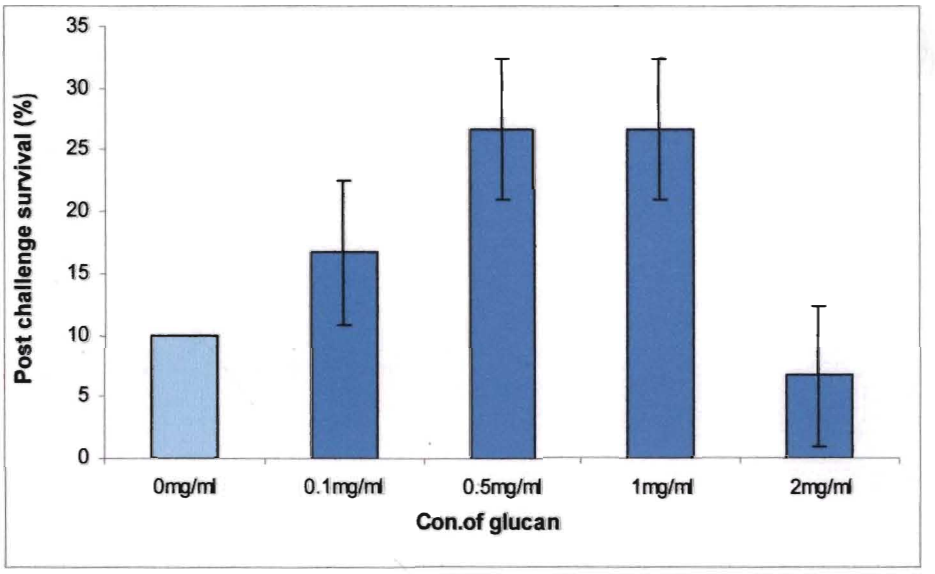


PCD1- Post challenge Day 1; PCD2- Post challenge Day 2; PCD3- Post challenge Day 3

PCD4- Post challenge Day 4; PCD5- Post challenge Day 5; PCD5- Post challenge Day 5

PCD7- Post challenge Day 7;

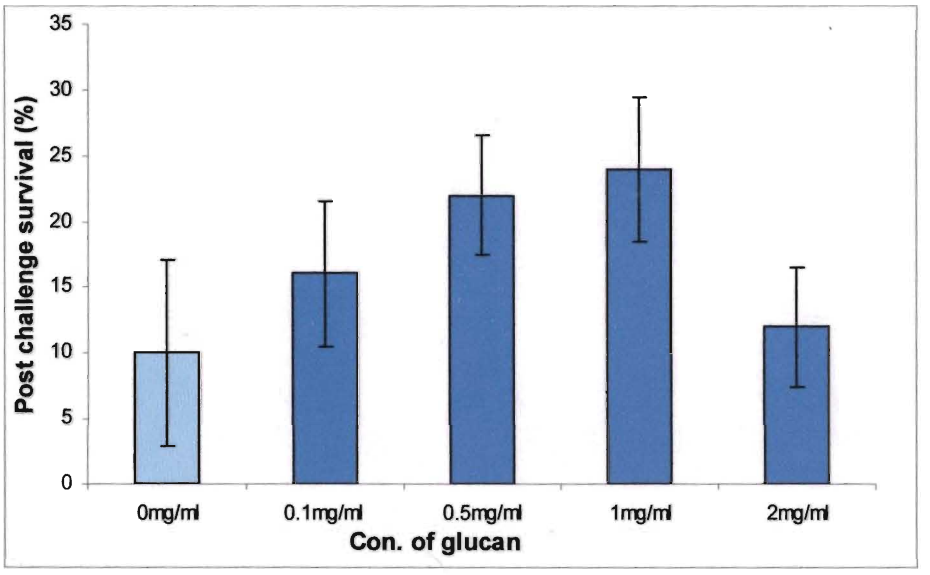
Fig. 6.2 Survival rates of *F. indicus* fed on diets containing graded levels of glucan (0.05, 0.1, 0.2, 0.3, and 0.4%) for 21 days and then challenged with WSSV.



Glucan (mg/ml)	Post challenge survival (%)*
Control	10 ± 0^{ab}
0.1mg/ml	16.67 ± 5.77^{b}
0.5mg/ml	26.67 ± 5.77^{c}
1mg/ml	26.67 ± 5.77^{c}
2mg/ml	6.67 ± 5.77^{a}

* Values with same superscript dose not vary significantly ($P<0.05$)

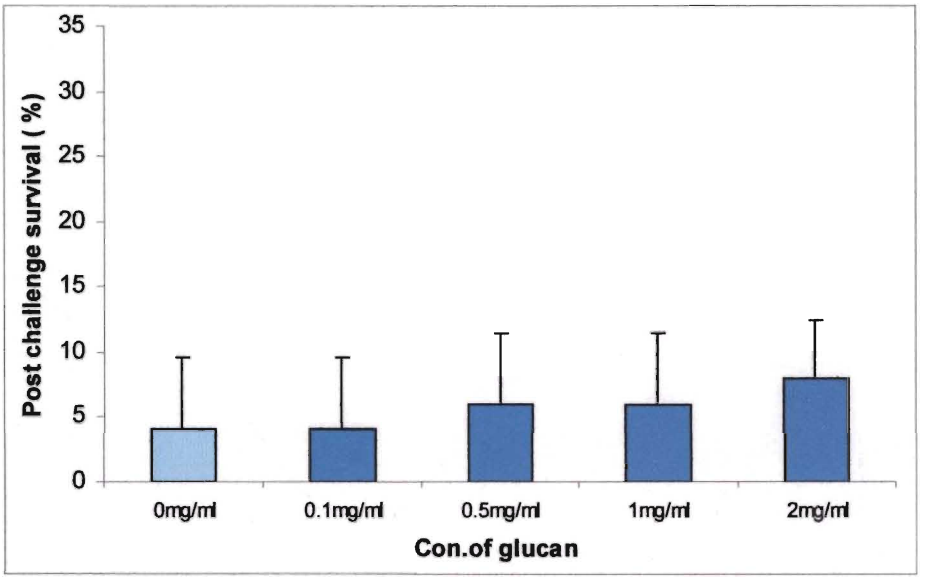
Fig 6.3. Post challenge survival (on 7th day) in *F. indicus* post larvae on challenge with WSSV after 10th day of dip treatment with different concentrations of yeast glucan solution.



Glucan (mg/ml)	Post challenge survival (%)*
Control	10 ± 0^{a}
0.1mg/ml	16 ± 5.48^{ab}
0.5mg/ml	22 ± 4.77^{bc}
1mg/ml	24 ± 5.48^{c}
2mg/ml	12 ± 4.47^{a}

* Values with same superscript dose not vary significantly ($P<0.05$)

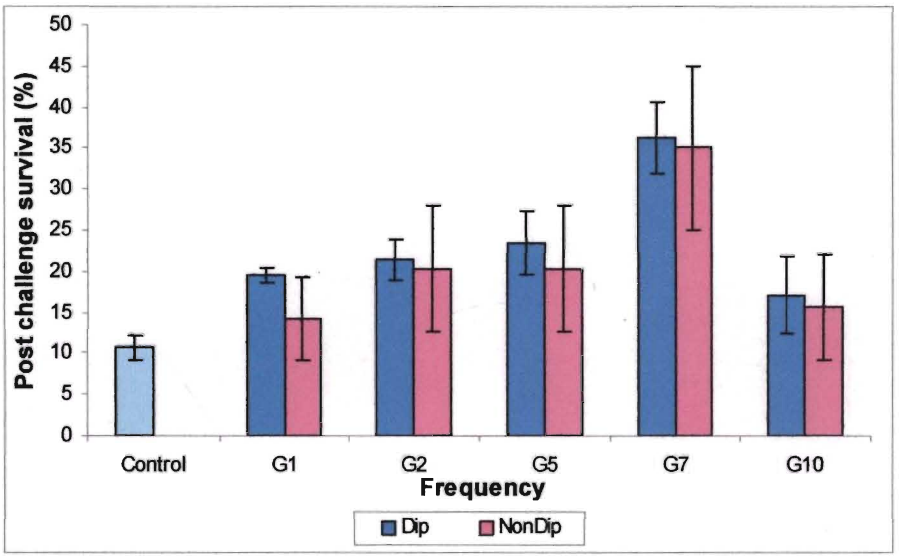
Fig 6.4. Post challenge survival (on 7th day) in *F. indicus* post larvae on challenge with WSSV after 15th day of dip treatment with different concentrations of yeast glucan solution.



Glucan (mg/ml)	Post challenge survival (%)*
Control	4 ± 5.48[a]
0.1mg/ml	4 ± 5.48 [a]
0.5mg/ml	6 ± 5.48 [a]
1mg/ml	6 ± 5.48 [a]
2mg/ml	8 ± 4.47 [a]

* Values with same superscript dose not vary significantly (P<0.05)

Fig 6.5. Post challenge survival in (on 7th day) *F. indicus* post larvae on challenge with WSSV after 20th day of dip treatment with different concentrations of yeast glucan solution.



Feed**	Post challenge survival (%)*	
	Dip	Non-Dip
Control	10.68 ± 1.47 [a]	10.68 ± 1.47 [a]
G1	19.55 ± 0.91 [b]	14.16 ± 5 [b]
G2	21.25 ± 2.5 [b]	20.28 ± 7.72 [b]
G5	23.38 ± 3.87 [b]	20.28 ± 7.72 [b]
G7	36.25 ± 4.33 [c]	35.00 ± 10 [c]
G10	17.05 ± 4.77 [b]	15.56 ± 6.48 [b]

* Values with same superscript dose not vary significantly (P<0.05)

** Frequency of glucan feeding : G1 – Daily Glucan feeding, G2 –Once in 2 days, G5- Once in 5 days, G 7- Once in seven days, G10- Once in 10 days

Fig 6.6. Post challenge survival in *F. indicus* post larvae (glucan dip treated and non-dip treated) when fed with 0.2% glucan diet at different frequencies and challenged with WSSV.

Chapter 7

Immunological profile of *Fenneropenaeus indicus* on administration of (1→3)-β-D-glucan of *Candida sake via* diet

7.1. Introduction

In recent years there have been conscious efforts to study and unravel the defence mechanisms in shrimps mainly because of its economic importance in aquaculture sector. The industry faces a serious threat from infectious and non-infectious diseases. This situation has grown worse with intensification of shrimp aquaculture, which gave less attention to shrimp physiology and its ecology. The causative agents of infectious disease in shrimps are mainly viruses and bacteria.

Bacterial infection of shrimp with *Vibrio* sp. is often related to injury, stress or disease caused by other pathogens (van de Braak *et al.*, 2002). Vibriosis particularly affects larval production in hatcheries whereas viruses are widespread in culture ponds and are responsible for mass mortalities and great economic losses. Of these the white spot disease virus (WSDV) of Asian countries gained much attention since they cause 100% mortalities within 2-10 days from the onset of infection (Chou *et al.*, 1995; Chang *et al.*, 1999). Strategies for prophylaxis and control of white spot virus include improvement of environmental condition, stocking disease resistant strains and use of probiotics and immunostimulants. Cell wall glucan obtained from yeast has been proved to be a good immunostimulant (Sung *et al.*, 1994, Chang *et al.*, 1999). Schizophyllum, a water-soluble β-1,3-glucan with some β-1,6-glucosidic side chain, obtained from *Schizophyllum commune* increases the resistance of *M. japonicus* against vibrio infection (Itami *et al.*, 1994). Enhancement of vibriosis resistance in *Penaeus vannamei* by supplementation of diets with yeast products has been reported by Scholz *et al.* (1999). Chang *et al.* (2003) reported that dietary β-1,3-glucan derived from *Schizophyllum commune* effectively improved immune profiles like phenoloxidase level and NBT activity and survival of *P. monodon* against white spot virus infection. In this chapter the glucan from *Candida sake* S165

was administered to adult *F. indicus* and the immunological parameters of the haemolymph of the animals were tested.

7.2.Materials and methods

7.2.1.Experimental diet

Cell wall β-Glucan was extracted from marine yeast *Candida sake* S165 as per the protocol described earlier (Section 5.2.1). The glucan was dried in vacuum, homogenized and coated on a standard diet (Table 2.3) using a commercially available binder 'BINDEX'. The final glucan concentration in the feed was 0.2%. Similarly a control feed was also prepared but without glucan addition. The feeds were dried at 50°C for 4 hrs and kept at -20°C in a freezer until use.

7.2.2. Experimental animals

A batch of apparently healthy adult *F. indicus* (mean body weight 23.5 ± 0.6g) were brought to the laboratory of School of Marine Sciences from a shrimp farm located at Kannamali, Cochin. The shrimps were randomly divided into four groups of 60 shrimps each into aquarium tank of 500 L capacity (Fig 4.1) and acclimatised to laboratory conditions for one week prior to experiment.

7.2.3. Feeding experiment

Among the four groups, first group received the basal diet without glucan and was considered as the control. The other groups were maintained on 0.2% glucan incorporated diets each of them with specific feeding frequencies. The second group fed with 0.2% glucan incorporated diet daily, third group once in seven days and the fourth group once in ten days. The rest of the days all test groups received basal control diet devoid of glucan. Feeding was done twice daily at 8 A.M and 7 P.M, at a rate of 10-15 % of total biomass in each treatment group. 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 (APHA, 1995) and maintained at

optimal level, by water exchange. The total culture period was 40 days. On 40th day all the animals were challenged with WSSV by oral administration of white spot virus infected prawn flesh. In the following days the animals were maintained on same diet as prior to challenge and, the haematological parameters were assayed at frequent intervals.

7.2.4.Assay of immunological parameters.

7.2.4.1.Collection of haemolymph

Haemolymph was collected from the rostral sinus as described earlier (4.2.5.1). The haemolymph samplings were carried out at the beginning of the feeding experiment (0 day/base line), day 40, besides post challenge day 1 (PCD1), post challenge day 2 (PCD2) and post challenge day 3 (PCD3). Samples were diluted three folds using shrimp salt solution (Vargas-Albores and Ochoa, 1992) and analysed individually without pooling together.

7.2.4.2. Total haemocyte count

Total haemocyte count (THC) was measured by using a Neubauer improved haemocytometer and expressed as THC per ml haemolymph.

7.2.4.3. Phenoloxidase (PO) activity

Phenoloxidase activity of haemolymph was measured spectrophotometrically by using L-3, 4-dihydroxyphenylalanine (L-DOPA) as substrate according to Soderhall (1981). 100µl of haemolymph was mixed with 100µl of 1% SDS for three minutes at 25°C. Then 1 ml of L-DOPA was added to the haemolymph. Increase in absorbance at 495 nm was measured for 3 min at an interval of 30 sec. using a UV-Visible Spectrophotometer (Hitachi U-2001). L-DOPA with distilled water was used as blank. Enzyme activity was expressed as increase in absorbance per minute per 100µl haemolymph.

7.2.4.4.Superoxide anion (NBT reduction) assay

Respiratory burst activity of haemocytes was measured spectrophotometrically as per the method described by Song and Hsieh (1994) with minor modifications. Nitro blue tetrazolium (NBT, SRL Chemicals, India) was used as substrate that gives a blue formazan colour due to its reduction by O_2^- produced during phagocytosis of heamocytes. 100 µl of haemolymph was taken into a microcentrifuge tube precoated with 0.2% poly- L-lysine (Sigma). Poly-L-lysine coating increases the haemocyte adhesion to the microcentrifuge tube. 100 µl NBT solution (2 mg/ml) prepared in Tris-HCl buffer (pH 7.6) was added to the haemolymph and incubated at room temperature for 30 min. Tubes were centrifuged at 300x g for 10 min. in a cooling centrifuge. Discarded the supernatant and stopped the reaction by adding 1ml absolute methanol followed by incubation for 10 min. Spun the tubes again, discarded the supernatant and left the tubes for air-drying for 30 min. The tubes were washed with 50% methanol for three times and a final washing was done using PBS of pH 7.6. 2M KOH (120 µl) followed by 140 µl dimethylsulphoxide (DMSO, SRL Chemicals) were added to the tubes. Finally 2ml distilled water was added. The optical density at 620nm was recorded by using UV-Visible Spectrophotometer (Hitachi. U-2001) against a blank consisting of reagents (KOH and DMSO) and 2 ml distilled water and expressed as NBT activity per 100µl haemolymph.

7.2.5.Statistical analysis

In order to determine significant differences if any, in immunological parameters between the different treatment groups the results were analyzed by one way analysis of Variance (ANOVA) and Duncan's multiple comparison of the means by using SPSS 10.0 for windows. Significant differences were indicated at $p<0.05$.

7.3. Results

7.3.1.Total haemocyte count (THC)

Total haemocyte count was found to be significantly high for the group fed with glucan once in seven days when compared to all other groups. Animals, which were fed with glucan daily or once in seven days did not show, marked variation with the control before or after challenge with white spot virus. Generally haemocyte count was found to be maximum on day 3 post challenge compared to all other days (Fig 7.1 and Table 7.1 of appendix). Uniform decrease in THC could be observed on post challenge day 1 for all treatment groups followed by an elevation in count culminating in the maximum on post challenge day 3. Variation in the haemocyte count was not found to be significantly different except in the case of once in seven days fed group.

7.3.2.Phenoloxidase activity

The phenoloxidase value was significantly higher in shrimps fed with glucan-incorporated feed once in seven days (Fig 7.2 and Table 7.2 of appendix) compared to all other groups. The PO level of this group was found to be significantly different from all other groups on post challenge day 3. Also the PO values of group III on 40^{th} day, PCD1 and PCD2 were found to differ from both control and group IV significantly. Maximum value for PO was recorded on 3^{rd} day of post challenge (0.921± 0.215) in the treatment group III. Generally a post challenge decrease in PO activity could be witnessed in all the treatment groups followed by a gradual increase showing the maximum activity on PCD3.

7.3.3.NBT reduction assay

NBT values were also significantly higher in shrimps fed with glucan diets once in seven days. A gradual increase in NBT values could be observed during the post infection period recording a maximum on PCD 3 for all treatment groups (Fig 7.3 and Table 7.3 of appendix). It is observed that

NBT did not show a decrease on post challenge day 1 as found in the case of other immunological parameters.

7.4. Discussion

Currently many commercial products are available in shrimp aquaculture under the label of immunostimulants and are extensively used by the shrimp farmers. But the nature of these compounds, the dose required for eliciting immuno-stimulation at optimum level, duration of protection conferred, route of application etc. are still uncertain. These lacunae in the scientific knowledge pertaining to such practices necessitate a comprehensive study on products before they are launched into the market.

Immunostimulants should be used as marginal aids for health rather than as substitute for good husbandry practice. Hence the application of immunostimulants either through feed or immersion should be under professional control based on the proper documentation like formulation, dose, application time, and the target organism.

In the present study the total haemocyte count on the 40th day in the case of daily glucan fed group showed a reduction in THC. Administration of higher dose of glucan might have caused the reduction in total haemocyte count by excessive degranulation making the host organism susceptible to infection during subsequent infection. Host homeostasis seems to be impaired due to this high concentration of glucan, a condition very similar to an acute infection by a pathogen leading to the reduction in circulating haemocytes. In such situations the mechanism to restore the haemocyte number probably by mobilizing the haemocytes from the reservoirs in haemal crypts or upregulation in cell division in the in haematopoietic tissue and proliferation within the haemolymph take place (Ghiretti-Magaldi *et al.*, 1977; Hose *et al.*, 1992). Recently Soderhall *et al.* (2003) reported that injection of β1,3-glucan into the haemocoel of a crustacean results in an accelerated maturation of haemocyte precursors in the haematopoietic tissue followed by release into

the circulation of new cells. Whereas in the case of a chronic long term application of glucan for 40 days or so might have worn out the reserve cells and the haematopoietic tissue may become 'stressed' due to uncontrolled induction for a longer period. Moreover it is not clear to what extent the new cell produced in this way is immunologically mature or competent and how long it will take for the complete restoration of immune equilibrium (Smith *et al*, 2003).

In the present study PO level showed an all time high value for group III, the one, which received glucan, feed once in seven days. After 40 days feeding the PO value of this group was 0.785 ± 0.251, while the base line value (ie. at the beginning of feeding experiment) was only 0.240 ± 0.04. This elevation of PO indicates an increase in immune status of the animals due to glucan administration. This also holds true to certain extent for group II and group IV, which received glucan feed daily and once in 10 days respectively. The peak activity of PO was recorded in group III, on the 3rd day of post challenge (0.921 ± 0.215) while that of group I was only 0.599 ± 0.222, which was very similar to the control (0.528±0.056).

The reduced value of PO for group IV vindicates the requirements of higher frequency of glucan administration. The decreased level of PO for the group II when compared to group III may be due to the over stimulation of immune system during the 40 days culture period with 0.2 % glucan feed. This over stimulation resulting in the exhaustion of the immune system might be the reason for low PO values during the experimental infection with WSSV. The present observations were in corroboration with the findings of Chang *et al.* (2000), who reported a reduced immunity in *P. monodon* after continuous feeding of glucan at a concentration of 2g kg^{-1} in feed for 40 days. Study of Soderhall and Cerenius (1998) highlights this fact that invertebrates can recognize and respond to picograms per litre of lipopolysaccharides or peptidoglycans from bacteria and β-1,3-glucan from fungi. Recently Hauton and Smith (2004) reported that β-1,3-glucan when used as an

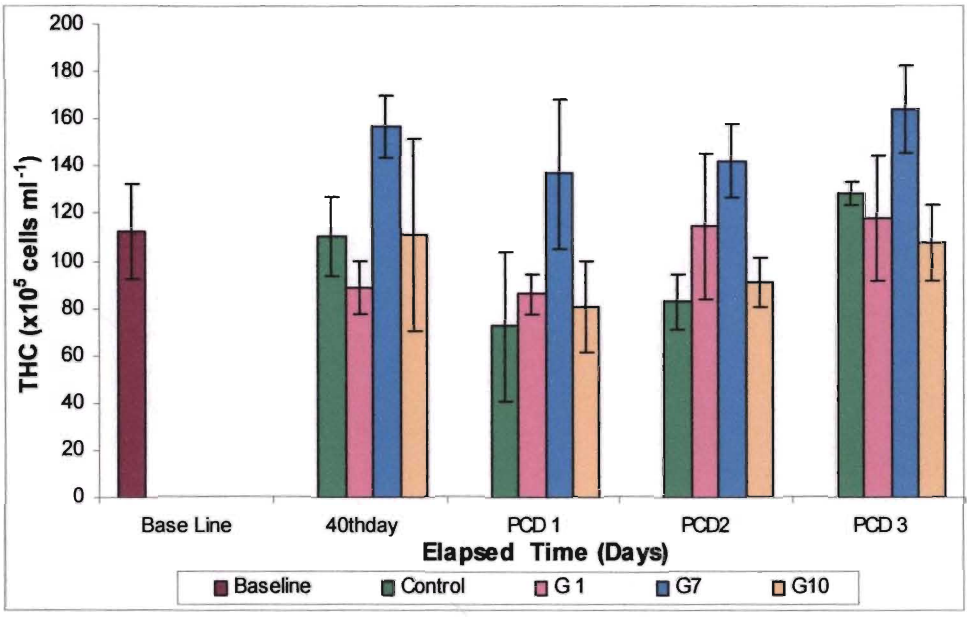
immunostimulant at a dose of 0.005% was shown to be toxic to granulocytes of *Homarus gammarus*.

Nitro blue tetrazolium (NBT) staining has been used for both quantitative and qualitative analysis of superoxide anion production. Respiratory burst by the haemocytes of *P. monodon* has been correlated to its phagocytic activity (Song and Hsieh, 1994). In the present study the baseline superoxide anion production was found to be 0.290 ± 0.068. After 40 days glucan administration NBT value increased to 0.932± 0.249 in group III, a three fold increase from baseline. This shows an enhanced O_2^- production on feeding glucan diet for 40 days, at an interval of once in seven days. But for group II (daily glucan feeding), the NBT value (0.365± 0.103) was almost similar to the control group (0.310± 0.065). Chang *et al.* (2000) reported that a long-term administration of dietary glucan to *P. monodon* at a concentration of 2g kg^{-1} for 40 days or so reduced the superoxide anion production by haemocytes from the 24^{th} day of feeding onwards. Robertsen *et al.* (1994) reported that the increase in respiratory burst activity of glucan treated fish macrophage was maximal at glucan concentration of 0.1-1 µg/ml, whereas at 10 µg/ml showed no effect and at 50 µg/ml glucan was inhibitory. These results are in agreement with the finding of Floch *et al.* (1987) who states that immunostimulant do not show a linear dose/effect relationship instead they most often show a distinct maximum at a certain intermediate concentration and high dosage may end up with no result or even adverse effect on the animal.

Group III, which received glucan, feed once in a week showed a higher activity for NBT throughout the experiment compared to the group maintained on glucan diet daily. This may be due to the overdose of glucan, which might have resulted host tissue injury and stress to the animals due to over production of superoxide anions. A similar observation was made by Yoshida *et al.* (1995) who reported that the number of NBT-positive cells in African catfish decreased due to prolonged use of glucan in feed. In the

present work the group IV that received glucan feed once in 10 days for a period of 40 days also failed to elicit a good response. This can be attributed to the sub-optimal level of glucan received by this group during the 40 days culture period. Studies by Robertsen (1990) and Matsuyama *et al.* (1992) reported that low dose of β-1,3 glucans (2 to 10 mg /kg) injected into salmon gave better disease resistance after 7 days, whereas a high concentration (100 mg/kg) gave no protection after 3 to 4 weeks. Rainbow trout treated with peptidoglycan orally for 56 days did not show protection after challenge with *V. anguillarum*, although fish treated for 28 days showed increased protection (Matsuo and Miyazono 1993).

The study reveals that optimal frequency of administration of glucan is once in seven days and over administration results in immune fatigue of the animals. Therefore dose and frequency of administration is of paramount importance in immunostimulation. These parameters are to be optimized for every compound with respect to the host animal in order to save cost and crop. Indiscriminate usage of immunostimulant without knowing the active ingredient, proper dosage and frequency could never be an effective management strategy, instead may witness a minimum protection to the animals in case of infection, leading to mass mortality in spite of the money and effort expended.



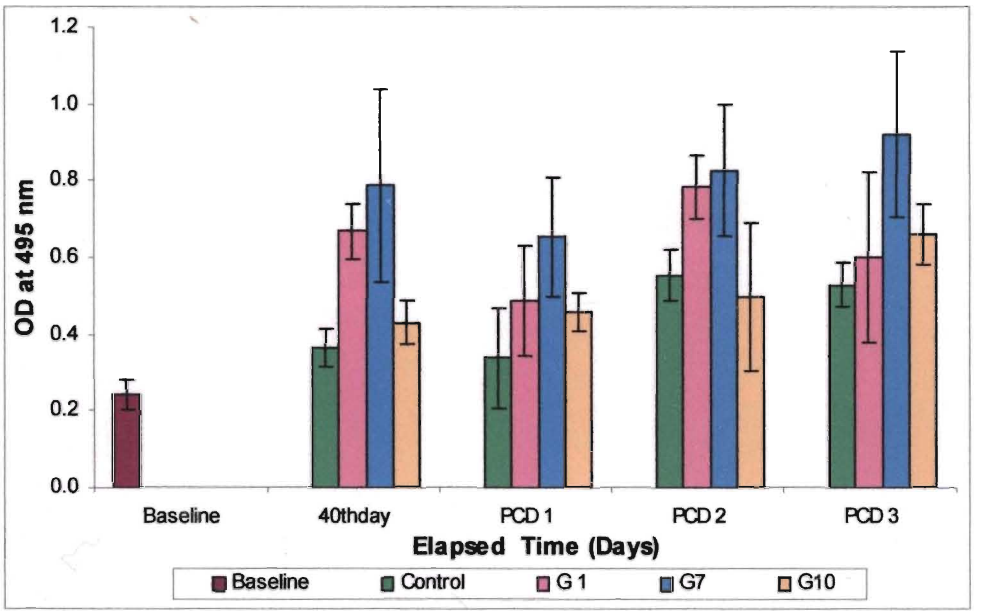
Feed	THC (x10^5 cells ml^{-1})*				
	Base line	40thday	PCD 1	PCD 2	PCD 3
Control	112.03±19.87^a	109.97±16.51^a	72.26±31.60^a	82.65±11.74^a	128.50±4.75^a
G 1	112.03±19.87^a	88.45±11.11^a	85.87±8.34^a	114.53±30.52^{ab}	117.95±26.49^a
G7	112.03±19.87^a	156.67±12.86^b	137.00±31.51^b	142.00±15.39^b	164.20±18.40^b
G10	112.03±19.87^a	110.90±40.65^a	80.40±19.31^a	90.85±10.36^a	107.56±15.66^a

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

PCD 1- Post challenge Day 1, PCD 2- Post challenge Day 2, PCD 3- Post challenge Day 3

G1- Glucan diet daily, G7- Glucan diet once in seven days , G10- Glucan diet once in ten days

Fig 7.1. Mean (± S.D) THC of *F. indicus* fed on diets containing glucan at different frequencies for 40 days and then challenged with WSSV.



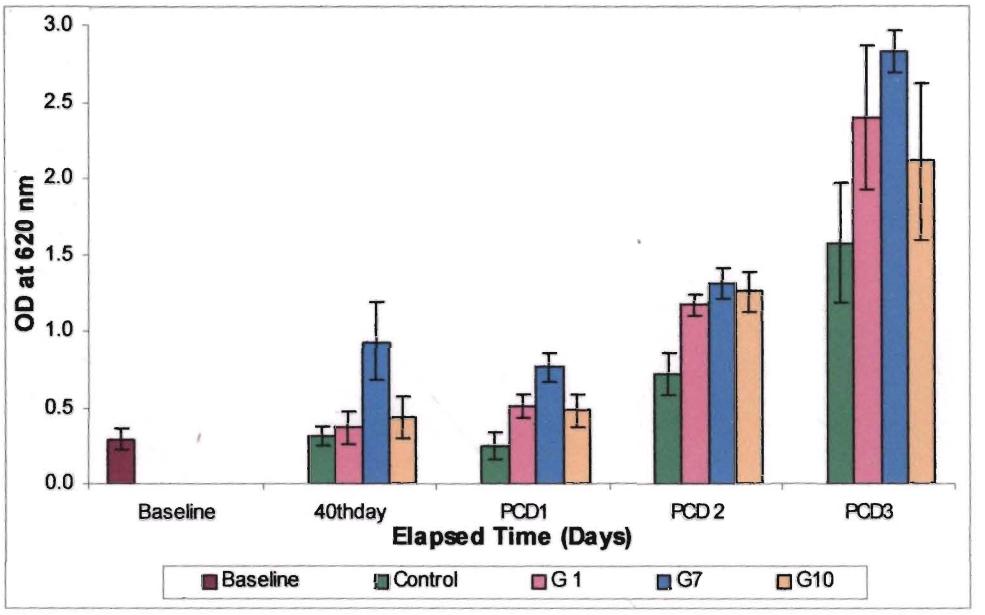
Feed	Phenoloxidase activity (Increase in OD at 495 nm min^{-1}/100µl Haemolymph				
	Baseline	40thday	PCD 1	PCD 2	PCD 3
Control	0.240 ± 0.04^{a}	0.363 ± 0.050^{a}	0.338 ± 0.130^{a}	0.552 ± 0.066^{a}	0.528 ± 0.056^{a}
G 1	0.240 ± 0.04^{a}	$0.667 \pm 0.072b^{c}$	0.486 ± 0.143^{ab}	0.783 ± 0.084^{bc}	0.599 ± 0.222^{a}
G7	0.240 ± 0.04^{a}	0.785 ± 0.251^{c}	0.654 ± 0.155^{b}	0.825 ± 0.172^{c}	0.921 ± 0.215^{b}
G10	0.240 ± 0.04^{a}	0.430 ± 0.057^{ab}	0.457 ± 0.048^{ab}	0.496 ± 0.194^{ab}	0.660 ± 0.079^{ab}

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

PCD 1- Post challenge Day 1, PCD 2- Post challenge Day 2, PCD 3- Post challenge Day 3

G1- Glucan diet daily, G7- Glucan diet once in seven days , G10- Glucan diet once in ten days

Fig. 7.2. Mean (± S.D) phenoloxidase (PO) value of *F. indicus* fed on diets containing glucan at different frequencies for 40 days and then challenged with WSSV.



Feed	NBT activity (OD at 495 nm/100µl Haemolymph)				
	Baseline	40thday	PCD 1	PCD 2	PCD 3
Control	0.290 ± 0.068 [a]	0.310± 0.065 [a]	0.244± 0.084 [a]	0.717± 0.132 [a]	1.573± 0.393 [a]
G 1	0.290 ± 0.068 [a]	0.365± 0.103 [a]	0.501± 0.074 [b]	1.167± 0.063 [bc]	2.394± 0.467 [b]
G7	0.290 ± 0.068 [a]	0.932± 0.249 [b]	0.760± 0.091 [b]	1.308± 0.098 [c]	2.827± 0.140 [b]
G10	0.290 ± 0.068 [a]	0.432± 0.132 [a]	0.480± 0.105 [b]	1.254± 0.134 [b]	2.108± 0.515 [ab]

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

PCD 1- Post challenge Day 1, PCD 2- Post challenge Day 2, PCD 3- Post challenge Day 3

G1- Glucan diet daily, G7- Glucan diet once in seven days , G10- Glucan diet once in ten days

Fig. 7.3. Mean (± S.D) NBT value of *F. indicus* fed on diets containing glucan at different frequencies for 40 days and then challenged with WSSV

Chapter 8

Comparative efficacy of whole cell marine yeast *Candida sake* and its cell wall component (1→3)-β-D-glucan as immunostimulants to *Fenneropenaeus indicus*

8.1. Introduction

The occurrence of infectious diseases in shrimp culture has reduced production levels during the last decade. The increase in production in the areas that have recovered from diseases has not been able to compensate for the decrease in production in the areas that are still suffering from disease outbreaks. The prevention and control of diseases are now considered priorities for shrimp aquaculture in the vast majority of the shrimp producing countries. Use of probiotic bacteria, based on the principle of competitive exclusion, and the use of immunostimulants are two of the most promising preventive methods developed in the fight against disease during the last few years (Raa, 1996; Gatesoupe, 1999). Bacteria that have been used successfully as probiotic belongs to the genus *Vibrio* (Griffith, 1995; Garriques and Arevalo, 1995) and *Bacillus* spp. (Moriarty, 1998; Rengpipat *et al.*, 1998). Several mechanisms have been suggested as modes of action for probiotic bacteria. The competitive exclusion mechanism, based of the substitution of the pathogen by the beneficial population, has been considered to be important by many authors (Fuller, 1992; Moriarty, 1998; 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. Also, stimulation of the immune system using probiotic strains has been reported by Rengpipat *et al.* (2000) and Gullian *et al.* (2003).

Immunostimulation is an alternative strategy to alert the shrimp defence system increasing the resistance against pathogenic agents (Rodriquez and Le Moullac, 2000). In shrimp, several microbial compounds have been reported as the main stimulants of cellular functions, such as β-glucan, lipopolysaccharides and peptidoglycans (Sung *et al.*, 1994; Song *et al.*, 1997; Chang *et al.*, 1999, 2000, 2003). These compounds have been researched to evaluate the usefulness of their supplementation against vibrios and White spot syndrome virus (Smith *et al.*, 2003).

Oral administration of Schizophyllum, a β-glucan derived from *Schizophyllum commune* at an optimal level of 10g kg^{-1} diet for 20 days effectively enhanced the immunity indices such as total haemocyte count, phenoloxidase, superoxide anion production and superoxide dismutase activity of *P. monodon* (Chang *et al.*, 2003). Chotigeat *et al.* (2004) reported that administration of fucoidan extracted from *Sargassum polycystum* to *P. monodon* increases the haemolymph antibacterial activity and phagocytic potential of experimental animals. Selvin *et al.* (2004) observed the immunomodulatory potential of selected marine secondary metabolites in *Penaeus monodon*. Sodium alginate, consisting of β-1,4-D-mannuronate and α-1,4-L-guluronate, obtained from giant kelp *Macrocystis pyrifera* has been reported to increase the phenoloxidase activity and respiratory burst activity of *Litopenaeus vannamei* (Cheng *et al.*, 2004). In this chapter the comparative efficacy of whole cell marine yeast *Candida sake* S165, its cell wall component β-glucan and cell wall β-glucan from a baker's yeast *Saccharomyces cerevisiae* MTCC36 as immunostimulants to *Fenneropenaeus indicus* was tested.

8.2. Materials and methods

8.2.1.Experimental diet

Based on the experiments carried out in section 4.2 and 7.2, 10% *C. sake* S165 incorporated feed and 0.2% glucan (*C. sake* S165) incorporated feed another of experimental feed containing 0.2% glucan of *S. cerevisiae* MTCC36 were used for a comparative study. A feed without yeast or glucan was used as control diet.

8.2.2.Experimental animals

Healthy adult *F. indicus* (mean body weight 20 ± 2g) were used for the study. The animals were brought from a shrimp farm located at Kannamali, Cochin. The shrimps were randomly divided into four groups of 60 shrimps each into

aquarium tank of 500L capacity (Fig 4.1) and acclimatised to laboratory conditions for one week prior to experiment.

8.2.3.Feeding trials

Of the four groups of test animals, the first group was fed with the control diet, the second group with 10% yeast *C. sake* S165 incorporated diet, third group with *C. sake* S165 glucan incorporated diet and the fourth group with *S. cerevisiae* MTCC 36 glucan incorporated diet. The feeding frequency of the glucan diets were fixed as once in seven days and during rest of the days control feed was given. Feeding was done twice daily at 8 A.M and 7 P.M, at a rate of 10-15 % of total biomass in each treatment group. 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 (APHA, 1995) and maintained at optimal level by water exchange. The total culture period was extended up to 40 days. On 40^{th} day of experiment all the animals were challenged with WSSV by oral administration of white spot virus infected prawn flesh. During post challenge days the animals were maintained on corresponding diets. The haematological parameters were assayed at definite intervals and the post challenge survival rate was also noted.

8.2.4.Assay of immunological parameters.

8.2.4.1.Collection of haemolymph

Immunological assays were performed using experimental animals to study the comparative efficacy of immunostimulants. Haemolymph was collected as described elsewhere (section 4.2.5.1). The haemolymph samplings were done at the beginning of the feeding experiment (0 day/base line) and day 40, besides post challenge day 1 (PCD1), post challenge day 2 (PCD2), post challenge day 3 (PCD3), post challenge day 5 (PCD5), post challenge day 7 (PCD7) and post challenge day 10 (PCD10). Samples were diluted to three

folds by using shrimp salt solution (Vargas-Albores and Ochoa, 1992) and analysed individually without pooling together.

8.2.4.2.Total haemocyte count

Total haemocyte count was measured by using a Neubauer improved haemocytometer and expressed as THC per ml haemolymph as described in Section 4.2.5.2.

8.2.4.3. Phenoloxidase (PO) activity

Phenoloxidase activity of haemolymph was measured spectrophotometrically as per section 4.2.5.3

8.2.4.4. Superoxide anion (NBT reduction) assay

Respiratory burst activity of haemocytes was measured spectrophotometrically as per section 4.2.5.4

8.2.4.5.Peroxidase assay

The peroxidase activity was measured by the method of Reddy *et al.* (1981). Pipetted out 3.0 ml of 0.05 M pyrogallol solution and 0.1 ml of haemolymph in a test tube. Adjusted the spectrophotometer to read '0' at 430 nm. Added 0.5 ml of 1 % H_2O_2 in a test cuvette. Mixed and recorded the change in absorbance for every 30 seconds up to 3 min. The difference in OD change per minute with and without haemolymph addition was measured as peroxidase activity. The activity is expressed in terms of increase in OD at 430 nm.

8.2.5. Statistical analysis

In order to determine significant difference if any, in immunological parameters between the different treatment groups the results were analysed using one way analysis of variance (ANOVA) and Duncan's

multiple comparison of the means by using SPSS 10.0 for windows. Significant differences were indicated at $p<0.05$.

8.3. Results

Total haemocyte count was maximum in prawn fed with whole cell yeast diet during entire experimental period, followed by glucan of *C. sake* S165 fed group and *S. cerevisiae* MTCC36 glucan fed groups (Fig 8.1 and Table 8.1 of appendix). On 40th day of feeding experiment the whole cell yeast fed group showed a THC of 145.91 x 10^5 ± 6.64 cells ml^{-1}, which was significantly different from control group on the same day ($P<0.05$). The haemocyte count was found to be maximum on PCD 3 for all treatment groups followed by PCD2 with the count almost at the same level. However the number decreased slowly and assumed the same level prior to that of infection.

Phenoloxidase activity was also uniformly higher in shrimps fed with whole cell yeast diet. After 40 days of feeding, the yeast fed group showed a value of 0.860±0.186 at OD 495 which was significantly different ($P<0.05$) from the control. PO value was maximum on post challenge day 3 followed by a decrease during subsequent days (Fig 8.2 and Table 8. 2 of appendix). The maximum performance exhibited by whole cell yeast was followed by *C. sake* S165–glucan and *S. cerevisiae* MTCC36-glucan.

Through out the experiment NBT activity in whole cell yeast fed group was significantly higher than all other test groups. NBT response in yeast fed group attained the highest level 6.373±1.821 at OD 630nm on third day of post challenge. The value showed a decreasing trend thereafter (Fig 8.3 and Table 8. 3 of appendix). The other three groups also showed similar pattern, where glucan of *C. sake* S165 fed group showed higher activity than that fed with glucan from *S. cerevisiae* MTCC36. On post challenge day 7 and 10 the NBT level reduced to that observed prior to WSSV infection.

As in other immunological parameters, peroxidase level also showed a higher value in yeast fed groups. The maximum activity was recorded on post challenge day 2 where the yeast fed group showed a value of 2.289 ± 0.381 at OD 435 nm which was followed by *C. sake* S165-glcaun fed group and *S. cerevisiae* MTCC36 fed group (Fig 8.4 and Table 8. 4 of appendix). No significant difference in the performance could be observed in terms of peroxidase activity on post challenge day 2 and day 3. On post challenge day 10 the peroxidase value in all groups dropped, almost in level with the baseline value.

Post challenge survival against WSSV challenge was recorded till 10^{th} day and yeast fed group showed better survival (68%), which was followed by groups fed with glucan of *C. sake* S165 (32%) (Fig 8.5). The group fed with glucan of *S. cerevisiae* MTCC36 showed a survival of 15.39% and the control 4.77%.

8.4. Discussion

Enhancement of disease resistance in shrimp was reported after oral administration of yeast or yeast products (Scholz *et al.*, 1999; Burgents *et al.*, 2004). Reports on enhanced survival and evidence of immunostimulation due to purified cell wall components like β-glucan and LPS are more numerous than for yeast (Itami *et al.*, 1998). In the present experiment the immunostimulatory potential of whole cell yeast *C. sake* S165 was compared with two other glucan incorporated feeds (*C. sake* S165- glucan, *S. cerevisiae* MTCC36–glucan). Of these three test feeds whole cell yeast fed group showed better immunostimulation during the feeding experiment both before and WSSV challenge. This was evident in the immunity profile of the animals tested. *C. sake* S165-glucan was second in order of performance followed by *S. cerevisiae* MTCC36-glucan diet and the control feed.

The total haemocyte count in the group fed with whole cell yeast was higher than that of all other experimental feeds including control. Even though there occurs a marginal drop in total haemocyte count after WSSV infection the whole cell yeast fed group maintained a higher cell count till the end of 10^{th} day post challenge. The animals fed with diet containing 0.2% glucan of *C. sake* showed better haemocyte count than groups fed with diet containing glucan of *S. cerevisiae*. The phenoloxidase level also showed a similar pattern where yeast fed group showed better value both before and after challenge with WSSV. NBT value also reflected the same pattern, where the maximum was recorded on third day post challenge. Throughout the experimental period NBT value was maximum with yeast incorporated diet. The peroxidase value was found to be maximum on the 2^{nd} day of post challenge in various treatment groups and the yeast fed group showed the maximum. Peroxidase enzyme is associated with cellular defence reactions in white blood cells of vertebrates and invertebrates. Hydrogen peroxide can be converted to hypochlorus acid *via* the myeloperoxidase system (Harrison and Shultz, 1976), which plays an important role in the antimicrobial activity of the phagocytic cells (Odell and Segal, 1988).

Post challenge survival was maximum for yeast fed groups and found to be significantly different ($p<0.05$) from all other groups. *C. sake* S165-glucan fed group ranked second whereas the *S. cerevisiae* MTCC36-glucan fed group showed comparatively lesser survival. Higher immunity indices and post challenge survival in whole cell yeast fed group compared to animals fed with glucans of same yeast shows that not only the glucan but also the other cellular constituents of the yeast imparts immunostimulatory property in shrimps. Microalgae, bacteria and yeast are frequently used as feed ingredients for fish and shellfishes as they are rich in proteins, vitamins, pigments and complex carbohydrates such as glucans (Tacon, 1994), wherein yeast gained more importance.

Nucleotide contents of yeast are of recent interest in fish nutrition as an immunostimulant (Li *et al.*, 2004; Li and Gatlin, 2005). Exogenous nucleotide may be involved in cell signalling pathways as well besides serving as nutrients for biosynthesis. Certain genes of immune system of fishes and shellfishes were induced to express by nucleotides (Low *et al.*, 2003; Chuo *et al.*, 2005).

The extraction of cell wall glucan from yeast requires harsh chemical and physical treatment with alkali and acid. During this procedure most of the nutrients from yeast cell wall may drain out leaving the particulate cell wall glucan as the main product. The better performance of yeast diets compared to the glucan diet could be due to the presence of nucleotides and other cellular component in the former. Scholz *et al.* (1999) compared the immune response of *P. vannamei* fed with yeast *Phaffia rhodozyma* and yeast glucan from *S. cerevisiae* and showed that *Phaffia rhodozyma* incorporated feed showed better performance. They are of the opinion that presence of carotenoid pigments in *P. rhodozyma* might have caused an over all increase in the performance of the animals. Carotenoid pigments are receiving increased attention in shrimp nutrition and is playing an important role in the physiology and over all improvement in the health of animals (Torrissen,1990). The present finding is in agreement with the earlier works by Torrissen (1990) and Scholz *et al.* (1999). Scholz *et al.* (1999) reported that a diet containing 0.1% glucan over 7 weeks significantly reduced the ability of shrimp to clear live *Vibrio harveyi* from the haemolymph when compared to control and to animals reared on diet containing 1% yeast *S. cerevisiae*. A few workers have reported the ability of live microbial culture in enhancing the disease resistance of shrimp. Rengpipat *et al.* (2000) reported greater immunity indices, (total haemocyte count, phagocytic activity, phenoloxidase level and serum antibacterial activity) in *Penaeus monodon* fed with live bacterium *Bacillus* S II. The authors speculate that *Bacillus* S II 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.

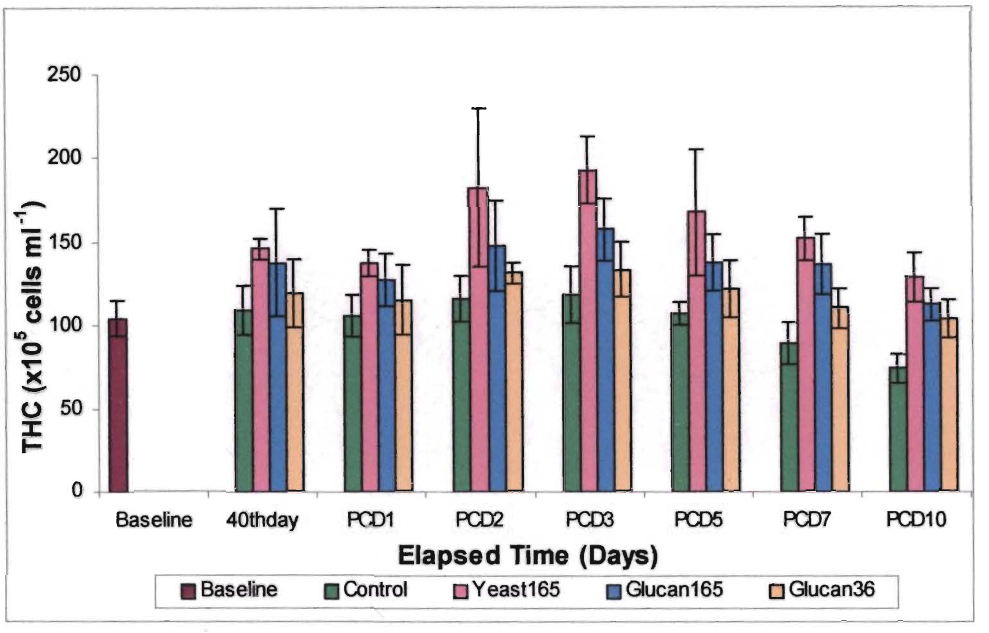
A similar study was done by Gullian *et al.* (2003) in which immunostimulatory effect of a probiotic bacteria *Bacillus* sp was reported in *P. vannamei* whereas another probiotic bacteria *Vibrio* P62 fails to produce any immunostimulatory effect. Alavandi *et al.* (2004) reported that two bacterial strains *Pseudomonas* sp and *Vibrio fluvialis* showed deprived immunity index in *P. monodon* in spite of their probiotic nature. The authors emphasise the need for a new selection protocol incorporating immunostimulation potential for identifying and employing probiotics for penaeid cultural systems.

Sorensen *et al.*(1998) reported that the cell wall preparations from yeasts *Pichia fabianii, Rhodotorula glutinis, Cryptococcus laurentii, Candida sake* and *Trichosporon capitatum* showed enhanced immune response in rat alveolar macrophage, but the magnitude of the response varied with species.

The cell wall preparation of *C. sake* S165 showed better immunostimulation than that of *S. cerevisiae* in the present investigation. The probable explanation for this difference could be the difference in the structure of cell wall glucan of the two strains of yeasts. So far numerous studies have revealed that the molecular weight (MW) and water solubility of beta glucan, which depend on the degree of branching (DB) and the chemical modifications were the critical properties in determining their use as immunostimulants (Williams *et al.*, 1992). So the structure–activity relationship of the glucans are worth studying in view of the difference in their immunostimulant potential in shrimp. The NMR spectra obtained for the two glucan samples, *C. sake* S 165 and *S. cerevisiae* (Fig 5.2 to 5.4) indicates the presence of mannan in the cell wall preparation of *C. sake*, but it was absent in the case of *S. cerevisiae* preparation. The cell wall

components like β-glucan, LPS and mannan of the yeast and other microorganisms are recognised by proteins, generally called pattern recognising proteins (PRP) present in the haemolymph of crustaceans (Soderhall and Thornqvist, 1997). Carbohydrate specific lectins were reported from arthropod haemolymph, which include β-glucan binding proteins (BGBP), mannan binding proteins (MBP), etc (Marques and Barracco, 2000). These lectins upon binding with corresponding carbohydrate will activate the proPO system of animals. The cell wall preparation of *C. sake* S165 containing both glucan and mannan, might have contributed to better immunostimulation in penaeid shrimps compare to cell wall preparation from *S. cerevisiae* MTCC36, which contained glucan alone.

Incorporation of yeast biomass as such in feed contributes both to nutrition and immunostimulation whereas glucans contribute only to immuno-stimulation. The present study shows that better immunostimulation as well as protection could be effected in prawns with yeast diets compared to glucan diets. Thus the incorporation of yeast biomass as such in diets get priority glucan incorporation into diet saving time, energy, money etc. However, this has to be confirmed working with more number of yeast cultures and applying them at field level.



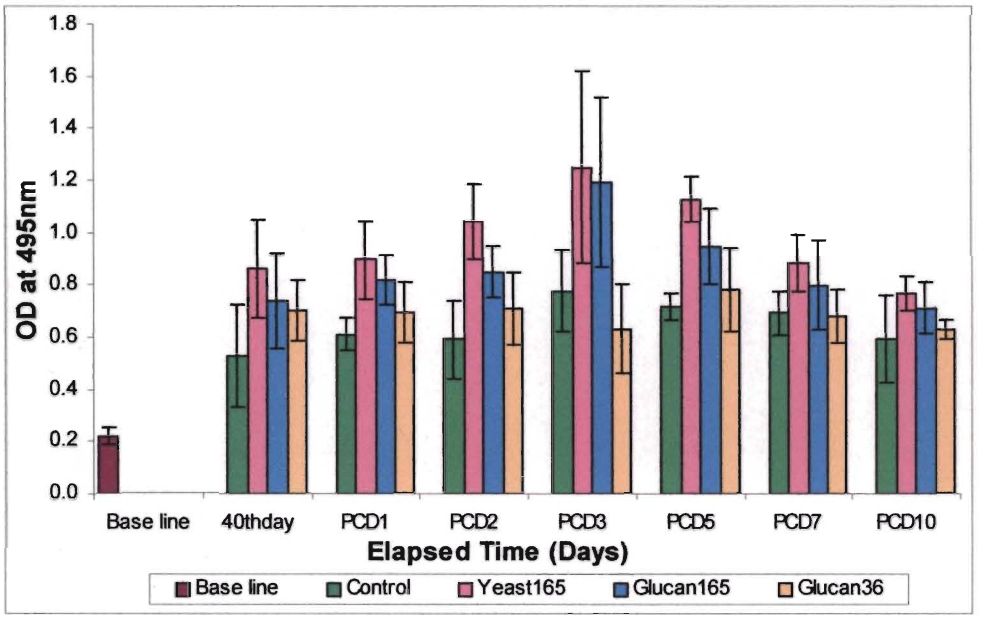
Group	THC (x10^5 cells ml^{-1})*								
	Feed	Baseline	40thday	PCD1	PCD2	PCD3	PCD5	PCD7	PCD10
GI	Control	103.98±10.6^a	109.35±14.9^a	105.92±12.79^a	115.75±13.73^a	118.42±16.54^a	107.32±6.84^a	89.11±12.65^a	74.09±8.45^a
GII	Y165	103.98±10.6^a	145.91±6.64^b	137.72±7.78^b	182.47±47.50^b	192.88±19.95^c	167.49±38.03^b	151.84±12.9^a	128.18±14.3^b
GIII	G165	103.98±10.6^a	137.69±32.2^{ab}	127.11±15.46^{ab}	147.39±26.90^{ab}	157.26±18.97^b	137.23±16.6^{ab}	136.22±18.0^b	112.15±9.4^{bc}
GIV	G36	103.98±10.6^a	118.90±20.3^{ab}	115.25±20.81^{ab}	131.56±6.28^a	133.12±16.53^{ab}	121.95±16.86^a	110.30±11.8^b	103.88±11.2^c

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

PCD : Post challenge Day

Yeast 165 : *Candida sake* (S165) incorporated diet
G 165 : *Candida sake* (S165) glucan incorporated diet
G 36 : *S. cerevisiae* (MTCC36) glucan incorporated diet.

Fig. 8.1. Mean (± S.D) Total haemocyte count (THC) of *F. indicus* fed with yeast and glucan diets and challenged with WSSV.



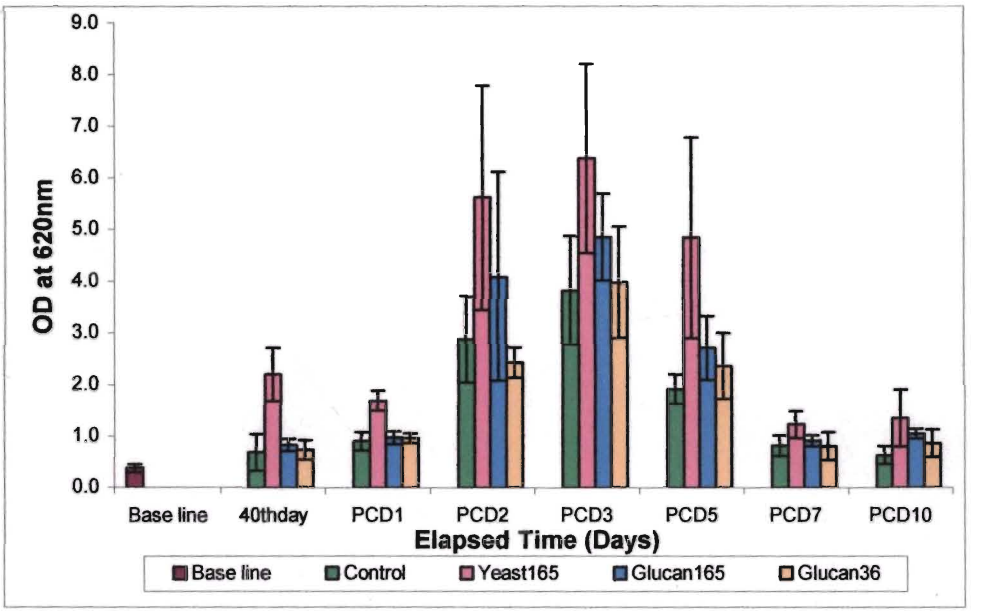
Group	Phenoloxidase activity (Increase in OD at 495 nm min^{-1}/100µl Haemolymph)*								
	Feed	Base line	40thday	PCD1	PCD2	PCD3	PCD5	PCD7	PCD10
GI	Control	0.219 ± 0.03^{a}	0.526 ± 0.19^{a}	0.611 ± 0.06^{a}	0.590 ± 0.14^{a}	0.775 ± 0.15^{a}	0.715 ± 0.05^{a}	0.693 ± 0.08^{ab}	0.593 ± 0.16^{a}
GII	Y165	0.219 ± 0.03^{a}	0.860 ± 0.18^{b}	0.895 ± 0.14^{c}	1.040 ± 0.14^{c}	1.250 ± 0.37^{c}	1.128 ± 0.08^{c}	0.881 ± 0.10^{b}	0.767 ± 0.06^{b}
GIII	G165	0.219 ± 0.03^{a}	0.740 ± 0.18^{ab}	0.816 ± 0.09^{bc}	0.849 ± 0.09^{bc}	1.193 ± 0.32^{bc}	0.948 ± 0.14^{bc}	0.799 ± 0.17^{ab}	0.712 ± 0.10^{ab}
GIV	G36	0.219 ± 0.03^{a}	0.703 ± 0.11^{ab}	0.692 ± 0.11^{ab}	0.707 ± 0.13^{ab}	0.632 ± 0.16^{a}	0.781 ± 0.15^{ab}	0.681 ± 0.10^{a}	0.631 ± 0.03^{ab}

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

PCD : Post challenge Day

Yeast 165 : *Candida sake* (S165) incorporated diet
G 165 : *Candida sake* (S165) glucan incorporated diet
G 36 : *S. cerevisiae* (MTCC36) glucan incorporated diet.

Fig. 8.2. Mean (± S.D) phenoloxidase (PO) value of *F. indicus* fed with yeast and glucan diets and challenged with WSSV.



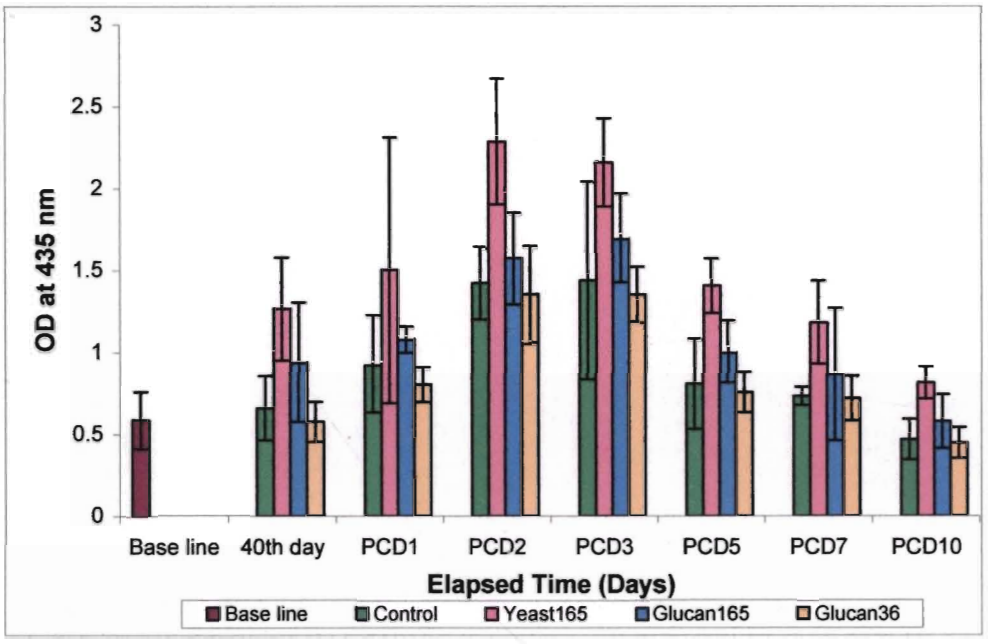
Group	NBT activity (OD at 620 nm/100µl Haemolymph)*								
	Feed	Base line	40thday	PCD1	PCD2	PCD3	PCD5	PCD7	PCD10
GI	Control	0.370± 0.08^{a}	0.678± 0.35^{a}	0.889± 0.17^{a}	2.870± 0.83^{a}	3.816±1.04^{a}	1.907± 0.27^{a}	0.805± 0.19^{a}	0.625± 0.17^{a}
GII	Y165	0.370± 0.08^{a}	2.182± 0.51^{b}	1.668± 0.20^{b}	5.605± 2.16^{b}	6.373±1.82^{b}	4.835±0.93^{b}	1.211± 0.26^{b}	1.344 ± 0.55^{b}
GIII	G165	0.370± 0.08^{a}	0.817± 0.11^{a}	0.956± 0.12^{a}	4.085±2.013^{ab}	4.849± 0.83^{ab}	2.70 ± 0.61^{a}	0.902± 0.10^{ab}	1.041± 0.09^{ab}
GIV	G36	0.370± 0.08^{a}	0.726± 0.18^{a}	0.945± 0.09^{a}	2.416± 0.29^{a}	3.975±1.07^{a}	2.353± 0.63^{a}	0.796± 0.27^{a}	0.851± 0.26^{ab}

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

PCD : Post challenge Day

Yeast 165 : *Candida sake* (S165) incorporated diet
G 165 : *Candida sake* (S165) glucan incorporated diet
G 36 : *S. cerevisiae* (MTCC36) glucan incorporated diet.

Fig. 8.3. Mean (± S.D) NBT value of *F. indicus* fed with yeast and glucan diets and challenged with WSSV.



Group	Peroxidase (OD at 435 nm /100µl Haemolymph)*								
	Feed	Baseline	40th day	PCD1	PCD2	PCD3	PCD5	PCD7	PCD10
GI	Control	0.584±0.17^{a}	0.660±0.19^{a}	0.931±0.29^{a}	1.425±0.21^{a}	1.439±0.601^{a}	0.809±0.27^{a}	0.735±0.05^{a}	0.469±0.12^{a}
GII	Y165	0.584±0.17^{a}	1.267±0.31^{b}	1.500±0.81^{b}	2.289±0.38^{b}	2.161±0.26^{b}	1.407±0.16^{b}	1.187±0.24^{b}	0.815±0.09^{b}
GIII	G165	0.584±0.17^{a}	0.941±0.36^{ab}	1.083±0.07^{ab}	1.574±0.27^{a}	1.697±0.27^{ab}	1.006±0.18^{a}	0.866±0.40^{ab}	0.579±0.16^{a}
GIV	G36	0.584±0.17^{a}	0.577±0.12^{a}	0.804±0.10^{a}	1.353±0.29^{a}	1.353±0.16^{a}	0.758±0.12^{a}	0.720±0.13^{a}	0.448±0.09^{a}

* Data at the same exposure time with different superscripts are significantly different ($p<0.05$).

PCD : Post challenge Day

Yeast 165 : *Candida sake* (S165) incorporated diet
G 165 : *Candida sake* (S165) glucan incorporated diet
G 36 : *S. cerevisiae* (MTCC36) glucan incorporated diet.

Fig. 8.4. Mean (± S.D) peroxidase value of *F. indicus* fed with yeast and glucan diets and challenged with WSSV.

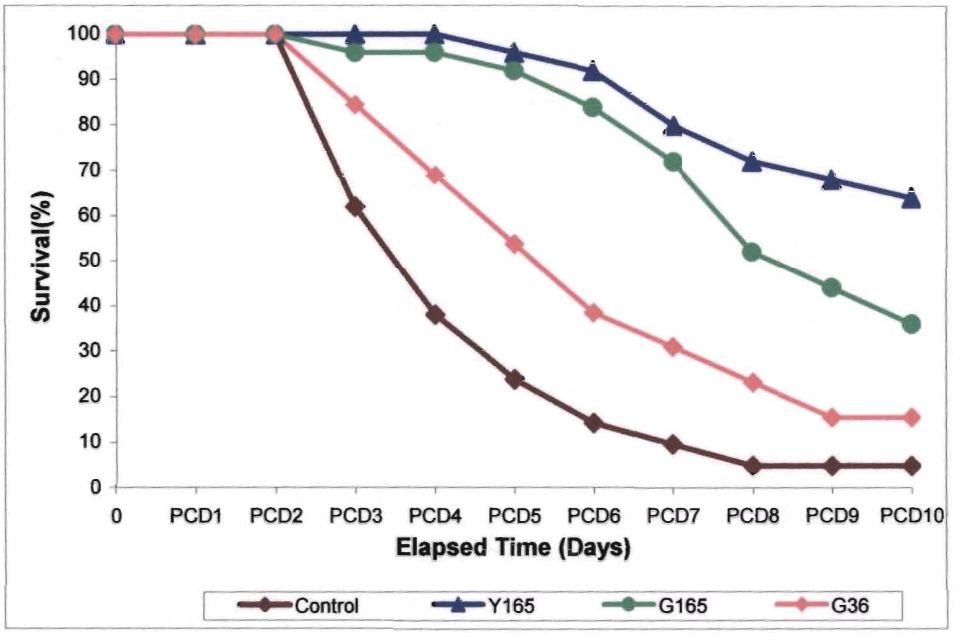


Fig 8.5.Post challenge survival in *F. indicus* fed with yeast and glucan diets and challenged with WSSV.

Chapter 9

Conclusion

Aquaculture has developed rapidly over the last three decades and has become an important industry as today's demand for fish exceeds the natural supply. However, disease out break is being increasingly recognized as a significant constraint in shrimp aquaculture production. The most important diseases of cultured penaeid shrimps have had viral or bacterial aetiologies. Many shrimp farms around the world have been badly hit by epidemics of White Spot Syndrome Virus (WSSV) since it emerged first in Asia around 1993. Rapid dissemination of viral disease invites concern over its effective control, rapid diagnosis and treatments. A proactive disease management strategy, at least in shrimp aquaculture, is a multidisciplinary subject where ecology, environment, nutrition, physiology and genetics of the organism have to be taken care of. There is no obvious biological basis for making vaccines for crustaceans. The most realistic approach at present to control disease in shrimp aquaculture therefore is to combine good husbandry and good feed along with the use of prophylactic agents including immunostimulants. Immunostimulants are chemical compounds that activate the immune system of animals and render them more resistant to infections by viruses, bacteria, fungi, and parasites. Immunostimulants have been obtained from diverse natural sources where, microbial cell wall acts as the main source. Cell wall components like lipopolysaccharides and peptidoglycan of certain bacteria and β-glucan from yeast and fungal cell wall are proved to be effective immunostimulants. Currently many commercial products are available in shrimp aquaculture sector under the label of immunostimulants and are extensively used by the shrimp farmers. But in many cases the nature of these compounds, the dose required for eliciting immunostimulation at optimum level and duration of protection conferred are still uncertain. These lacunae in the scientific knowledge pertaining to such practices necessitate a comprehensive study on products before they are launched into the market. In this scenario, the present work was carried out with the following objectives.

1. Screening of marine yeasts to identify potent strains showing immunostimulant and growth enhancing property in *Fenneropenaeus indicus.*
2. Optimisation of yeast biomass concentration in feed for effective protection against experimental infection with White Spot Syndrome Virus.
3. Optimisation of culture conditions of the selected marine yeast for biomass production.
4. Extraction and partial H-NMR structural characterization of (1→3)-β-D-glucan from selected yeasts
5. Efficacy of (1→3)-β-glucan from marine yeast as an immunostimulant to Indian white prawn *Fenneropenaeus indicus.* Optimisation of the dose, frequency and mode of glucan administration to *Fenneropenaeus indicus* .
6. Assessing the immunological profile of *Fenneropenaeus indicus* on administration of whole cell marine yeast/cell wall glucan

The salient findings of the study are summarised as follows;

- Seven marine yeasts were screened for growth promoting and immunostimulant property in *F. indicus. Candida sake* S165 was found to be best in terms of its support for growth and protection against white spot virus infection.

- Yeast biomass incorporation at 10% level in the feed was found to be optimal for immunostimulation in *F. indicus.* Feeds incorporated with mercapto-ethanol treated yeast biomass supported the best growth and protection against WSSV infection followed by autoclaved and untreated yeast biomass.

- *Candida sake* S165 preferred 15ppt salinity, pH 4.5 and incubation temperature 30^0C for optimal growth. Molasses at a concentration of 2mg/ml (sugars) was found to be optimal for growth. Ammonium sulphate at a concentration of 0.3% was found to be the best nitrogen source in the medium. Potassium phosphate, 0.3%, Magnesium sulphate, 0.03% and Calcium carbonate, 0.2% were found to be optimal for growth of *C. sake* S165.

- Immunological studies with Adult *F. indicus* proved that 10% yeast in feeds supported maximum immunostimulation.

- Total haemocyte count, phenoloxidase and nitro blue tetrazolium values were found to be maximum in *F. indicus* maintained on 10% yeast diets followed by 20% and 1% yeast diets and control diet. Haematological parameters were found to be maximum on Post Challenge Day 3.

- H-NMR spectra of the glucan extracted from *C. sake* and *S. cerevisiae* proved that the compounds are glucan and the extract from *C. sake* was found to contain small proportion of mannan.

- Dip treatment of *F. indicus post* larvae with different concentrations of glucan suspension showed that 0.2 mg/ml was the optimum in terms of protection against WSSV.

- Protection imparted to the *F. indicus* post larvae through glucan dip treatment against WSSV challenge was found to last only for about two weeks as evidenced from the survival percentage of post larvae.

- Frequency of administration of glucan at 0.2 % level in the diet was found to be optimum at once in seven days administration in *F. indicus* post larvae.

- Confirmation of the optimal frequency of administration of glucan in *F. indicus* adults through immunological assays showed that glucan administration *via* diet once in a week was giving maximum immunostimulation and thereby protection against WSSV.

- Total haemocyte count, phenoloxidase activity and nitro blue tetrazolium reduction were maximum for *F. indicus* maintained on glucan diet administered once in a week.

- A significant increase in the haematological parameters could be noticed on Post Challenge Day 3 in *F. Indicus*, maintained on glucan diets and challenged with WSSV.

- Test on the comparative efficacy of marine yeast (whole cell) and its cell wall component β-1,3-glucan as immunostimulant in *F. indicus* showed that whole cell yeast supported maximum immunostimulation compared to the glucans.

- Haematological parameters were found to be maximum on Post Challenge Day 3 except for peroxidase in *F. indicus* when maintained on various yeast and glucan incorporated diets and challenged with WSSV. On Post Challenge Day 2 also haematological values were closer to that on Day 3. Post Challenge Day 1, Day 5, Day 7 and Day 10 presented comparatively lesser values in all treatment groups.

- Marine yeast *Candida sake* S165 fed group showed maximum survival (68%) when challenged with WSSV compared to all other treatment groups. *Candida sake* glucan was next in order in terms of the protection (32%) imparted to *F. indicus* followed by *S. cerevisiae* glucan.

The study revealed that marine yeast *Candida sake* can be effectively used as potential source of immunostimulants for application in penaeid prawns culture systems. The study emphasise the fact that the dose and frequency of application of immunostimulants are to be standardised and validated before commercialisation to achieve optimum stimulation of the immune system and to avoid immune fatigue die to over dose. Marine yeast (whole cell) was found to support better immunostimulation compared to its cell wall component β-1,3-glucan. This study shows that administration of marine yeast (whole cell) or β-1,3-glucan as immunostimulants in aquaculture would definitely help in protection of the stock to a few more days even though total protection is not being imparted. This partial protection itself would be highly helpful to the farming industry so that they can get sufficient time to plan for a safe harvest and save the crop from cent percent mortality.

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Appendices

Appendix 1

Table 4.1. THC of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV. Data of the same treatment groups at various time intervals with different letters are significantly different ($p<0.05$).

Time Interval	THC ($x10^5$ cells ml^{-1})*			
	Control	1Y	10Y	20Y
Base line	66.77 ± 10.09^{ab}	66.77 ± 10.09^{a}	66.77 ± 10.09^{a}	66.77 ± 10.09^{a}
15th Day	53.25 ± 2.88^{a}	73.42 ± 14.39^{a}	102.67 ± 22.25^{abc}	62.08 ± 15.90^{a}
28thday	80.50 ± 25.50^{ab}	110.67 ± 24.13^{b}	121.33 ± 18.76^{bc}	101.17 ± 12.96^{b}
PCD1	83.75 ± 13.52^{b}	85.30 ± 8.73^{ab}	96.25 ± 9.59^{ab}	51.17 ± 12.41^{a}
PCD2	71.67 ± 20.36^{ab}	91.17 ± 17.32^{ab}	107.37 ± 20.20^{bc}	72.17 ± 13.82^{a}
PCD3	79.25 ± 2.88^{ab}	94.79 ± 18.69^{ab}	140.00 ± 32.57^{c}	115.67 ± 16.74^{b}

Table 4.2 Phenoloxidase (PO) value of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV. Data of the same treatment groups at various time intervals with different letters are significantly different ($p<0.05$).

Time Interval	PO activity (Increase in OD at 495 nm min^{-1}/100µl Haemolymph)*			
	Control	1Y	10Y	20Y
Base line	0.376 ± 0.052^{a}	0.376 ± 0.052^{a}	0.376 ± 0.052^{a}	0.376 ± 0.052^{a}
15th Day	0.575 ± 0.164^{ab}	0.722 ± 0.155^{bc}	1.143 ± 0.126^{b}	1.215 ± 0.2^{d}
28thday	0.427 ± 0.073^{a}	0.558 ± 0.116^{ab}	1.015 ± 0.234^{b}	0.902 ± 0.67^{bc}
PCD1	0.703 ± 0.126^{bc}	0.863 ± 0.235^{c}	0.987 ± 0.198^{b}	0.837 ± 0.1^{b}
PCD2	0.810 ± 0.113^{bc}	0.998 ± 0.173^{cd}	1.257 ± 0.126^{b}	1.06 ± 0.11^{cd}
PCD3	0.942 ± 0.210^{c}	1.195 ± 0.166^{d}	1.540 ± 0.166^{c}	1.212 ± 0.08^{d}

Table 4.3 NBT value of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV. Data of the same treatment groups at various time intervals with different letters are significantly different ($p<0.05$).

Time Interval	NBT activity (OD at 620 nm/100µl Haemolymph)*			
	Control	1Y	10Y	20Y
Baseline	0.457 ± 0.075^{a}	0.457 ± 0.075^{a}	0.457 ± 0.075^{a}	0.457 ± 0.075^{a}
15th Day	0.470 ± 0.153^{a}	0.645 ± 0.146^{a}	1.182 ± 0.186^{b}	0.967 ± 0.375^{b}
28thday	0.452 ± 0.103^{a}	0.625 ± 0.090^{a}	0.920 ± 0.110^{ab}	0.852 ± 0.091^{ab}
PCD1	0.542 ± 0.132^{a}	0.562 ± 0.113^{a}	0.890 ± 0.21^{ab}	0.810 ± 0.103^{ab}
PCD2	0.630 ± 0.080^{a}	1.293 ± 0.585^{b}	2.005 ± 0.771^{c}	0.767 ± 0.416^{ab}
PCD3	1.512 ± 0.722^{b}	2.308 ± 0.155^{c}	3.705 ± 0.374^{d}	2.648 ± 0.196^{c}

Appendix 2

Table 7.1 THC of *F. indicus* fed on diets containing glucan for 40 days at different frequencies and then challenged with WSSV. Data of the same treatment groups at various time intervals with different superscripts are significantly different ($p<0.05$).

Time interval	THC ($x10^5$ cells ml^{-1})*			
	Control	G 1	G7	G10
Baseline	112.03 ± 19.87^{bc}	112.03 ± 19.87^{a}	112.03 ± 19.87^{a}	112.03 ± 19.87^{a}
40thday	109.97 ± 16.51^{bc}	88.45 ± 11.11^{a}	156.67 ± 12.86^{b}	110.90 ± 40.65^{a}
PCD 1	72.26 ± 31.60^{a}	85.87 ± 8.34^{a}	137.00 ± 31.51^{ab}	80.40 ± 19.31^{a}
PCD 2	82.65 ± 11.74^{ab}	114.53 ± 30.52^{a}	142.00 ± 15.39^{ab}	90.85 ± 10.36^{a}
PCD 3	128.50 ± 4.75^{c}	117.95 ± 26.49^{a}	164.20 ± 18.40^{b}	107.56 ± 15.66^{a}

Table 7.2. Phenoloxidase (PO) value of *F. indicus* fed on diets containing glucan for 40 days at different frequencies and then challenged with WSSV. Data of the same treatment groups at various time intervals with different superscripts are significantly different ($p<0.05$).

Time interval	PO activity (Increase in OD at 495 nm min^{-1}/100µl Haemolymph)*			
	Control	G 1	G7	G10
Baseline	0.240 ± 0.04^{a}	0.240 ± 0.04^{a}	0.240 ± 0.04^{a}	0.240 ± 0.04^{a}
40thday	0.363 ± 0.050^{a}	0.667 ± 0.072^{bc}	0.785 ± 0.251^{b}	0.430 ± 0.057^{b}
PCD 1	0.338 ± 0.130^{a}	0.486 ± 0.143^{b}	0.654 ± 0.155^{b}	0.457 ± 0.048^{b}
PCD 2	0.552 ± 0.066^{b}	0.783 ± 0.084^{c}	0.825 ± 0.172^{b}	0.496 ± 0.194^{bc}
PCD 3	0.528 ± 0.056^{b}	0.599 ± 0.222^{bc}	0.921 ± 0.215^{b}	0.660 ± 0.079^{c}

Table 7.3.NBT value of *F. indicus* fed on diets containing glucan for 40 days at different frequencies and then challenged with WSSV. Data of the same treatment groups at various time intervals with different superscripts are significantly different ($p<0.05$).

Time interval	NBT activity (OD at 620 nm/100µl Haemolymph)*			
	Control	G 1	G7	G10
Baseline	0.290 ± 0.068^{a}	0.290 ± 0.068^{a}	0.290 ± 0.068^{a}	0.290 ± 0.068^{a}
40thday	0.310 ± 0.065^{b}	0.365 ± 0.103^{a}	$0.932\pm 0.249^{a\,b}$	0.432 ± 0.132^{a}
PCD 1	0.244 ± 0.084^{a}	0.501 ± 0.074^{a}	$0.760\pm 0.091^{a\,b}$	0.480 ± 0.105^{a}
PCD 2	0.717 ± 0.132^{b}	1.167 ± 0.063^{b}	1.308 ± 0.098^{b}	1.254 ± 0.134^{b}
PCD 3	1.573 ± 0.393^{c}	2.394 ± 0.467^{c}	2.827 ± 0.140^{c}	2.108 ± 0.515^{c}

Appendix 3

Table 8.1. THC of *F. indicus* fed with yeast and glucan diets and challenged with WSSV. Data of the same treatment groups at various time intervals with different letters are significantly different ($p<0.05$).

Time Interval	THC ($x10^5$ cells ml^{-1})*			
	Control	Y165	G165	G36
Baseline	103.98±10.60^{bc}	103.98±10.60^{a}	103.98±10.60^{a}	103.98±10.60^{a}
40thday	109.35±14.93^{c}	145.91±6.64^{bcd}	137.69±32.20^{bc}	118.90±20.33^{ab}
PCD1	105.92±12.79^{bc}	137.72±7.78^{abc}	127.11±15.46^{abc}	115.25±20.81^{ab}
PCD2	115.75±13.73^{c}	182.47±47.50^{de}	147.39±26.90^{c}	131.56±6.28^{b}
PCD3	118.42±16.54^{c}	192.88±19.95^{e}	157.26±18.97^{c}	133.12±16.53a^{b}
PCD5	107.32±6.84^{bc}	167.49±38.03^{cde}	137.23±16.6^{bc}	121.95±16.86^{ab}
PCD7	89.11±12.65^{ab}	151.84±12.95^{bcd}	136.22±18.01^{bc}	110.30±11.83^{ab}
PCD10	74.09±8.45^{a}	128.18±14.36^{ab}	112.15±9.42b^{ab}	103.88±11.28^{c}

Table 8.2 PO value of *F. indicus* fed with yeast and glucan diets and challenged with WSSV. Data of the same treatment groups at various time intervals with different letters are significantly different ($p<0.05$).

Time Interval	PO activity (Increase in OD at 495 nm min^{-1}/100µl Haemolymph)*			
	Control	Y165	G165	G36
Base line	0.219 ± 0.033^{a}	0.219 ± 0.033^{a}	0.219 ± 0.033^{a}	0.219 ± 0.033^{a}
40thday	0.526 ± 0.196^{a}	0.860 ± 0.186^{bc}	0.740 ± 0.181^{b}	0.703 ± 0.114^{b}
PCD1	0.611± 0.063^{a}	0.895± 0.147^{bc}	0.816± 0.092^{b}	0.692± 0.116^{b}
PCD2	0.590± 0.147^{c}	1.040± 0.144^{bcd}	0.849 ± 0.095^{b}	0.707± 0.138^{b}
PCD3	0.775 ± 0.155^{d}	1.250 ± 0.370^{d}	1.193 ± 0.323^{c}	0.632 ± 0.169b
PCD5	0.715 ± 0.050^{b}	1.128 ± 0.089^{cd}	0.948 ± 0.142^{b}	0.781± 0.159^{b}
PCD7	0.693 ± 0.082^{a}	0.881 ± 0.108^{bc}	0.799 ± 0.170^{b}	0.681± 0.102^{b}
PCD10	0.593 ± 0.164^{a}	0.767 ± 0.063^{b}	0.712 ± 0.101^{b}	0.631± 0.035^{b}

G9029

Table 8.3. NBT value of *F. indicus* fed with yeast and glucan diets and challenged with WSSV. Data of the same treatment groups at various time intervals with different letters are significantly different ($p<0.05$

Time Interval	NBT activity (OD at 620 nm/100µl Haemolymph)*			
	Control	Y165	G165	G36
Base line	0.370 ± 0.080^a	0.370± 0.080^a	0.370± 0.080^a	0.370± 0.080^a
40thday	0.678± 0.351^a	2.182± 0.514^a	0.817± 0.118^a	0.726± 0.186^a
PCD1	0.889± 0.175^a	1.668± 0.200^a	0.956± 0.124^a	0.945± 0.094^a
PCD2	2.870± 0.831^c	5.605± 2.169^b	4.085±2.013^c	2.416± 0.290^b
PCD3	3.816±1.047^d	6.373±1.821^b	4.849± 0.833^c	3.975±1.076^c
PCD5	1.907± 0.277^b	4.835±0.938^b	2.70 ± 0.616^b	2.353± 0.639^b
PCD7	0.805± 0.197^a	1.211± 0.267^a	0.902± 0.102^a	0.796± 0.270^a
PCD10	0.625± 0.170^a	1.344 ± 0.550^a	1.041± 0.094^a	0.851± 0.266^a

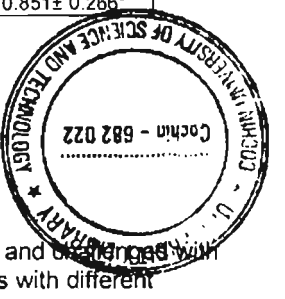

Table 8.4. Peroxidase value of *F. indicus* fed with yeast and glucan diets and challenged with WSSV. Data of the same treatment groups at various time intervals with different letters are significantly different ($p<0.05$)

Time Interval	Peroxidase (OD at 435 nm /100µl Haemolymph)*			
	Control	Y165	G165	G36
Base line	0.584±0.173^a	0.584±0.173^a	0.584±0.173^a	0.584±0.173^{ab}
40th day	0.660±0.197^a	1.267±0.310^c	0.941±0.363^{ab}	0.577±0.122^{ab}
PCD1	0.931±0.296^a	1.500±0.810^c	1.083±0.075^b	0.804±0.106^b
PCD2	1.425±0.218^b	2.289±0.381^d	1.574±0.277^c	1.353±0.294^c
PCD3	1.439±0.601^b	2.161±0.264^d	1.697±0.271^c	1.353±0.167^c
PCD5	0.809±0.275^a	1.407±0.162^c	1.006±0.188^b	0.758±0.123^b
PCD7	0.735±0.055^a	1.187±0.247^{bc}	0.866±0.403^{ab}	0.720±0.137^b
PCD10	0.469±0.123^a	0.815±0.098^{ab}	0.579±0.164^a	0.448±0.094^a