BIOACTIVE GLUCAN AND MELANIN FROM MARINE YEASTS: CHARACTERIZATION AND EVALUATION

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by

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To the Lord Almighty.....

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Certificate

This is to certify that the thesis entitled ***"Bioactive Glucan and Melanin from Marine Yeasts: Characterization and Evaluation"*** *is an authentic record of the research work carried out by* ***Ms. Wilsy Wilson*** *under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of* ***Doctor of Philosophy in Microbiology*** *under the Faculty of Marine Sciences of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral committee have been incorporated in the thesis.*

Dr. Rosamma Philip
(Supervising Guide)

Kochi-16
December 2016

Declaration

I hereby declare that the thesis entitled ***"Bioactive Glucan and Melanin from Marine Yeasts: Characterization and Evaluation"*** *is a genuine record of research work done by me under the supervision and guidance of Dr. Rosamma Philip in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.*

Wilsy Wilson

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Wilsy Wilson

Contents

Chapter 1

GENERAL INTRODUCTION

The marine biotope occupies almost three quarters of the earth's surface. It harbours a huge variety of marine organisms that are diverse in their physiology and adaptations. These marine inhabitants serve as an excellent model for various studies and its full potential is far from being discovered. Marine environment is a complex ecosystem characterized by special environmental conditions and the biodiversity of this unique environment make it a treasure of bioactive compounds. Since 1920s, when the first antibiotic, Penicillin was discovered by Fleming, it was believed that soil microorganisms are the largest source for novel drugs. At present marine microorganisms are promising and sustainable sources of bioactive compounds in an environment friendly perspective.

By the turn of the century, marine natural products had become an established sub discipline and several thousand compounds have been identified. Moreover, there is a rich pipeline of clinical and preclinical marine compounds to propose their sustained application in human medicine. The advancement in cultivation technologies and molecular biology techniques have led to the discovery of bioactive molecules from marine microorganisms and it may lead to the most exciting novel directions of marine science (Romano et al., 2016). The microbial communities from the marine environment are one of the major sources for compounds of unknown magnitude with great potentials. To adapt and survive in the complex marine ecosystem, marine microorganisms including marine yeasts accumulate structurally unique bioactive secondary metabolites not found in terrestrial organisms (Bhakuni and Rawat, 2005;

Schumacher et al., 2011). The present study is exclusively focused on the bioactive potential of marine yeasts, which is still an underexploited microbial community for its bioactive potential over their terrestrial counterparts.

Yeasts are eukaryotic, non-motile, unicellular fungi that reproduce either primarily by budding and transverse division or sexually through spore formation. Generally, yeast cells are larger than bacteria and the size vary considerably. They are commonly spherical, ovoid or elongated in shape and may vary depending on age and environment (Wayman and Parekh, 1990). Even though yeasts are unicellular, some species of yeast forms may become multicellular through the formation of strings of connected budding cells known as pseudohyphae or false hyphae, as seen in most molds. Yeast can produce pigments of varying colour ranging from yellow to black. Yeasts have ultrastructural features similar to that of higher eukaryotic cells. It is used as a model organism in molecular and cell biology due to their ease of growth, amenability to biochemical, genetic and molecular biological analyses.

Yeasts are predominant members of the fungal ecosystem and at present approximately 1,500 species have been described which are distributed between the ascomycetes and the basidiomycetes (Kurtzman and Fell, 2006). New species are being characterized in a regular manner and there is considerable unexploited yeast biodiversity on Earth. Yeasts are ubiquitous in nature and have been isolated from different sources such as soil, water, plants, animals and insects. They can grow as symbiotic or parasitic in association with animals (Fonseca and Inacio, 2006; Xu et al., 2011; Yurkov et al., 2012).

Marine yeasts, defined as the yeasts that are isolated from marine environments, are able to grow better on medium prepared using seawater rather than freshwater (Chi et al., 2010). The first marine yeasts were isolated from the

Atlantic Ocean by Fischer in 1894. Those were red and white yeasts and identified as *Torula* sp. and *Mycoderma* sp. respectively. Subsequently marine yeasts were isolated from different sources such as estuaries, sediments, seawater, seaweeds, invertebrates, seabirds, marine fishes, mammals etc. Yeasts grouped as obligate marine yeasts are those which grow and sporulate solely in marine or estuarine habitat whereas facultatives are those originated from terrestrial habitats but are able to grow and sporulate in the marine environment (Kohlmeyer and Kohlmeyer, 1979).

Different samplers have been designed for the collection of samples depending on the locations and depth of the sea (Dorschel, 2007; Singh, 2011). For deep sea sample collection, research submarines can also be used. Researchers use a number of media with different compositions for the isolation of marine yeasts. Wickerham's yeast malt medium is the most commonly used medium for the isolation of marine yeasts. Moreover, malt extract agar medium, seawater nutrient agar medium, YM agar medium, modified Sabauroud's dextrose agar medium and YPD agar medium are some of the media used by researchers for the isolation of marine yeasts (Wickerham, 1951; Nagahama et al., 1999; Loureiro et al., 2005; Masuda et al., 2008; Sarlin and Philip, 2011). In most of the media preparations, components were suspended in natural or artificial sea water. Antibiotics such as chloramphenicol, streptomycin or penicillin are commonly added in the medium to inhibit the growth of bacteria. To inhibit the growth of moulds, various inhibitors have been added to the media viz., rose Bengal, dichloran and propionate (Jarvis, 1973; King et al., 1979; Kutty, 2009).

Generally, yeast species were phenotypically described based on morphology, physiological and biochemical analysis. Recently, various molecular techniques have been applied to differentiate or identify yeast species. The advancement in molecular biology and emergence of new methods have made the

identification and characterization more systematic. Instead of traditional identification methods, genetic characterization through molecular techniques imparts more powerful means of strain identification and differentiation amongst strains (Recek et al., 2002).

The previous study report states that marine yeasts do not belong to a specific genus or group, but are represented by a wide variety of well-known genera, such as *Candida, Cryptococcus, Debaryomyces, Pichia, Hansenula, Rhodotorula, Saccharomyces, Trichosporon, Torulopsis, Sporobolomyces* and black yeasts (Bhat and Kachwalla, 1955; Fell et al., 1960; Fell and van Uden, 1963; Godinho et al., 1978; Kohlmeyer and Kohlmeyer, 1979; Nagahama et al., 2001; Gadanho and Sampaio, 2005; Kutty and Philip, 2008; Burgaud et al., 2010; Konishi et al., 2010; Galkiewicz et al., 2012). *Candida, Cryptococcus, Debaryomyces* and *Rhodotorula* are the most frequently observed genera and among this *Candida, Debaryomyces* and *Rhodotorula* exhibited a cosmopolitan distribution.

The presence of black yeast has been reported by Van Uden and Castelo-Branco (1963) from Pacific sub surface waters and deep waters of Loma Trough, off San Diego. *Hortaea werneckii, Phaeotheca triangularis* and *Aureobasidium pullulans* are the black yeast strains isolated from salt pans at the Adriatic coast (Zalar et al., 1999). Moreover, most of the black yeast strains *Hortaea werneckii* were isolated from the hypersaline waters and it is known as halotolerant eukaryotic model organism (Gunde-Cimerman et al., 2000; Butinar et al., 2005; Cantrell et al., 2006). The presence of black yeast, *Hortaea werneckii* in Arabian Sea and Bay of Bengal have been reported (Kuriakose, 2012; Kutty et al., 2013a) and these isolates were capable of producing the pigment melanin. The distribution studies of marine yeasts are mostly restricted to coastal waters of the Atlantic, Pacific and Indian Oceans.

Marine organisms have considerable importance as new promising sources of enormous number of biologically active compounds (Newman and Cragg, 2004; Lordan et al., 2011; Chen et al., 2016). Extensive researches have been done to explore the bioactive potential of marine microbes and the results are promising. The extreme environmental conditions such as variation in temperature, salinity, pH and many other factors have been fortified the marine microorganisms comprising marine yeasts to produce potentially active biomolecules. The biomolecules produced by these organisms have gained great biotechnological potential and its use as cosmeceuticals and nutraceuticals. It has been reported that marine yeasts are able to produce many bioactive substances such as amino acids, glucans, glutathione, toxins, enzymes, phytase and vitamins with potential application in the food, pharmaceutical, cosmetic and chemical industries as well as for marine culture and environmental protection (Chi et al., 2009; Sarkar et al., 2010). Due to the exhausted resources, most of the terrestrially isolated bioactive compounds appear repetitive and research has been extended to the marine environment.

Since ancient times, yeasts were used in many industrial processes such as production of alcohol and carbon dioxide, which are important to the brewing, alcohol distillation and baking industries. Yeast, also used as single cell protein (SCP) which is highly substantial in aquaculture owing to the nutritional quality and its possible utilization as feed for animals or in aquaculture. Similarly, the ability of yeasts to produce different extracellular enzymes such as amylase, alkaline protease, acid protease, cellulase, phytase, lipase, xylanase and inulinase were extensively studied (Ogrydziak, 1993; Pandey et al., 2001; Buzzini and Martini, 2002; Gupta et al., 2003; Vakhlu and Kour, 2006; Rocha et al., 2006; Chi et al., 2007; Bhadra et al., 2008;). Most of the enzymes may find potential application in food, feed, pharmaceutical, maricultural and fermentation

industries. Furthermore, yeast cells are good sources of a number of vitamins such as thiamine, pantothenic acid, riboflavin, vitamin B6, and vitamin B12, pyridoxine, choline, glutathione, folic acid and p-amino benzoic acid. Polyamines, one of the biomolecule which involves in many of the cellular functions also have been noticed in *Debaryomyces* (Tovar et al., 2002; Reyes-Becerril et al., 2011). Ergosterol, another beneficial biomolecule with varying functions such as an important pharmaceutical intermediate, precursor of vitamin D2 and cortisone, the main material to produce sterol drugs such as cortisone and progesterone was also extracted from several yeasts (Young et al., 2003; He et al., 2007; Gomez-Lopez et al., 2011). Production of melatonin in yeast *S. cerevisiae* has been reported by Sprenger et al. (1999). It is known as a regulator of circadian rhythms and also acts as an antioxidant. The diverse functions of melatonin have been reported and most of its effect suggests a clear neurohormonal activity (Jacob et al., 2002; Pohanka, 2011; Rodriguez-Naranjo et al., 2012).

Killer toxins are one of the remarkable compounds produced by several yeast strains which can be used as natural biocontrol agent. *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula, Kluyveromyces, Metschnikowia, Pichia, Saccharomyces, Ustilago, Torulopsis, Williopsis* and *Zygosaccharomyces* are some of the identified yeast genera capable of producing killer toxins (Young and Yagiu, 1978; Staib, 1999; Pfeiffer et al., 2004; Oro, 2014; Mehlomakulu et al., 2014). Furthermore, antifungal effects of yeast strains belonging to *Cryptococcus albidus*, *Wickerhamomyces anomalus, Metschnikowia pulcherrima* and *Aureobasidium pullulans* against some pathogenic fungi such as *Botrytis* sp., *Aspergillus carbonarius*, *Penicillium expansum*, *P. digitatum* and *Monilia fructicola* were also reported through *in vitro* and *in vivo* wound assay (Oro, 2014). Yeasts or its metabolites can be used

as biocontrol agents and it will be a valid alternative to fungicides in controlling pre- and post-harvest decay of many fruits.

The cell wall polysaccharides of yeast also have been demonstrated as bioactive due to the diverse functions/applications. In yeast, the cell wall is made up of glucans, mannans, mannoproteins and minor component of chitin. The cell wall polysaccharides can be separated from each other by alkaline extraction. These polysaccharides have various applications in food, feed and health industries. These polysaccharides are known as biological response modifiers (BRMs) due to their ability to trigger a usually non-specific reaction of the immune system against tumor cells, inflammations, viral and bacterial infections. Kogan et al. (2008) reported the antioxidant, antimutagenic and antigenotoxic activities of the yeast polysaccharides and suggest their potential application in anticancer prevention/therapy.

β-Glucans are homopolysaccharides of glucose, present in the yeast cell wall (~50-60 %) and are potent stimulators of nonspecific defense mechanisms in animals. A variety of biological functions of β-glucan have been reported. The first reported major function of β-glucans was its antitumor activity (Chihara et al., 1970). Many other biological activities have also been reported; including antifungal, antiinfection (Onderdonk et al., 1992), radioprotective (Gu et al., 2005) and cholesterol reduction (Wolever et al., 2011). Yeasts strengthen the immunity towards various diseases (Hofer and Pospisil, 2011) and fight against pathogens that are already active in humans and animals. Anti-inflammatory, antioxidant and cytotoxic activities of β-glucan-rich extract from *Geastrum saccatum* mushroom have also been reported (Dore et al., 2007). *In vitro* cytotoxic study of β-glucan from Maitake mushroom in human prostatic cancer cells exhibited strong anticancer effect (Fullerton et al., 2000). β-glucans extracted from various mushrooms exhibited a positive impact against a range

of cancers (Okamura et al., 1986; Kimura et al., 1994; Kodama et al., 2002). Due to its emulsion-stabilizing effects and antioxidant activity, yeast β-glucans can prevent skin injuries caused by solar radiation and therefore are used in sun-screens, oils and gels (Mason, 2001; Michiko and Yutaka, 2007). Rorstad et al. (1993) reported that yeast glucan also showed an adjuvant effect when included in vaccines against furunculosis in Atlantic salmon (*Salmo salar* L.).

In recent years, bioactive polysaccharides isolated from natural sources have attracted much attention in the field of biochemistry and pharmacology. For instance, polysaccharides or their glycoconjugates were shown to exhibit multiple biological activities including anticarcinogenic, anticoagulant, immunostimulating, antioxidant etc. Similarly, polysaccharides have gained special attention in aquaculture sector as an immunostimulant against the severe threat by various pathogens. Substances used for immunostimulation therefore increases the resistance of the host, not by enhancing specific memory responses, but by enhancing nonspecific defense mechanisms (Sakai, 1999). It has been reported that polysaccharides from yeasts also confer protection and/or enhance disease resistance in shrimp, a process often referred to as immunostimulation.

Aquaculture is facing severe threat due to the continuous attack of various viruses and bacteria. Glucan enhances the defense potential of fish and shellfish against bacterial or viral infection and thus acts as immunostimulant/ immunomodulator (Chang et al., 2003). Marine yeast derived glucans have also been studied by previous researchers and proved its potential as an immunostimulant (Sajeevan et al., 2009; Sukumaran et al., 2010; Antony et al., 2011c). Various immunological parameters such as total haemocyte count (THC), phenoloxidase (PO) activity, respiratory burst activity, alkaline phosphatase activity and acid phosphatase activity have been used to detect the

immunological effect of glucan against bacterial or viral infections in aquaculture. Earlier studies report that glucan incorporated feeds could significantly enhance the survival of shrimps against WSSV attack, one of the serious risk to aquaculture industry. *Debaryomyces hansenii* S8, *D. hansenii* S169, *Candida tropicalis* S186, *C. haemulonii* S27 and *C. sake* S165 are some of the marine yeasts used for the study of glucan and its application in aquaculture (Sajeevan et al., 2009; Sukumaran et al., 2010; Antony et al., 2011c; Subramanian and Philip, 2013). The use of immunostimulants have gained a lot of attention as a valuable alternate to the use of antibiotics and chemotherapeutics in the fight against infectious diseases in aquaculture sector.

In addition to glucans; mannans and mannoproteins represent ~ 30-40% of the yeast cell wall. Yeast derived mannans/mannan-oligosaccharides (MOS) have important role in various biological activity. These are composed mainly of D-mannose, but sometimes contain minor saccharide components such as glucose, galactose and xylose. Mannans have been added in many of animal feed supplements and provide a range of beneficial effects. It helps to improve the immune system of the animal, growth and survival. Incorporation of these polysaccharides in feed helps to maintain a healthy gut flora and thus promotes gut health (Ponton et al., 2001; Grisdale-Helland et al., 2008; Refstie et al., 2010; Huu and Jones, 2014). Mannan can also function as prebiotic by favouring the growth of beneficial bacteria in the gut. These yeast cell wall polysaccharides are also used as adjuncts for fish and animal feeds. In general, these polysaccharides have been proposed to promote animal growth and health by various mechanisms, such as immunomodulation, oxidative status, interactions with gut constituents and binding of toxins and pathogens (Kurtzman et al., 2011).

In the same way, natural pigments have also become a choice of study not only due to the ability to increase the marketability of products; but also due

to advantageous biological activities as antioxidants and anticancer agents. Melanins are frequently used in medicine, pharmacology, and cosmetics preparations. Earlier studies revealed that, yeasts are also a promising candidate for the production of various pigments and it may find potential applications in industries.

Yeasts are able to produce varying hues of pigments and due to this property; pigmented yeasts are an interesting group from the biotechnological point of view. It produces different colours of pigments ranging from light yellow to bright orange, red, pink and dark brown or even black. The most common pigments produced by yeasts comprise melanin and carotene. Most of the pigmented yeasts belong to ascomycota and basidiomycota. Melanins are frequently associated with the cell wall of yeasts, which can provide a protective function against damages caused by ultraviolet (UV) light, oxidants, radiation and extreme climate conditions. These protective effects of melanin make them an important candidate in many of the pharmacology and cosmetics preparations. Photoprotection (Paramonov et al., 2002), antioxidation (Tu et al., 2009), free radical-scavenging (Rozanowska et al., 1999) and immunomodulatory effects (El-Obeid et al., 2006) are some of the biological effects of natural melanin.

Melanin is an ubiquitous pigment found in plants, animals and microorganisms. Melanin producing yeasts are generally known as 'black yeasts'. Pathogenic yeasts produce melanin as a protective measure against the host immune system. *Cryptococcus neoformans*, *Candida albicans, Paracoccidioides brasiliensis* and *Wangiella dermatitidis* are some of the melanin producing pathogenic yeasts (da Silva et al., 2009). Terrestrial fungus has been shown to produce melanin and apart from terrestrial habitat, marine environment is also a source of melanin producing yeast and most of them associated with high salt

concentration. The ascomycetous black yeasts, *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum* and *Aureobasidium pullulans* are halophilic fungi that inhabit hypersaline water of solar salterns. Among the marine black yeasts, *H. werneckii* has been identified as the dominant fungal species in hypersaline waters on three continents (Kogej et al., 2004; Gunde-Cimerman and Plemenitas, 2006).

Carotenoid is a pigment responsible for most of the yellow to red colours of fruits, flowers, birds, insects and marine invertebrates. The pleasant colour of this pigment make it a colourant in many of the foods, feeds and nutritional supplements. Apart from this, carotenoids also have extensive array of functions, especially in relation to human health and their role as biological antioxidants. *In vitro* cell culture experiments have shown that carotenoids have anticancer properties (Fraser and Bramley, 2004). Similarly, in aquaculture animal health, carotenoids play an important role in enhancing their antioxidant defense ability and resistance to diseases and environmental stressors (Chien et al., 2003; Pan et al., 2003; Amar et al., 2004; Yang et al., 2011). Recently, carotenoid pigment from yeast *Rhodotorula mucilaginosa* AY-01 with significant antioxidant and antibacterial activities have been reported (Yoo et al., 2016). Carotenoid producing yeasts from marine environment have been isolated and mostly they belong to basidiomycetous classes, including *Rhodotorula, Rhodosporidium, Sporidiobolus* and *Sporobolomyces* (Nagahama et al., 2001). Former studies have reported that natural pigments not only have the capacity to increase the marketability of products, but also exhibit advantageous biological activities such as antioxidant and anticancer properties.

Continuous outbreaks of diseases and the constant use of chemotherapeutics is a relevant problem in aquaculture. Mainstream disease causing infectious pathogens are various viruses and bacteria. Bacteria such as *Vibrio vulnificus*

(Sung et al., 1994), *V. campbellii*, *V. proteolyticus* (Marques et al., 2006), *V. alginolyticus* (Wang and Chen, 2005), *Aeromonas veronii* (Lin et al., 2011), *V. harveyi* (Amparyup et al., 2012) and viruses such as white spot syndrome virus (WSSV) (Chang et al., 2003; Sajeevan et al., 2009; Sukumaran et al., 2010), Taura syndrome virus (TSV) (Song et al., 2003), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Yeh et al., 2009) and infectious myonecrosis virus (IMNV) (Nunes, 2010) are some of the problematic disease causing agents in aquaculture. Among the diverse pathogens, WSSV is one of the most alarming shrimp pathogens, and it has caused severe devastation to the worldwide shrimp culture industry (Takahashi et al., 1994; Wang et al., 1995). Different strategies such as the use of probiotics, vaccines and immunostimulants have been proposed for the control and prevention of diseases in aquaculture. Administration of immunostimulants especially glucan in shrimp cultures have been considered as a potential mode of protection against WSSV infection. The use of immunostimulants has gained lot of attention as a valuable substitute to the use of antibiotics and vaccines in the fight against infectious diseases in shrimp farming (Subasinghe, 1997).

The emergence of new bioactive compounds from natural sources have paved the way for the treatment of many of the life threatening diseases including cancer. Even though many factors contribute to the development of cancer, one hallmark is the loss of balance between cell proliferation and cell death, resulting in increased cell replication and the failure of damaged cells to be removed through apoptosis (Hanahan and Weinberg, 2011). Networks of genes have been involved in the apoptosis, an active form of cell suicide (Wong, 2011). Anti-cancer effects of extracts from various natural sources have been emerged as a concern of many of the research study (Leone et al., 2003; Abdullaev and Espinosa-Aguirre, 2004; Park et al., 2006; Bachmeier et al., 2008; Zikri et al.,

2009; Leonardi et al., 2010; Kim et al., 2011). Similarly, pigment extracts from various natural sources have also been made an area of anticancer research (Giovannucci et al., 1995; Narisawa et al., 1996; Nahum et al., 2001).

Because of the intricacy of this disease, gene expression based approaches have been considered as a tool for the better understanding of the newly isolated compounds for their anticancer effects. Curcumin, major component of *Curcuma longa* L., modulates growth of tumor cells through regulation of multiple cell signaling pathways including cell proliferation pathway (cyclin D1, c-myc), cell survival pathway (Bcl-2, Bcl-x, cFLIP, XIAP, c-IAP1), caspase activation pathway (caspase-8, caspase-3, caspase-9), tumor suppressor pathway (p53, p21), death receptor pathway (DR4, DR5), mitochondrial pathways, and protein kinase pathway (JNK, Akt, and AMPK) (Ravindran et al., 2009). Ethanolic and spore oil extracts of the dark fungus *Ganoderma lucidum* have exhibited anticancer effects with significant changes in the expression of genes associated with cell cycle and apoptosis in human prostate cancer cell lines PC3 and DU145 (Kao et al., 2014). Recently, an *in vitro* study of the red pigment extracted from *Serratia marcescens* exhibited strong anticancer potential on a panel of three breast cancer cell lines viz., MCF-7, MDAMB231, T47D and prostate cancer cell lines viz., PC3, LNCaP and DU-145 by MTT assay (Athavale et al., 2013).

During the past 20 years, thousands of novel compounds and their metabolites with diverse biological activities ranging from antiviral to anticancer have been isolated from various marine sources (Garcia-Fernandez et al., 2002; Amador et al., 2003). Subsequently, marine-derived microorganisms have also become important sources of new bioactive molecules (Lam, 2007).

Recently Sekar et al. (2015) reported that selected compounds from crude culture extracts of marine yeast, *Candida albicans* can act as an anticancer agent

by the protein ligand docking study. A marine carotenoid pigment fucoxanthin subjected for anticancer studies in various cell lines, has shown that the pigment fucoxanthin exerts its antiproliferative activity through different molecules and pathways including the Bcl-2 proteins, MAPK, NFκB, Caspases, GADD45, and several other molecules that are involved in either cell cycle arrest, apoptosis or metastasis (Kumar et al., 2013a).

The cytotoxic activity of three different extracts of the marine yeasts *Candida albicans*, *Kuraishia capsulate* and *Saccharomyces cerevisiae* isolated from coastal mangrove ecosystem have been studied against human breast carcinoma cells (MCF7), human hepatocarcinoma cells (HepG2) and African Green Monkey kidney cell lines (VERO) using MTT assay (Senthilraja and Kathiresan, 2015). All extracts were able to inhibit the proliferation of the cancer cells (MCF-7, HepG2) and the normal Vero cell viability. Amongst three yeast strains, *S. cerevisiae* showed more than 80% cell viability in normal Vero cell lines and at the same time the IC_{50} value of *S. cerevisiae* extract indicate more efficiency against MCF7 cells suggesting anti-breast cancer activity. Authors suggest that this yeast species from coastal environment will be promising for further development as an anticancer drug.

Searching for natural products in relatively untapped sources will be expanding our ability to find novel, potent and selective bioactive compounds. Henceforth the marine environment, the largely unexplored reservoir may provide the novel bioactive compounds with immense possibility. In this perspective, the present study was undertaken to explore the potential of marine yeasts as a source of bioactive compounds. Glucan from Baker's yeast, *Saccharomyces cerevisiae* has already been proved to be a good source of immunostimulant. Efficacy of yeast glucans depends on the frequency of branching as well as the number of glucose molecules in the β- 1,6 branching

points. Earlier studies have shown yeast-wise variation in immunostimulation property and the survival in shrimps estimated by immunological assays and mortality data. The present study was undertaken to understand the efficacy of glucans from various marine yeasts in terms of immune gene expression in shrimps and selection of the potential strains as source of glucans for application in aquaculture.

Previous studies on melanins are mainly focused on plant/animal melanins. In this study, marine black yeast, *Hortaea werneckii* has been selected since the information on this melanin is very much limited. Physicochemical and functional characterization of the marine black yeast melanin has been undertaken to understand its property for possible commercial applications.

Objectives of the study are as follows:

- ✓ Extraction and characterization of glucan and melanin from marine yeast isolates
- ✓ Testing the bioactive potential of marine yeast glucan as an immunostimulant in *Penaeus monodon*
- ✓ Functional characterization of marine yeast glucan and melanin for exploring its bioactive potential

The Thesis is comprised of six chapters. A general introduction to the topic is given in Chapter1. Extraction of glucan from marine yeasts and primary screening of glucan as an immunostimulant in *P. monodon* postlarvae against WSSV infection is presented in Chapter 2. Chapter 3 deals with characterization and evaluation of selected glucans as immunostimulant in adult *P. monodon* against WSSV infection. Chapter 4 illustrates the extraction of melanin from marine black yeast, physicochemical characterization of melanin and evaluation of its bioactivity. Chapter 5 deals with the evaluation

of glucan and melanin as an anticancer agent by *in vitro* gene expression analysis. A summary and conclusion of the study is presented in Chapter 6, followed by references and appendices.

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Chapter 2

Screening of marine yeast glucans as immunostimulant in *P. monodon* post larvae

2.1 Introduction

Aquaculture is one of the fastest growing food sectors in the world. It plays a critical role in global food security and nutritional needs of people in developing and developed countries. In addition to its growing importance in food and nutritional security, many millions of people around the world find a source of income and livelihood in the aquaculture sector. Recent information acquired by FAO in 2014 indicates that shrimp continues to be the largest single commodity in value terms, accounting for about 15 percent of the total value of internationally traded fishery products in 2012. Globally, the farmed shrimp production volumes decreased in 2012 and particularly in 2013, mainly as a result of disease-related problems in some countries in Asia and Latin America. As the shellfish aquaculture industry grows, the necessity for disease prevention and control increases concurrently.

Disease outbreak has been a major threat to the shrimp farming industry causing huge economic loss during the last few decades. Generally, the early developmental stages of the animals are believed to be more susceptible to

pathogens than adults (Lightner et al., 1983a; Momoyama and Sano, 1989). *Penaeus monodon* is the most farmed crustacean species in many of the developing countries in Asia and is under persistent threat from viral infections. White spot syndrome virus (WSSV) infection is considered as one of the serious problems and is usually associated with high incidence of larval mortality reaching 100% within 3-10 days of infection (Liu et al., 2009a). The use of immunostimulants as prophylactic agents rather than chemotherapeutics in shrimp farming help to control infections and therefore lead to reduced mortality and protection from drug resistant pathogens. Yeast cell and cell wall components are used as immunostimulants in aquaculture over the years. Use of immunostimulants of biological origin such as β-glucan (Chang et al., 2003; Burgents et al., 2004; Rodriguez et al., 2007; Sajeevan et al., 2009; Sukumaran et al., 2010; Subramanian and Philip, 2013; Mastan, 2015); chitin (Wang and Chen, 2005); mannoproteins (Tizard et al., 1989; Abu-Elala et al., 2013); peptidoglycans (Boonyaratpalin et al., 1995; Zhang et al., 2014) and lipopolysaccharides (Takahashi et al., 2000) known as biological response modifiers (BRMs) (Yan et al., 2005; Leung et al., 2006) have been proved to enhance immune responses in organisms, rendering higher survival rate and resistance to infections.

2.1.1 Shrimp aquaculture

Shrimp culture has grown into one of the largest and most important aquaculture crops worldwide. All kinds of shrimps are highly desirable now in the world market. Most coastal countries have a harvest industry for shrimp and more than 50 countries practice shrimp aquaculture. Around 80 percent of cultured shrimps come from Asia with Thailand, China, Indonesia and India as the top producers. In the Western hemisphere, Ecuador is the major shrimp producing country. Shrimp aquaculture expanded significantly during the 1980s and now represents a multi-billion dollar industry. In 2002, the global shrimp farming industry produced an estimated 1.6 million metric tons of shrimp, and

production is projected to increase at a rate of 12-15% per year over the next several years (Rosenberry, 2003).

The giant tiger shrimp, *Penaeus monodon* accounts for more than half of the total shrimp aquaculture production. It is known as the largest and fastest growing of all shrimp species. Within three to six months, *P. monodon* can reach a marketable size of 20 cm and 35 g even at high stocking densities. Due to this advantage the commercial aquaculturists practice farming *P. monodon* for more rapid cash flow than other cultured fish and shellfish (Muir and Roberts, 1982). This species can reach a weight of over 150 g and length of over 33 cm, if allowed to grow to full size (Dore, 1994) and is also tolerant to wide range of salinities which make it more attractive to the farmers. Other important commercial species are *P. merguiensis, P. semisulcatus, P. japonicus, P. penicillatus, Fenneropenaeus indicus, F. chinensis, Litopenaeus vannamei, Marsupenaeus japonicus, Metapenaeus ensis, M. monoceros* and *M. brevicornis.*

2.1.2 Diseases in shrimp aquaculture

Disease outbreaks and catastrophic production losses are the major constraints on shrimp aquaculture. The emergence of diseases depends upon several factors such as environmental stress, poor water quality, high stocking density and various infectious microbial pathogens. Successive expansion of the industry and the increasingly globalised trade in broodstock, larvae and commodities arising from shrimp farming have also led to the emergence of several disease conditions (OIE, 2009; Stentiford et al., 2010; Lightner, 2012). The most significant diseases of cultured shrimps and losses are caused by viral pathogens followed by bacterial pathogens and a few of fungal and protozoan agents also (Flegel, 2006a; 2012). Viral pathogens appear to be the most significant constraint on the growth and survival of shrimps under culture conditions. Current estimates predict that up to 40% of tropical shrimp production (>$3bn) is lost annually, mainly due to viral pathogens.

Among the array of viral pathogens, white spot syndrome virus (WSSV) causing white spot disease (WSD) provides the single most striking example of a pathogen negatively impacting the production of food (and wealth) from the crustacean aquaculture sector. The first outbreak of WSSV was reported in 1992 in Taiwan, and then immediately spread to other shrimp farming countries (Lightner and Redman, 1998). Most of the Asian countries have been affected by WSSV and outbreaks were also reported in the USA (Lightner, 1996), Central America and South America (OIE, 2003). Based upon current annual production of $10bn, an estimated 10% of the output is lost each year due to WSD alone.

WSSV is a circular dsDNA (~300 kb) virus assigned to the genus, Whispovirus, in the family Nimaviridae (Mayo, 2002). Gross symptoms of WSSV infected shrimps include reduced feeding, lethargy, high colour variation and white spots under the cuticle due to abnormal calcium deposits (Stentiford et al., 2009) and transmission of the etiological agent can occur both vertically and horizontally (Lo and Kou, 1998; Bachere, 2000). WSSV has a wide host range and mainly infect marine penaeid shrimps (Rodriguez et al., 2003). In experimental studies WSSV causes mortalities in *P. vannamei, P. stylirostris, P. aztecus, P. duorarum* and *P. setiferus* (Lightner, 1996). Previous experimental study reports state that shrimp become susceptible to WSSV infection from PL 6, PL 10 or PL 30 onwards (Venegas et al., 1999; Flegel, 2007) and infection could not be induced in the early larval stages such as nauplii, zoea and mysis of *P. monodon* (Yoganandhan et al., 2003). In the course of infection most of the cells, tissues and organs such as haemocytes, cuticular epidermis, hematopoietic tissue, connective tissue, gills, lymphoid organs, foregut (including the stomach) and oocytes were found to be hit by WSSV. Cellular changes such as nuclear hypertrophy, chromatin margination and homogeneous eosinophilic to dense basophilic intranuclear inclusions were found in infected haemocytes and

tissues (Chou et al., 1995; Huang et al., 1995; Wongteerasupaya et al., 1995; Durand et al., 1997; Wang et al., 1999).

Yellow head disease by Yellow head Virus (YHV) (Chantanachookin et al., 1993); Infectious Hypodermal and Hematopoietic Necrosis by Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) (Lightner et al., 1983b); Taura syndrome by Taura syndrome virus (TSV) (Lightner et al., 1995) and infectious myonecrosis by Infectious myonecrosis virus (IMNV) (Poulos et al., 2006) are the prevailing viral diseases other than WSD in shrimp aquaculture. So far, more than twenty viral diseases have been reported to infect shrimps and prawns among that these five viral pathogens of penaeid shrimps are currently listed by the World Organization for Animal Health (OIE, 2009).

Among the bacterial diseases, vibriosis caused by vibrio species is the most common disease in Penaeid shrimps (Baticados et al., 1990; Lightner, 1996). *V. harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. penaeicida* (Nash et al., 1992; Chen, 1992; Ishimaru et al., 1995) are some of the commonly reported species causing vibriosis. One of the fungal pathogen from the genus *Fusarium* has been reported in penaeid shrimp and disease may affect all developmental stages of penaeid shrimp (Ramaiah, 2006). Fungi *Lagenidium callinectes* has also been reported to cause larval mycosis of cultured penaeid shrimps (Lightner and Fontaine, 1973). Protozoans also cause diseases at a less intensity in penaeid culture and it is mainly due to high density of prawns and poor water quality and management system.

2.1.3 Crustacean immune system

To fight against various diseases a healthy immune system is prerequisite for any living organisms. Crustaceans mainly rely on innate immunity whereas vertebrates have both adaptive and innate immune system. To fight against infectious diseases, invertebrates mainly depend on non-specific or innate immune response (Kurtz and Franz, 2003). Invertebrates lack the genes, proteins

and cells that build up high antigen specific recognition and immune memory as exist in vertebrates (Flajnik and Du Pasquier, 2004). As a part of this innate or non-specific immunity, shrimps possess both cellular and humoral immunity even though not much specialized as in vertebrates. The first line of defense in innate immunity in shrimps is the external barriers which include cuticle, tegumental glands, epithelial immunity, branchial podocytes, autotomy of appendages and regeneration of appendages. Once the pathogen enters to the host hemocoel, it encounters the innate defense mechanisms which include cellular and humoral immune responses (Fig. 2.1).

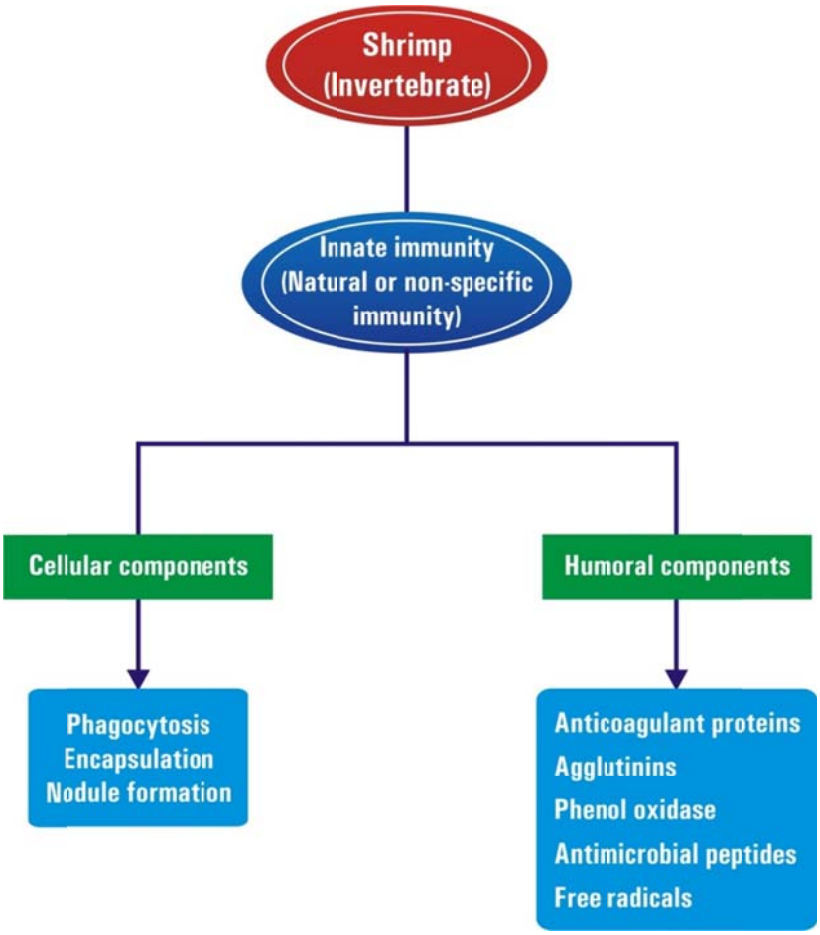


Fig.2.1 Shrimp defense mechanisms

2.1.3.1 Haemocytes

Haemocytes play an extremely important role in the immune response of shrimps by synthesis and exocytosis of a series of bioactive molecules (Smith and Chisholm, 1992; 2001). Basically, haemocytes execute inflammatory-type reactions such as haemocyte clumping, phagocytosis, encapsulation, cytotoxicity, production of reactive oxygen metabolites and the release of antimicrobial peptides. Three types of circulating haemocytes have been identified based on morphological and cytochemical characterization which includes hyaline, granular and semigranular cells (Bauchau, 1981). A number of immune molecules are produced and stored in the granules of haemocytes. At the time of infection or activation by bacterial and/or fungal cell wall components such as peptidoglycan (PG), lipopolysaccharides (LPS) and β-glucans (BGs), the immune molecules release in to the haemolymph. Pattern recognition proteins (PRPs) or pattern recognition receptors (PRRs) present on the host cells recognize and bind the microbial cell wall components known as pathogen-associated molecular patterns (PAMPs) and activate various immune responses in the host against various pathogens (Lee and Soderhall, 2002; Ji et al., 2009). Each cell types have been attributed with different functions such as coagulation, phagocytosis and release of proPO system (Johansson and Soderhall, 1985; Omori et al., 1989; Gargioni and Barracco, 1998).

2.1.3.2 Cellular immune responses

The major defense reactions with the involvement of haemocytes against foreign intruders include phagocytosis, nodulation and encapsulation.

2.1.3.2.1 Phagocytosis

Phagocytosis is one of the cellular mechanisms of innate immune systems that enable the blocking of invasion of any pathogen. This is achieved mainly

through the actions of haemocytes in shrimp. In phagocytosis, cell surface receptors recognize and bind the particles and cytoskeleton modification occurs in the phagocytic cell. Particles are then engulfed and internalized, forming phagosomes which are moved through a series of early and late endocytic membrane compartments and then intracellular destruction of the engulfed particle occurs through the phago-lysosomal pathway in the phgosomes (May and Machesky, 2001). A number of molecules involved in the regulation of phagocytosis were identified in shrimps such as Rab GTPases (Wu and Zhang, 2007; Wu et al., 2008); Ras nuclear related protein (Ran protein) (Liu et al., 2009b); ADP ribosylation factors (Arfs) (Zhang et al., 2010a). Opsonic factors that recognize microbial components and enhance the phagocytic rate also have important role in phagocytosis (Vargas-Albores et al., 1993).

2.1.3.2.2 Nodule formation

Nodule formation is followed by the heavy load of microorganisms. In nodulation numerous haemocytes act synergistically to trap microorganisms or big antigens that cannot be removed by phagocytosis. It is a method of pathogen removal from the circulating haemolymph of host by localisation of microorganisms. The microbes undergo subsequent destruction by the activation of the proPO system and melanization (Wang et al., 2001; Van de Braak, 2002). Nodules have been detected in gills, heart and hepatopancreas.

2.1.3.2.3 Encapsulation

Encapsulation is another cellular immune response and is carried out by the host when the parasite is too large to be engulfed by phagocytosis. Semigranular cells are primarily involved in the process. The parasite is killed with in the encapsulated capsule by the action of proPO system, melanization and various cytotoxic molecules. Some proteins also work as opsonins to

mediate the encapsulation process (Vargas-Albores and Yepiz-Plascencia, 1998).

The process of nodulation and encapsulation appear to be same but directed against different targets (Lavine and Strand, 2002). Nodulation immobilizes large amounts of biotic targets with the size of bacteria or fungal spores, whereas encapsulation is directed to bigger targets such as parasites or abiotic targets such as plastic beads (Ratcliffe and Gagen, 1977; Pech and Strand, 1996). Normally both the process ends with melanization of the capsule/nodule and the killing of the invader by asphyxiation and action of various toxic products.

2.1.3.3 Humoral immune responses

The humoral immune response begins with the recognition of microbial cell wall components by the pattern recognition proteins (PRPs), which will in turn trigger various immune reactions against the invading pathogens in the host. Haemocytes are the major sites for the storage and release of immune molecules in the humoral responses, which include the proPhenoloxidase (proPO) system, the clotting cascade and a wide array of antimicrobial peptides (AMPs) and reactive oxygen intermediates.

2.1.3.3.1 The clotting system

In humoral immune response, the clotting system is the first line of defense against pathogens. It also serves to prevent blood loss during injury and wound healing. Tranglutaminase (TGase) and clotting protein are the major molecules involved in the blood clotting system of shrimp (Maningas et al., 2008). Transglutaminase catalyses intermolecular or intramolecular bond formation between the side chains of free lysine and glutamine residues on certain proteins, resulting in protein polymerization and plays a role in blood coagulation (Song

and Li, 2014). In crustaceans, clottable protein found in several species have the ability to polymerize and form clot in the presence of Ca^{2+} and TGases released from haemocytes upon microbial attack (Kopacek et al., 1993).

2.1.3.3.2 Prophenoloxidase system (proPO system)

The proPhenoloxidase (proPO) system is one of the major innate immune systems in invertebrates. The activation of proPO system is facilitated by a serine proteinase cascade. proPO-activating proteinases (PAPs) or proPO-activating enzymes (PPAEs) are the serine proteases which catalyze the proteolysis of inactive proPO to active phenoloxidase (PO) (Sritunyalucksana and Soderhall, 2000). PO is the enzyme involved in melanin formation by a cascade of reactions and intermediates; PO catalyzes the oxidation of phenolic compounds to quinones, which then get converted to melanin through several non-enzymatic steps. The toxic intermediates including quinone substances and melanization process around the invading pathogens help in killing the pathogens directly.

The activation of this proPO system can be triggered by very low quantities of microbial cell wall components such as β-1,3 glucans, lipopolysaccharides or peptidoglycans (Soderhall, 1982; Soderhall and Cerenius, 1998). Pattern recognition proteins (PRPs) such as β-1,3-glucan binding proteins (BGBP), lipopolysaccharide binding proteins (LPSBP) and peptidoglycan binding proteins (PGBP) are the recognition molecules to recognize and respond against microbial invaders by the presence of signature molecules on the surface of the invaders (Janeway, 1989; Cerenius et al., 2008) and trigger the reactions. Enhancement of the proPO system was detected in haemocytes of *P. monodon* by treatment with β-glucans (Sung et al., 1994). To prevent the unwanted and premature activation of this complex enzymatic system, protease inhibitors like serpine or serine proteinase inhibitors and alpha-2-macroglobulin (α-2-M) have been identified in the haemolymph of crustaceans (Lin et al., 2008; Wetsaphan et al., 2013).

2.1.3.3.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small peptides that have been isolated from both vertebrates and invertebrates. The innate immune system of invertebrates also relies upon AMPs as a foremost part of immune response against microbial attack (Bachere, 2003). They have wide range of activity against Gram-positive and Gram-negative bacteria, fungi, protozoa and viruses. Till date, number of AMPs have been isolated and characterized that are active against range of pathogens. Crustins, penaeidins and anti-lipopolysaccharide factor (ALF) (Destoumieux et al., 1997; 2001; Bartlett et al., 2002; Supungul et al., 2004; Somboonwiwat, et al., 2005; Ponprateep et al., 2012) are the three types of AMPs that have been fully characterized from the haemocytes of penaeid shrimps so far.

Haemocytes are the site of production, and during stress or any pathogen attack these molecules are released in to the haemolymph by degranulation process where they act to destroy the invading microorganisms. Molecular analysis have revealed that Toll pathway and Immune deficiency (IMD) pathway are the two distinct pathways involved in regulating the gene expression of AMPs in *Drosophila* (De Gregorio et al., 2002). Toll pathway plays an important role to react against Gram-positive bacterial and fungal pathogens, whereas the IMD pathway specially identifies Gram-negative bacteria (Ferrandon et al., 2007; Leulier and Lemaitre, 2008). Mechanism of action of AMPs is mainly due to the interaction between the amphipathic peptides and microbial membranes. This cause the disruption of cells by the formation of ion channels, transmembrane pores and extensive membrane rupture. This phenomenon has been extensively studied using various model membranes (Matsuzaki et al., 1996; Ehrenstein and Lecar, 1977; Shai, 1999; Ladokhin et al., 2001; Yamaguchi et al., 2002). Studies have been undertaken regarding the application of various immunostimulants such as peptidoglycan or β-glucan to enhance the immune system by the up-regulation of

AMPs in shrimps (Rattanachai et al., 2005; Amparyup et al., 2008; Antony et al., 2011c).

2.1.3.3.4 Reactive oxygen compounds

As a part of innate immunity, production and release of reactive oxygen compounds from haemocytes is an important defense mechanism in invertebrates. When pathogens enter into the haemolymph, they are engulfed by haemocytes, and various reactive oxygen species (ROS) are produced against them. Superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are some of the ROS generated. The release of superoxide anions is commonly known as the respiratory burst, and it is known as an important microbicidal activity in the innate immune system (Munoz et al., 2000). Increased respiratory burst activity has been reported following the treatment with β-glucans in haemocytes of tiger shrimp *P. monodon* against bacterial and viral infections (Song and Hsieh, 1994). The generation of highly reactive oxygen species, may cause severe cytotoxic effects in the host. In order to overcome this problem, the effective elimination of ROS is necessary, and it is accomplished by a number of antioxidant defense mechanisms which includes superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase (Homblad and Soderhall, 1999; Dandapat et al., 2003).

2.1.3.3.5 Pattern recognition proteins (PRPs)

Pattern recognition proteins play an important role in the innate immune response of crustaceans. PRPs such as lipopolysaccharide binding protein (LPSBP), lipopolysaccharide and β-1,3-glucan binding protein (LGBP), β-1,3-glucan binding proteins (BGBP) or peptidoglycan binding proteins (PGBP) have been identified in various invertebrates (Lee and Soderhall, 2002; Cheng et al., 2005). These molecules elicit various biological functions including the

activation of prophenoloxidase (proPO) cascade when they bind to PAMPs to remove the pathogens. PAMPs are pathogen-associated molecular patterns, that are recognized as nonself molecules and which include bacterial cell wall components such as lipopolysaccharide (LPS), peptidoglycan (PG) and lipoteichoic acid, fungal cell wall component β-1,3-glucan, and double-stranded RNA of viruses. *Pm*LGBP is a PRP characterized from *P. monodon,* have been reported for its involvement in the activation of shrimp proPO system by the binding activity of *Pm*LGBP towards LPS and β-1,3-glucan in the haemocytes (Amparyup et al., 2012).

2.1.3.3.6 Lectins/Agglutinins

These are proteins or glycoproteins without catalytic activity and have the ability to bind to specific carbohydrates expressed on microorganisms. Lectins involve in diverse immune functions such as agglutination (Vazquez et al., 1996), nodule formation (Koizumi et al., 1999), phagocytosis (Luo et al., 2006), activation of prophenoloxidase system (Yu et al., 1999), cell adhesion (Vasta et al., 1999) and so on. Some lectins can also function as opsonins by mediating binding between haemocytes and pathogens and thus facilitate phgocytosis (Cerenius et al., 1994). Lectins exist in almost all living organisms including animals and plants (Sharon and Lis, 1989). Diverse groups of lectins have been identified in shrimp which include C-type, L-type, P-type, M-type, fibrinogen-like domain lectins, galectins, and calnexin/calreticulin (Wang and Wang, 2013). The characteristic carbohydrate recognition domain (CRD) with disulfide bonds present in the Calcium-dependent (C-type) lectins, play a key role in pathogen recognition. Two C-type lectins containing a single CRD have been identified and characterized from *P. monodon* (Luo et al., 2006). A novel C-type lectin from the shrimp *L. vannamei* have been identified recently

and do possesses antivirus activity against WSSV infection in shrimps, and also prolong the survival of shrimps (Zhao et al., 2009).

2.1.3.3.7 Peroxinectin

Peroxinectin, is an immuno-reactive factor stored in the haemocytes in an inactive state and activated in the presence of non-self-molecules. It is a multifunctional molecule with cell adhesion activity and apart from that, diverse functions have also been presented *in vitro* as an opsonin (Thornqvist et al., 1994), inducer of degranulation (Johansson and Soderhall, 1989), encapsulation enhancer (Kobayashi et al., 1990) and also peroxidase activity (Johansson et al., 1995). The cell adhesion and peroxidase activities of peroxinectin isolated and purified from crayfish *P. leniusculus* and tiger shrimp *P. monodon* in the presence of lipopolysaccharide (LPS) and β-1,3-glucans have been reported (Johansson and Soderhall, 1988; Sritunyalucksana et al., 2001). Liu et al. (2005a) reported the presence of peroxinectin in granular and semi granular cells of the white shrimp *L. vannamei* and an up-regulation of the gene transcript have been noticed upon bacterial infection.

2.1.4 Disease control strategies in shrimp aquaculture

Disease problems are the major constraint to the growth of many shrimp species, which may occur due to the attack of various pathogens, exposure to various stressful and adverse environmental conditions (Lightner, 1993; 2011; 2012; Flegel, 2006b). Disease management is an important criterion to overcome the substantial economic losses in this regard. Proper treatment of waste water, disposal of diseased dead shrimps from culture system and post-harvest processes should be intensively considered to minimize the disease occurrence and horizontal transmission of pathogens (Bondad-Reantaso et al., 2005).

2.1.4.1 Use of chemicals and antibiotics

Applications of chemicals and antibiotics in various developmental stages of shrimps have been followed from long back to control and prevent diseases. But the continuous uses of these chemicals have caused the emergence of antibiotic resistance of pathogens, chemical pollution to the environment and adverse effect on the normal flora (Primavera, 1998; Cabello, 2004; Le et al., 2005; Romero et al., 2012). Due to these adverse effects, the use of chemotherapeutics and antibiotics have been restricted in aquaculture.

2.1.4.2 Vaccination

As a part of different strategies put forth in shrimp aquaculture for the better survival of organisms from infections the term 'vaccination' is also used by researchers. There are a few reports regarding the vaccination study in shrimps using inactivated pathogens or recombinant proteins suggesting protective effects (Kurtz and Franz, 2003) although an adaptive immune response is yet to be proved in invertebrates. Immunological responses of *P. monodon* to DNA vaccine pVP28 and its efficacy to protect shrimps against WSSV have been studied (Kumar et al., 2008). VP28 is the most studied envelope protein of WSSV. It has been reported that inactivated virus preparation of WSSV could induce the expression of various biodefense genes in *P. monodon* challenged with WSSV (Sudheer et al., 2015). Further studies need to be performed on the practical application of these vaccines in fields to control infections.

2.1.4.3 Probiotics

The dietary supplementation of beneficial live microorganisms (probiotic bacteria or probiotics) is an alternative approach for the control of diseases in aquaculture management. Probiotics execute numerous beneficial effects by modulating the biological systems in the host (Cross, 2002). Studies using

various probiotic strains in shrimps are progressing to understand its effects. Probiotics belong to *Lactobacillus, Bacillus, Micrococcus, Pseudomonas, Vibrio* and *Aeromonas* have been proposed as biological control agents in aquaculture (Antony and Philip, 2008). The beneficial effect of probiotics have been emphasized by the antimicrobial peptide (AMP) gene expression and survival in *P. monodon* both pre- and post-challenge WSSV (Antony et al., 2011a; Antony et al., 2011c). Researchers put forth probiotics as an alternative strategy to the adverse effects of antibiotics and chemotherapeutics (Nayak, 2010; Hai, 2015); however detailed understanding of the mechanism of action and field trials are necessary for the successful commercial application of these products in shrimp farms.

2.1.4.4 Use of immunostimulants

In shrimp farming, the practice of immunostimulant application as prophylactic agents rather than chemotherapeutics helps to control infections and therefore lead to reduced mortality and protection from pathogens. The administration of immunostimulants prior to an infection helps the organism to activate various immune responses and thus aid a better survival and protection from the severity of the pathogen attack.

Several immunostimulants from different sources have been applied in invertebrate cultures to activate the immune system of animals and build up protection from diseases over the years. Bacterial, fungal and yeast cell wall components are commonly used as immunostimulants in aquaculture. β-glucan (Chang et al., 2003; Sajeevan et al., 2009); chitin (Wang and Chen, 2005); mannoproteins (Tizard et al., 1989); peptidoglycans (Boonyaratpalin et al., 1995) and lipopolysaccharides (Takahashi et al., 2000) are some of the cell wall components which have been used as an immunostimulant. Various plant

extracts have also been tested for its immunostimulant potential (Chotigeat et al., 2004; Balasubramanian et al., 2008; Sudheer et al., 2011). The efficacies of yeast β-glucan as an immunostimulant in aquaculture have been reported using different commercially available β-glucan brands in the market (Ringo et al., 2012). Among the various immunostimulants, β-glucan has gained special attention and research is still on progress to understand the detailed mechanism of action under molecular level and thereby help to refine their use.

2.1.4.5 Bacterial cell wall components as immunostimulant

2.1.4.5.1 Peptidoglycan

Peptidoglycans (PG) are the major bacterial cell wall component especially in Gram-positive bacteria and have been studied for its potential effect as an immunostimulant. It has been reported that PG confers protection or enhance disease resistance in animals. Sritunyalucksana et al. (1999) reported that addition of PG in the diet led to an increase in the immune response and antibacterial activities in *P. monodon*. In another report PG derived from *Bifidobacterium thermophilum* resulted in enhanced resistance against *Vibrio penaeicida* and increased the phagocytic activity in *Marsupenaeus japonicus* (Itami et al., 1998). Administration of PG also caused an increase in the expression of few immune-related genes involved in crustacean immune system in *M. japonicus* (Rattanachai et al., 2005; Fagutao et al., 2008).

2.1.4.5.2 Lipopolysaccharides

Lipopolysaccharides (LPS) are the major component of the outer membrane of Gram-negative bacteria. LPS, at low doses stimulate the immune system and thereby improve disease resistance against pathogens (Noworthy, 1983). In aquaculture the immunostimulatory effect of LPS have been demonstrated in fish and shrimp (Neumann et al., 1995; Felix, 2005).

Rungrassame et al. (2013) reported that shrimp groups (*P. monodon*) fed with LPS containing diet exhibited higher survival rates than the control group when exposed to *V. harveyi*. LPS could also induce immune-related gene transcripts such as anti-lipopolysaccharide factor 3 (ALF3), C-lectin and mucin-like peritrophin. From these findings authors provide the evidence that LPS is also a candidate as an immunostimulant in shrimp farming.

2.1.4.6 Glucan as an immunostimulant in aquaculture

Yeast cell wall is one of the major source of β-1,3-glucan and is known to have potential to stimulate the immune system of fishes and crustaceans. β-1,3-glucans is a well-documented class of immunostimulants used in fish and shellfish aquaculture to enhance the defense against various bacterial or viral infections (Sakai, 1999). Due to their ability to modulate the immune system of the host, glucans belong to the group of substances known as biological response modifiers (BRMs). Yeast cell wall comprises ~50-60% of glucan. β-glucans are homopolysaccharides, comprising poly-glucose molecules that has β-(1,3)-D-linkages in the backbone, and also possess varying degree of side chains with β-1,6 branching. The molecular weight and degree of branching of β-glucans may differ. The immune modulating and immune stimulating ability of β-1,3/1,6 glucans depend on its branched structure and branching frequency (Bohn and BeMiller, 1995; Raa, 2015). β-glucans, isolated from the yeasts may be either soluble or in particulate forms and can exhibit various bioactivities. Among the various glucans and glycans tested, particulate β-1,3/1,6 baker's yeast glucan was found to be most active (Seljelid et al., 1981).

It has been reported that 1,3-β-glucan by binding to a specific receptor on the plasma membrane of macrophages, enhances the innate host defense responses against the attack of infectious agents (Di Luzio, 1985; Mueller et al., 2000). In crustacean immunity, proPO system have important role in the disease

resistance of animals. β-1,3-glucans have been reported to be a specific activator of proPhenoloxidase in several experimental studies (Sritunyalucksana et al., 1999). The immune responses are triggered by minute quantities of pathogen associated molecular patterns (PAMPs) that include β-1,3-glucans, bacterial lipopolysaccharides and peptidoglycans. Recognition and interaction of PAMPs by a spectrum of pattern recognition proteins (PRPs) present on the haemocytes cause the activation of several key pathways including proPO and accomplish the release of a battery of active effector molecules (Vargas-Albores et al., 2000; Amparyup et al., 2012). An array of reports have been documented regarding the enhancement of immune responses in shrimps after the administration of cell wall β-glucan from yeast and mushroom that include enhanced phenoloxidase activity, superoxide anion, superoxide dismutase, phagocytosis, total number of haemocytes and enhanced protection from WSSV infection (Huang et al., 1999; Chang et al., 1999; 2000; 2003; Thanardkit et al., 2002). Different methods of glucan treatment have been adopted by researchers that include immersion, dip treatment, oral administration and intramuscular injection.

2.1.4.7 Marine yeasts as source of immunostimulants

Extensive search for bioactive compounds with potential applications, marine yeast has gained special attention. The immunostimulant potential of marine yeast cell and cell wall components especially in aquaculture is noteworthy. Sajeevan et al. (2006) reported the efficacy of marine yeast isolate *Candida sake* S165 as source of immunostimulant to Indian white shrimp *F. indicus* by means of immune parameters such as total haemocyte count, phenoloxidase and respiratory burst activity of haemocytes. It was also found that *F. indicus* fed with marine yeast *C. sake* exhibited significantly higher post-challenge survival against WSSV compared to the control group. Similarly, β-mercapto-ethanol treated marine yeasts showed better protection against white spot syndrome virus infection in Indian white shrimp *F. indicus*

(Sajeevan et al., 2010). In a comparative study between marine yeasts and baker's yeast, it was found that marine yeast fed experimental groups exhibited better survival against WSSV infection in *F. indicus*. Marine yeasts also performed better immune response than the baker's yeast *S. cerevisiae* in terms of various immune assays like total haemocyte count, phenol oxidase, NBT reduction, alkaline phosphatase and acid phosphatase (Sarlin and Philip, 2011). Recently Babu et al. (2013) also reported the effectiveness of marine yeasts *C. aquaetextoris* S527 as a potent immunostimulant in black tiger shrimp *P. monodon* against WSSV infection. Hematological assays and immune gene expression profiles supported the findings that marine yeast confers protection through the enhancement of immune responses.

Marine yeast glucan also has proved its potential as an immunostimulant in aquaculture. Previous researchers have extracted glucan from various marine yeasts and the extracted glucan exhibited potential immunostimulant property in peneaid shrimps against WSSV infection (Sarlin, 2005; Sajeevan et al., 2009; Sukumaran et al., 2010; Antony et al., 2011c; Subramanian and Philip, 2013) were assessed by means of various immune parameters such as total haemocyte count, phenoloxidase activity, respiratory burst activity, peroxidase activity, alkaline phosphatase and acid phosphatase activity. Sajeevan et al. (2009) optimized the dose and frequency of glucan administration in *F. indicus* and the data states that shrimps fed 0.2% marine yeast glucan showed significantly higher immune response against WSSV infection. Structural characterization of marine yeast glucan by proton nuclear magnetic resonance (NMR) indicated that structures containing a backbone chain composed of (1-3)-linked anhydroglucose repeat units (AGRUs) with (1-6)-linked AGRUs in side chains (Sukumaran et al., 2010). The study also ascertained that glucan extracted from marine yeast isolates with a higher molecular weight and a lower degree of branching acts as better immunostimulants in *P. monodon* post larvae against WSSV than did the glucan

from Baker's yeast *S. cerevisiae*. Recently, Antony et al. (2011c) reported that marine yeasts and glucans could enhance the up-regulation of crustin-like AMP gene in haemolymph and various tissues of *P. monodon* against WSSV infection.

In shrimp culture, the most widely used immunostimulants are glucan based and thus apart from the basic hematological parameters, the immunostimulant effect of these glucans at molecular level is also essential. Only very few reports are available depicting the immunostimulant effects of marine yeast glucan at gene expression level. Attempts to understand the molecular responses in the highly susceptible post larval stage of shrimps are rare. For sustainable shrimp farming, efficient prophylactic approaches such as the use of immunostimulants especially at early stages of shrimps to control diseases are of great importance. Therefore an understanding of molecular level responses and defense mechanisms of various immunostimulants against the viral pathogens in shrimp cultures is significant.

The present study is focused on testing the efficacy of marine yeast glucans in shrimps at molecular level. The immunostimulatory activity of these glucans were assessed in terms of gene expression pre- and post-challenge WSSV in *P. monodon* post larvae by analysing the expression profile of immune genes and post-challenge survival.

2.2 Materials and Methods

2.2.1 Microorganisms used for glucan extraction

Eight marine yeasts (*Candida parapsilosis* R20, *Hortaea werneckii* R23, *C. spencermartinsiae* R28, *C. haemulonii* R63, *C. oceani* R89, *Debaryomyces fabryi* R100, *D. nepalensis* R305 and *Meyerozyma guilliermondii* R340) maintained in the Microbiology Laboratory of the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, India were used in the present work (Fig. 2.2). These

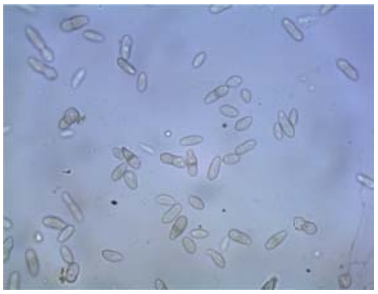
cultures were isolated from the sediment samples collected from Arabian Sea and Bay of Bengal.

Culture media and growth conditions: Malt Extract Agar (malt extract, 20 g; mycological peptone, 5 g; agar, 20 g; 20 ‰ seawater, 1000 ml; pH 6) plates were prepared and swab inoculation of the overnight grown yeast cultures were done. Incubation was done for 5 days at room temperature (28±2°C) and the biomass was harvested using sterile seawater.

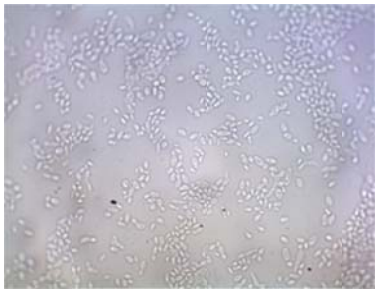
2.2.2 Glucan extraction

The harvested yeast biomass was separated by centrifugation at 10,000 rpm for 20 min at 4°C in a refrigerated centrifuge (Kubota, Japan) and dried under vacuum. Glucan was extracted from the dried yeast biomass following the method of Williams et al. (1991) with slight modifications. Briefly, 2 g dried yeast biomass suspended in 40 ml 3% NaOH was maintained at 100°C for 3 h in a water bath and was kept at room temperature (RT) over night. The suspension was centrifuged at 8000 rpm for 15 min to collect the insoluble residue, the residue was resuspended in 3% NaOH and the procedure was repeated (2x). The residue collected was treated with 0.5 N acetic acid and heat to boil for 6 h and the residue was separated by centrifugation. The procedure was repeated (2x). This residue was treated with distilled water at 100°C and centrifuged at 8000 rpm for 10 min to collect the insoluble residue. The procedure was repeated (5x). The insoluble fraction was resuspended in ethanol, boiled and centrifuged at 8000 rpm to collect the residue and the procedure was repeated (3x). The residue was thoroughly washed with distilled water (3x) and dried the residue under vacuum. The final product (glucan) was used for the present study.

***Candida parapsilosis* R20**

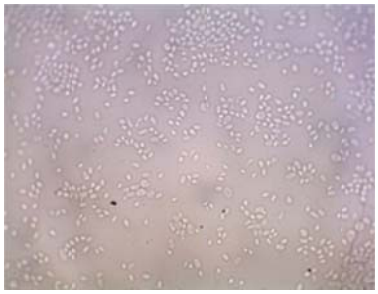


Hortaea werneckii R23

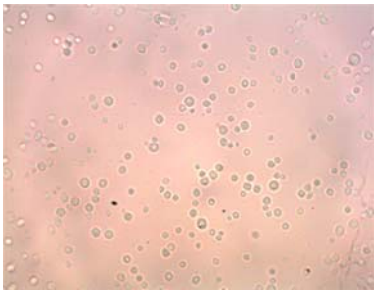


***Candida spencermartinsiae* R28**

***Candida haemulonii* R63**



***Candida oceani* R89**



***Debaryomyces fabryi* R100**

***Debaryomyces nepalensis* R305**

***Meyerozyma guillermondii* R340**

Fig. 2.2 Microscopic appearance of eight different marine yeast strains used for glucan extraction

2.2.3 Structural characterization of the cell wall glucans

Spectral data were collected on a Bruker Avance III 600 NMR spectrometer using a CH cryoprobe operating at 345 °K (72°C). 10-25 mg of the glucan was dissolved in 1 ml of DMSO-d_6 at 80°C. A few drops of trifluoroacetic acid-d (99.8% deuterated, Cambridge Isotope Laboratories) were added to the solution to shift the water and hydroxyl proton resonances downfield. NMR chemical shifts were referenced to the residual DMSO-d_6 multiplet proton resonance at 2.50 ppm. The NMR spectral collection and processing parameters are as follows: 25 ppm spectral width centered at 5.0 ppm, 32, 768 data points, 15 s relaxation delay, 32 scans, and 0.2 Hz exponential apodization. Branching frequency and side chain length were calculated as reported previously (Lowman et al., 2003).

2.2.4 Determination of biocompatibility

To determine the biocompatibility, XTT (2,3-Bis [2-Methoxy-4-Nitro-5-Sulfophenyl]-2H-Tetrazolium-5-Carboxanilide) assay was performed for assessing the glucan induced metabolic inhibition on HEp-2 cells. For this soluble yeast glucan was prepared following Williams et al. (1992) with slight modifications. 0.5 g of glucan was dissolved in 12.5 ml of Me_2SO containing 9 g of 8 M urea. In another flask, Me_2SO (12.5 ml) and concentrated H_2SO_4 (1.25 ml) were thoroughly mixed, and this mixture was added drop-wise to the previously mixed glucan-Me_2SO-urea solution with continuous stirring. This solution was heated to 100°C in a water bath with continuous stirring, and the reaction was carried out for 4 h. The solution was cooled to room temperature (28±2 °C), diluted in 500 ml of MilliQ water, and passed through a Millipore prefilter (1.2 µl) to remove microparticulate glucan. This glucan solution was purified with an ultrafiltration system using a 10,000 MW cut-off filter, pH was adjusted to 7 and concentrated by lyophilisation.

Approximately, 1×10^6 HEp-2 cells were inoculated into each well of a 96 well tissue culture plate containing MEM (minimal essential medium) supplemented with 10% fetal bovine serum (FBS) and incubated for 12 h at 37°C. After incubation, the cells were washed twice with phosphate buffered saline (PBS), and the medium was exchanged with fresh MEM containing various concentrations of the glucans (0.05 to 6.4 mg ml^{-1}). The cells were incubated for 24 h at 37°C, and the assays were performed following manufacturer's protocol (Cytotox-PAN I, Xenometrix, Germany). Briefly, each experimental glucan treated cells were incubated with 50 µl pre-warmed XTT at 37°C for 4 h, mixed the formazan formed in each well and absorbance was measured at 480 nm in a microplate reader (TECAN Infinite Tm, Austria) with a reference wavelength at 690 nm and the percentage inhibition of mitochondrial dehydrogenase activity and the IC_{50} were calculated (Scudiero et al., 1988).

2.2.5 Preparation of glucan incorporated diet

The experimental feeds were prepared by incorporating 0.2% homogenised glucan to a standard shrimp diet (Table 2.1) based on a previous study by Sajeevan et al. (2009) on standardisation of dose and frequency of administration of the immunostimulants in shrimp culture. Eight experimental feeds and a control feed (without glucan) were used for the study. The glucan diet prepared from different marine yeast strains are referred as YG20 (*C. parapsilosis* R20), YG23 (*H. werneckii* R23), YG28 (*C. spencermartinsiae* R28), YG63 (*C. haemulonii* R63), YG89 (*C. oceani* R89), YG100 (*D. fabryi* R100), YG305 (*D. nepalensis* R305) and YG340 (*M. guilliermondii* R340). The feed preparations were stored at -20°C until use.

Table 2.1 Composition of experimental feeds

Ingredients	Control feed (g)	Experimental glucan feed (g)
Fish meal powder	28	28
Prawn shell powder	20	20
Rice bran	10	10
Soybean meal	10	10
Ground nut oil cake	10	10
Refined wheat flour	20	19.8
Yeast glucan	0	0.2
Vitamin mineral mix (ml)	2	2

2.2.6 Experimental animals and rearing conditions

P. monodon post larvae (PL 10-15) with a mean body weight 15-20 mg and PCR-negative for WSSV were used for the present study. The PL were bought from a shrimp hatchery at Kannamali, Cochin, India and acclimatized to 30 L fibre reinforced plastic tanks containing 15‰ seawater for one week. Continuous aeration was provided in all tanks during the experiment. They were fed standard shrimp diet (Table 2.1) twice daily at a rate of 10-15% body weight and the unused feed and faecal matter were siphoned out daily. Water quality was maintained by 25% water exchange every second day just before feeding.

2.2.7 Experimental design and WSSV challenge

The experimental animals were randomly divided into nine groups of 25 *P. monodon* post larvae (PL 10-15). Triplicate tanks were maintained for each group. Eight experimental groups were fed glucan incorporated diets once in seven days based on the previous studies by Sajeevan et al. (2009) and rest of the days, the animals were fed the control diet. Group was fed the control diet alone, and all groups were fed twice daily. Five animals from each group were sampled after 45 days. Only those in the intermoult stage were sampled during the experiment. All the experimental groups were orally challenged with white spot syndrome virus WSSV by feeding WSSV infected

shrimp (*P. monodon*) tissue on the 46th day and thereafter maintained on respective feeding schedule for the following days. After 48 h, five animals each from all the groups were sampled for gene expression studies. At the same time the percentage survival of the PL of all the nine groups were also recorded up to 7th day post-challenge.

2.2.8 Gene expression analysis

2.2.8.1 Total RNA extraction

Total RNA was extracted from the whole tissue using TRI Reagent (Sigma) following the manufacturer's protocol. Briefly, the whole tissue was homogenized in 1 ml TRI Reagent. Samples were allowed to stand for 5 min at room temperature for the complete dissociation of the nucleoprotein complexes. Chloroform was added to the homogenate (0.2 ml per 1 ml TRI Reagent), vigorously shaken for 15 sec and allowed to stand for 2-15 min at room temperature. The following mixture was centrifuged at 12,000 x g for 15 min at 4°C. Centrifugation separates the mixture into three phases containing protein in red organic phase, DNA in interphase and RNA in colourless upper aqueous phase. The aqueous phase was transferred to a fresh tube and added 0.5 ml isopropanol per ml TRI Reagent. The samples were kept at room temperature for 5-10 min and then centrifuged at 12000 x g for 10 min at 4°C. The RNA precipitated out in the side and bottom of the tube was washed by adding 1 ml 75% ethanol per 1 ml of TRI Reagent. The samples were vortexed and centrifuged at 7500 x g for 5 min at 4°C. RNA pellets were air dried and dissolved in RNase free water at 55-60°C for 10-15 min.

Quantity and purity of the RNA were checked by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratio (A260:A280) $\geq$ 1.8 were used for further experiments.

2.2.8.2 Reverse transcription

First strand cDNA was generated in a 20 µl reaction volume containing 5 µg total RNA, 1x RT buffer, 2 mM dNTP, 2 µM oligo d(T_{20}), 20 U of RNase inhibitor and 100 U of Mu-MLV reverse transcriptase. The reaction was conducted at 42°C for 1 h subsequently an inactivation step at 85°C for 15 min.

2.2.8.3 Semi-quantitative RT-PCR analysis

Expression of the target genes when supplemented with different glucans were analyzed employing semi-quantitative RT-PCR. β-actin and elongation factor (ELF) were used as internal controls for expression analysis. Five times diluted cDNA was used and PCR amplification of 1 µl of cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primers and 1U Taq DNA polymerase. Amplification was performed using the respective primers of the target genes (Table 2.2). The thermal profile used for the amplification of target genes was an initial denaturation at 94°C for 2 min followed by 28 cycles of denaturation at 94°C for 15 s, extension at 68°C for 30 s and a final extension at 68°C for 10 min. The annealing temperature varied for different genes.

2.2.8.4 Agarose gel electrophoresis

The PCR products were visualized on 1.5% agarose gel. Agarose gel was prepared in 1x TBE buffer (Tris-base - 10.8 g, Boric acid - 5.5 g, 0.5 M EDTA - 4 ml, double distilled water - 100 ml, pH - 8.0). Ethidium bromide (2 µl) was added to the melted agarose. After cooling to 45°C, the agarose was poured on to gel tray and was kept to solidify. The gel try was transferred into a buffer tank and was immersed in 1x TBE buffer. Five microlitre of PCR product was mixed with 3 µl of 6 x gel loading buffer (1 % Bromophenol blue - 250 µl, 1 % xylene

cyanol - 250 µl, glycerol - 300 µl, double distilled water - 200 µl) and loaded onto the well. Electrophoresis was performed at a voltage of 3-5 volt/cm till the bromophenol blue dye front migrates to the middle of the gel. The gel was visualized on a UV transilluminator and documentation was done.

2.2.8.5 Expression analysis of target genes

Semi-quantitative RT-PCR method was employed to analyse the expression profile of AMP genes (ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5); immune genes (α-2-macroglobulin, astakine, caspase, catalase, glutathione peroxidase, glutathione-S-transferase, haemocyanin, peroxinectin, pm Cathepsin C, prophenoloxidase, superoxide dismutase, transglutaminase) and WSSV genes (DNA polymerase, endonuclease, immediate early gene, latency related gene, protein kinase, thymidine kinase and VP28). β-actin and ELF were used as internal controls. Expression profiles of the target genes when supplemented with different glucans were analyzed pre- and post-challenge WSSV. Shrimps fed standard diet and challenged with WSSV served as the positive control and the unchallenged shrimps as the negative control. The PCR cycles had been optimized so that the target and housekeeping gene amplification were at logarithmic phase. The PCR reaction of each sample was carried out in triplicates and the amplicons were analyzed using agarose gel electrophoresis. The intensity of the gel bands was measured using Image J analysis software to assess the level of expression.

2.2.9 Statistical analysis

All the tests were performed in triplicates, and the data of all experiments were recorded as mean ± SD. In order to determine significant differences between the various experimental groups, the results were analyzed using One Way Analysis of Variance (ANOVA) and Duncan's multiple comparison of the

means using SPSS 21.0 for windows. Significant differences were considered at $p < 0.05$. The IC_{50} values were calculated by probit analysis of SPSS.

Table 2.2 Primers used for the study

Target gene	Sequence (5'-3')	Product size (bp)	Annealing Temp.(°C)
1. Control genes			
ELF	F-atggttgtcaactttgcccc R-ttgacctccttgatcacacc	600 bp	60
Beta actin	F-cttgtggttgacaatggctccg R-tggtgaaggagtagccacgctc	520 bp	55
II. AMP genes			
ALF	F-caagggtgggaggctgtgg R-tgagctgagccactggttgg	300 bp	62
Crustin-1	F-cgcacagccgagagaaacactatcaagat R-ggcctatccctcagaacccagcacg	456 bp	60
Crustin-2	F-tgttcccacgacttcaagtgtgc R-caaagattcaactaaataaacag	299 bp	60
Crustin-3	F-tccctggaggtcaattgagtg R-agtcgaacatgcaggcctatcc	233 bp	60
Penaeidin-3	F-aggatatcatccagttcctg R-acctacatcctttccacaag	240 bp	60
Penaeidin-5	F-acctgaccctcacctgcagaggcc R-ttcgttgtcttctccatcaacc	300 bp	60
III. Immune genes			
Alpha-2-macroglobulin	F-atggccaatcccgagaggtacctactg R-cggcgttgggaatgttgta	500 bp	65
Asatakine	F-gtcgcgcatttaacaaggag R- ccctgtggattgagctcact	455 bp	56
Caspase	F-ggaggaacctgcgaagaac R-agcgtcgagtggatgtaagg	825bp	57
Catalase	F-actcccattgctgttcgt R-atcccaatttccttcttctg	130 bp	47
Glutathione Peroxidase	F-agtcgatgtcaacgggtcaac R-gctgaacctcttaaacgcctg	985 bp	57
Glutathione-S-transferase	F-ttcgccggagacaagctaacc R-gcgatcgtaaactgagcgtac	247 bp	57
Haemocyanin	F-gtcgacgaacttcactggga R-gttcagtgtcatcaacggca	598 bp	56
Peroxinectin	F-cgaagcttcttgcaactacca R-gcaggctgattaaactggctt	547 bp	56
Pm Cathepsin C	F- gattctgaccagcaaccacca R-tacaggctccatagtaacctccaa	240 bp	56
Prophenol oxidase	F-tggcactggcacttgatcta R-gcgaaagaacacagggtctct	590 bp	56
Superoxide dismutase (cytosolic MnSOD)	F-cgaggcttgcgcgtcac R-tttggttgccccgaggagtc	924 bp	61

Transglutaminase	F-tgggycttcgggcagtt R-cgaagggcacgtcgtac	627 bp	56
IV. WSSV genes			
DNA polymerase	F-tgggaagaaagatgcgagag R-ccctccgaacaacatctcag	586 bp	54
Endonuclease	F-tgacgaggaggattgtaaag R –ttatggttctgtatttgagg	408 bp	50
Immediate early gene	F-gactctacaaatctctttgcca R-ctacctttgcaccaattgctag	502 bp	54
Latency related gene	F-cttgtgggaaaagggtcctc R-tcgtcaaggcttacgtgtc	647 bp	53
Protein kinase	F-tggagggtggggaccaacggacaaaac R-caaattgacagtagagaattttgcac	512 bp	55
Thymidine kinase	F-gagcagccatacgggtaaac R-gcgagcgtctaccttaatcc	412 bp	54
VP28	F-ctgctgtgattgctgtattt R-cagtgccagagtaggtgac	555 bp	54

2.3 Results

2.3.1 NMR spectra and glucan yield of different marine yeast isolates

The yield of glucan extracted from various marine yeasts ranged from 12.5 to 27.5% with the minimum from *C. spencermartinsiae* (R28) and the maximum from *H. werneckii* (R23) and *D. fabryi* (R100) (Fig. 2.3). The Proton NMR spectra of carbohydrate region of water insoluble particulate glucans from eight different marine yeast isolates viz., *C. parapsilosis* R20, *Hortaea werneckii* R23, *C. spencermartinsiae* R28, *C. haemulonii* R63, *C.oceani* R89, *D. fabryi* R100, *D. nepalensis* R305 and *M. guilliermondii* R340 is shown in Fig. 2.4. The major component of these glucan isolates were identified as (1-6)-branched (1-3)-β-D-glucan. However, there were differences in the branching frequency (1-6-branching) and the side chain length of the glucans. Six of the glucans were found to be linear (R20, R28, R63, R89, R305, R340) and one (R100) was found to be cyclic. Structure of R23 could not be elucidated from the NMR data (Table 2.3).

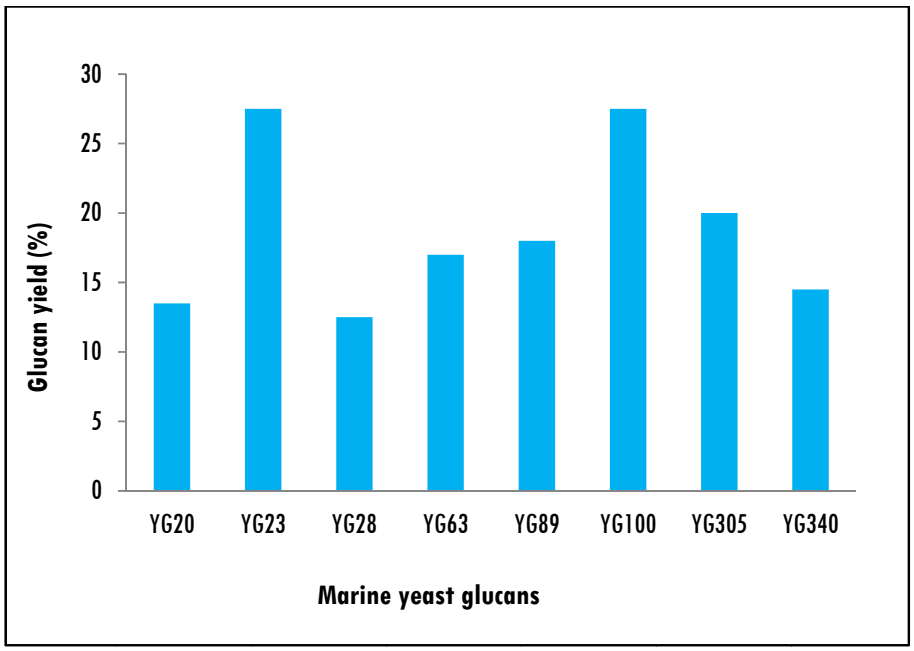
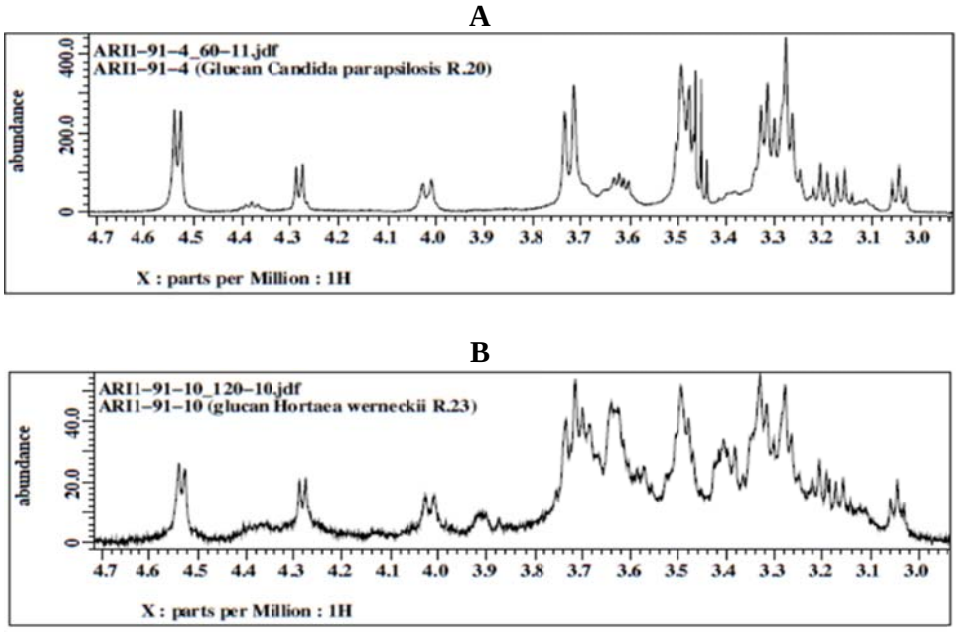


Fig. 2.3 Glucan yield from different marine yeast isolates



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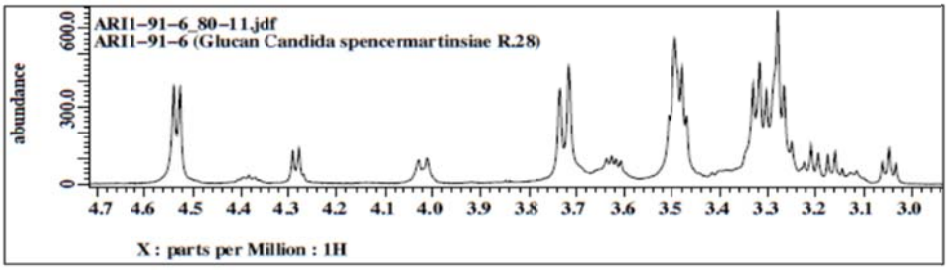
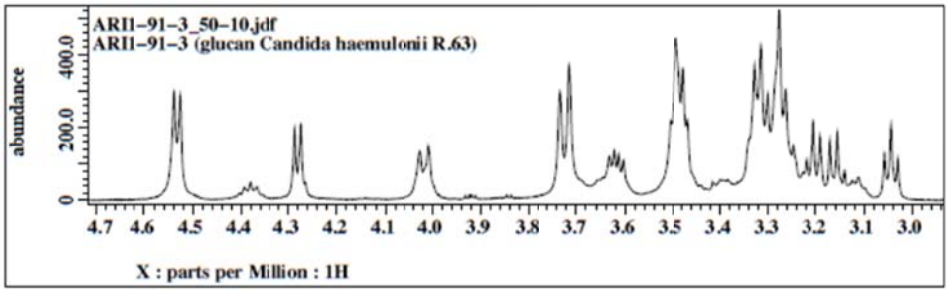
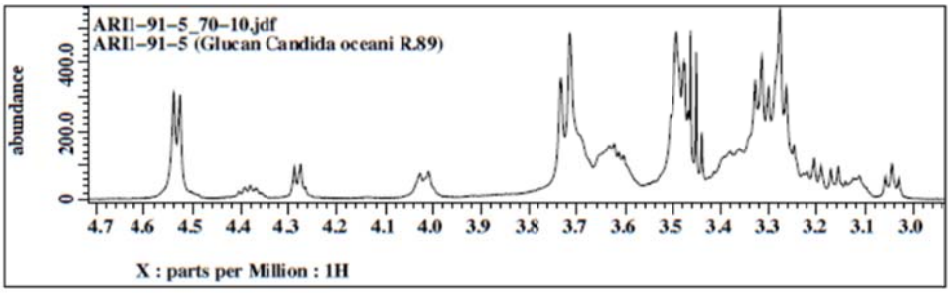

ARII-91-6_80-11.jdf
ARII-91-6 (Glucan Candida spencermartinsiae R.28)
abundance
600.0
300.0
0
4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0
X : parts per Million : 1H

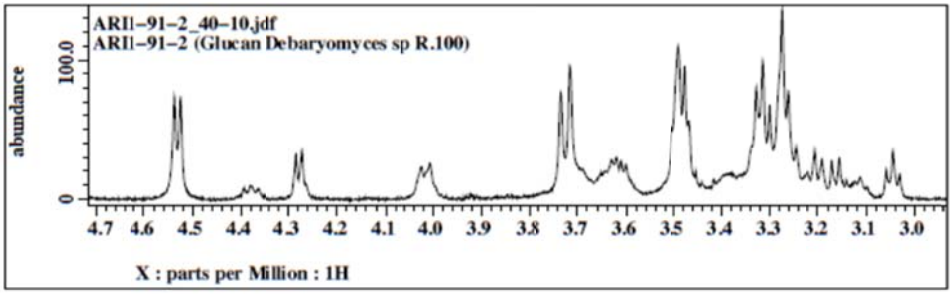
D


ARII-91-3_50-10.jdf
ARII-91-3 (glucan Candida haemulonii R.63)
abundance
400.0
200.0
0
4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0
X : parts per Million : 1H

E


ARII-91-5_70-10.jdf
ARII-91-5 (Glucan Candida oceani R.89)
abundance
400.0
200.0
0
4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0
X : parts per Million : 1H

F


ARII-91-2_40-10.jdf
ARII-91-2 (Glucan Debaryomyces sp R.100)
abundance
100.0
0
4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0
X : parts per Million : 1H

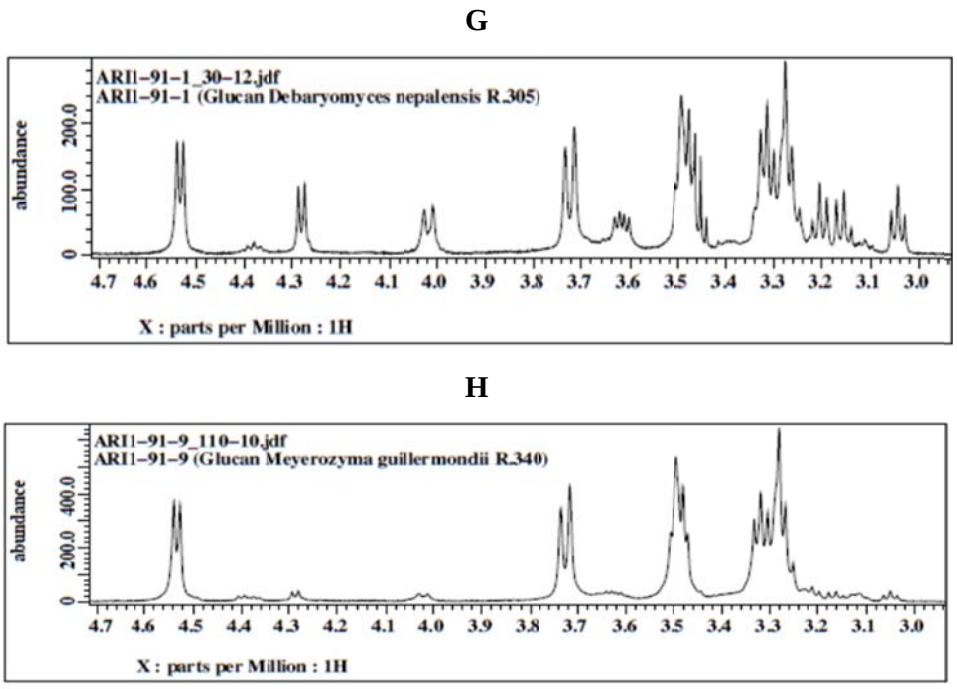


Fig. 2.4 The Proton NMR spectra of carbohydrate region of water insoluble particulate glucan from eight different marine yeast isolates (A) *Candida parapsilosis* R20 (B) *Hortaea werneckii* R23 (C) *Candida spencermartinsiae* R28 (D) *Candida haemulonii* R63 (E) *Candida oceani* R89 (F) *Debaryomyces fabryi* R100 (G) *Debaryomyces nepalensis* R305 (H) *Meyerozyma guilliermondii* R340

Table 2.3 Characteristics of the glucans isolated from various marine yeasts

Marine yeast Isolates	Glucan yield (%)*	Side chain length	Branching frequency	Structural arrangement
Candida parapsilosis (R20)	13.5	5.9	15.2	Linear
Hortaea werneckii (R23)	27.5	5.6	9.7	-
Candida spencermartinsiae (R28)	12.5	5.1	15.1	Linear
Candida haemulonii (R63)	17	5.7	9.3	Linear
Candida oceani (R89)	18	4.1	11.7	Linear
Debaryomyces fabryi (R100)	27.5	4.4	5.8	Cyclic
Debaryomyces nepalensis (R305)	20	7.9	15.1	Linear
Meyerozyma guilliermondii (R340)	14.5	2.8	25.6	Linear

* Expressed as the percentage of glucan extracted from dried yeast biomass

2.3.2 Biocompatibility of yeast glucans

Cytotoxicity assay (XTT) of the eight glucans revealed its biocompatibility with eukaryotic cells. All glucans showed an IC_{50} between 6.07 and 7.86 mg ml^{-1} with the lowest in YG20 (6.07 ± 2.92 mg ml^{-1}), followed by YG340 (6.09 ± 4.16 mg ml^{-1}), YG305 (6.78 ± 4.84 mg ml^{-1}), YG63 (7.22 ± 6.03 mg ml^{-1}); YG23 (7.57 ± 6.24 mg ml^{-1}); YG100 (7.63 ± 7.11 mg ml^{-1}); YG89 (7.78 ± 4.87 mg ml^{-1}) and YG28 (7.86 ± 6.60 mg ml^{-1}) (Fig. 2.5).

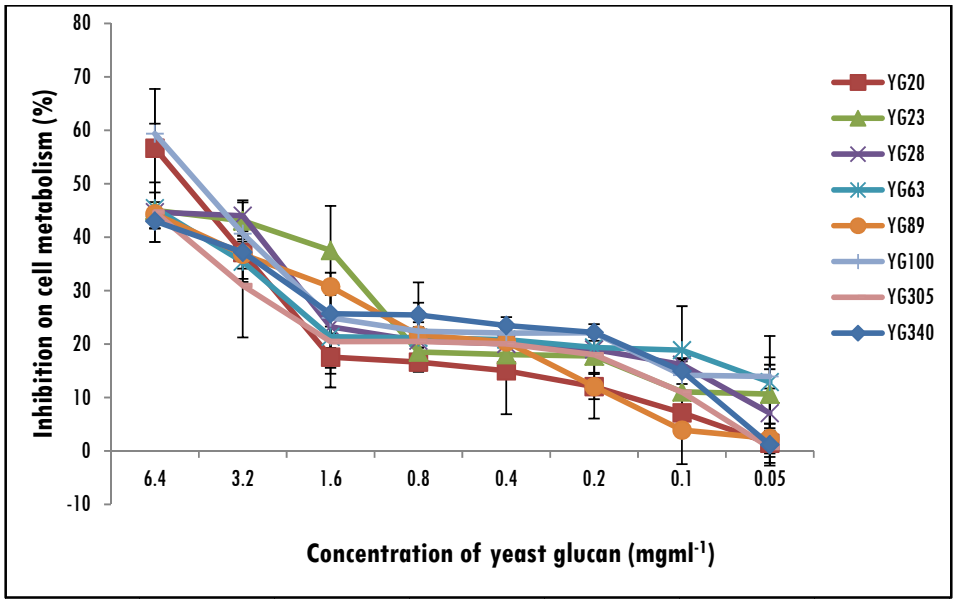


Fig. 2.5 Biocompatibility assay of yeast glucan on HEp-2 cells. The percentage inhibition on cell metabolism was calculated based on mitochondrial dehydrogenase activity using XTT assay (n = 3)

2.3.3 Estimation of the immunostimulant potential of yeast glucans

Gene expression profiling and post-challenge survival data showed that the glucans extracted from eight different marine yeasts used in the present study possessed immunostimulatory activities. Post-challenge survival was found to be significantly higher in glucan administered groups than the control

group. Among the various treated groups, *H. werneckii* R23 (YG23) glucan fed group showed the highest survival rate (70.27%) followed by YG20 from *C. parapsilosis* R20 (66.66%), YG28 from *C. spencermartinsiae* R28 (60.97%), YG89 from *C. oceani* R89 (58.53%), YG100 from *D. fabryi* R100 (54.05%), YG63 from *C. haemulonii* R63 (48.64%), YG305 from *D. nepalensis* R305 (45.7%) and YG340 from *M. guilliermondii* R340 (43.24%) (Fig. 2.6).

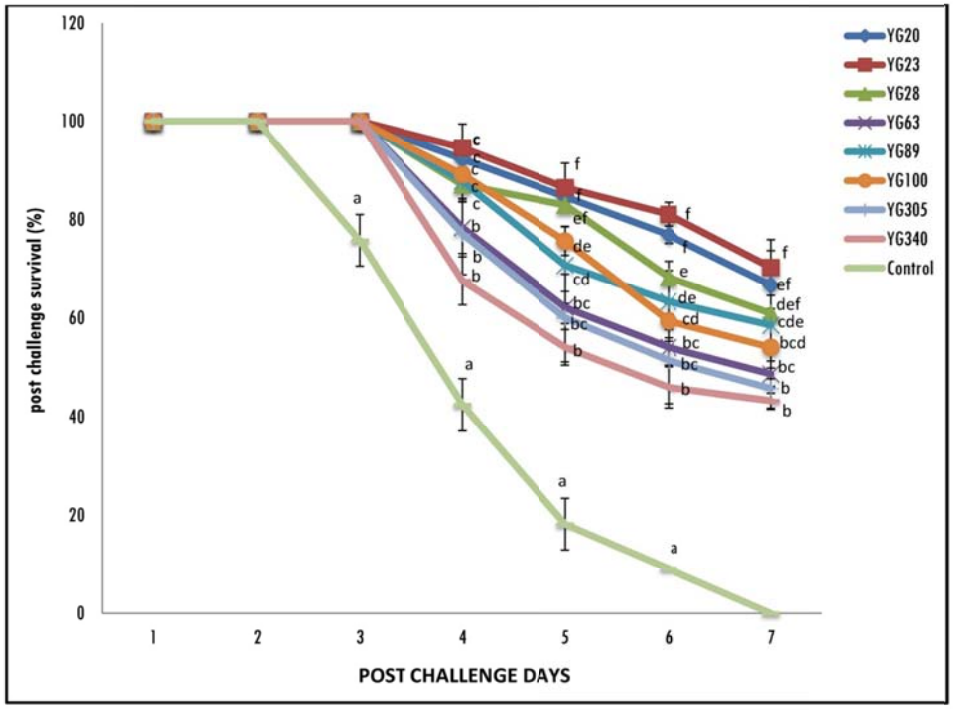


Fig. 2.6 Post challenge survival of *P. monodon* post larvae when fed with different marine yeast glucan incorporated diets and challenged with WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group, Control – Control feed administered group)

2.3.4 Expression profile of control genes in *P. monodon* post larvae in response to various marine yeast glucan and WSSV challenge

Expression profile of two control genes viz., β-actin and ELF were analyzed (Fig. 2.7 - 2.9).

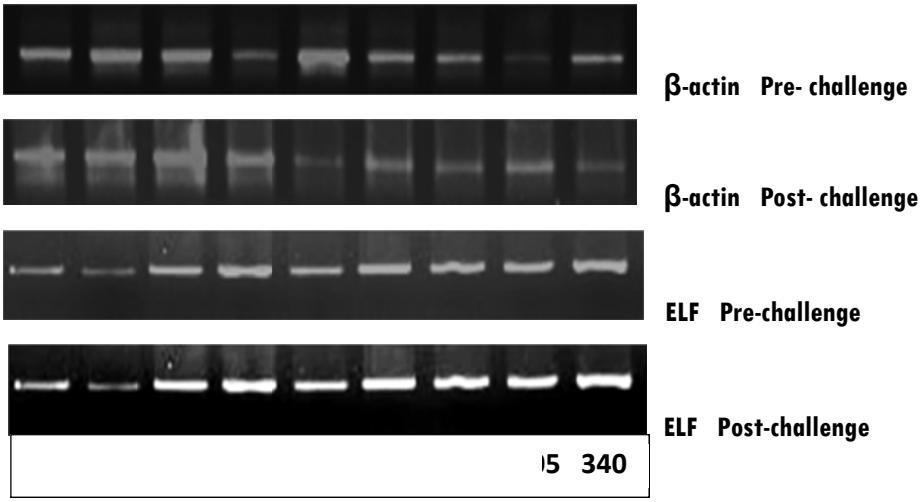


Fig. 2.7 Expression profile of β-actin and ELF (internal control genes) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV (Agarose gel electrophoretogram)

(C – Control feed administered group, 20 – Yeast glucan 20 administered group, 23 – Yeast glucan 23 administered group, 28 – Yeast glucan 28 administered group, 63 – Yeast glucan 63 administered group, 89 – Yeast glucan 89 administered group, 100 – Yeast glucan 100 administered group, 305 – Yeast glucan 305 administered group, 340 – Yeast glucan 340 administered group)

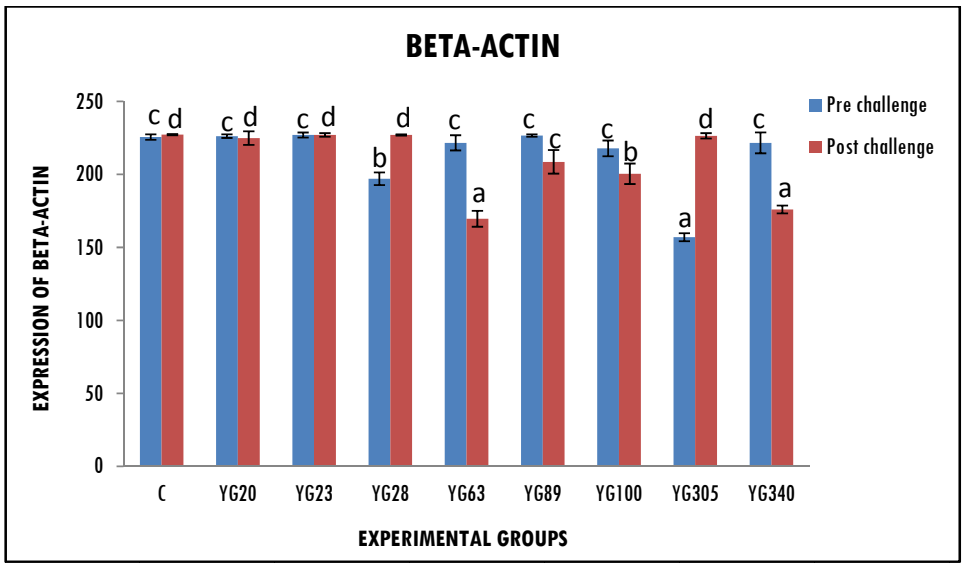


Fig. 2.8 Expression profile of β-actin (control gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

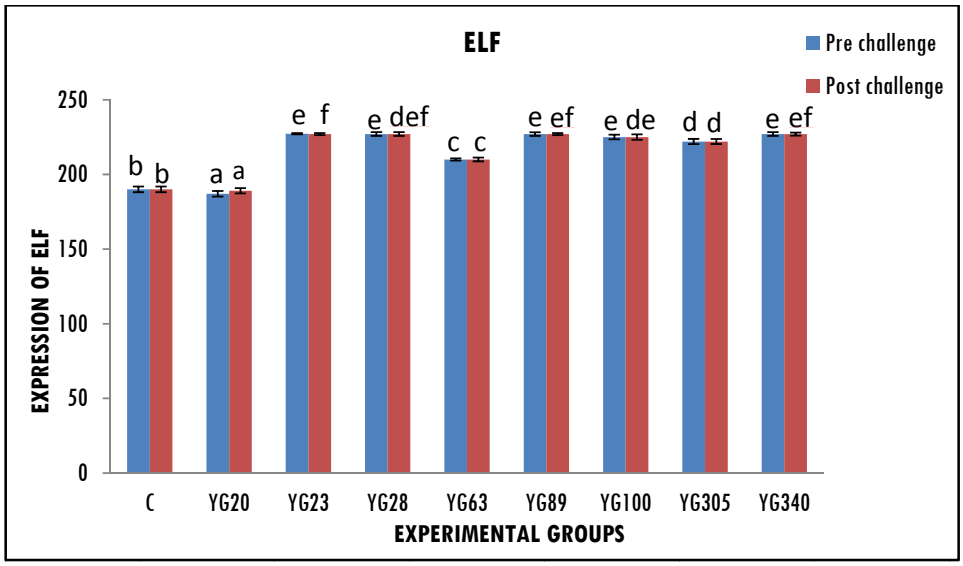


Fig. 2.9 Expression profile of ELF (control gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

2.3.5 Expression profile of AMP genes in *P. monodon* post larvae in response to various marine yeast glucan and WSSV challenge

Glucans YG20, YG23, YG28 and YG100 were found to be the effective glucans in terms of the AMP expression in shrimps. Glucan administration was found to up-regulate all the AMP genes, especially the crustins and anti-lipopolysaccharide factor. There was significant down-regulation of the AMPs in the control shrimps post challenge 48 h with WSSV. Penaeidin-5 was the only AMP gene that did not respond to any of the experimental glucan diets post-challenge, except its expression in YG28 pre- challenge (Fig. 2.10 & Fig. 2.11-2.16).

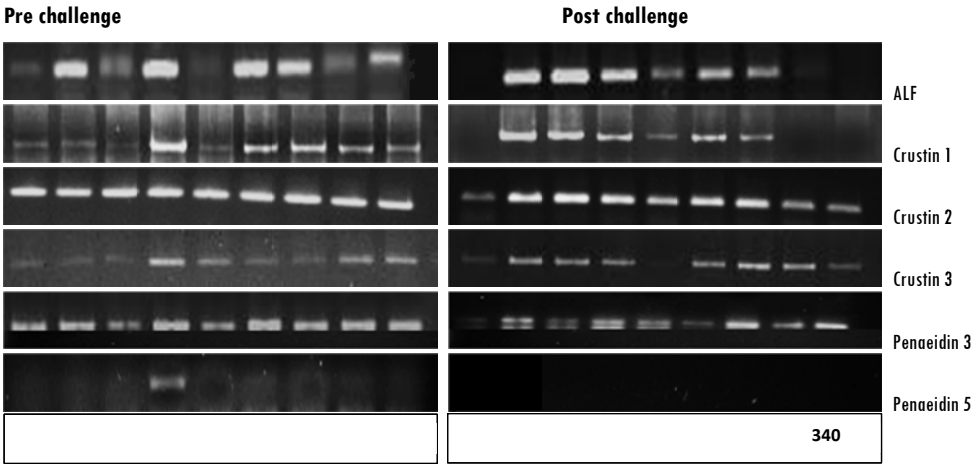


Fig. 2.10 Expression profile of AMP genes (ALF, crustin-1, crustin-2, penaeidin-3, and penaeidin-5) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV (Agarose gel electrophoretogram).

(C – Control feed administered group, 20 – Yeast glucan 20 administered group, 23 – Yeast glucan 23 administered group, 28 – Yeast glucan 28 administered group, 63 – Yeast glucan 63 administered group, 89 – Yeast glucan 89 administered group, 100 – Yeast glucan 100 administered group, 305 – Yeast glucan 305 administered group, 340 – Yeast glucan 340 administered group)

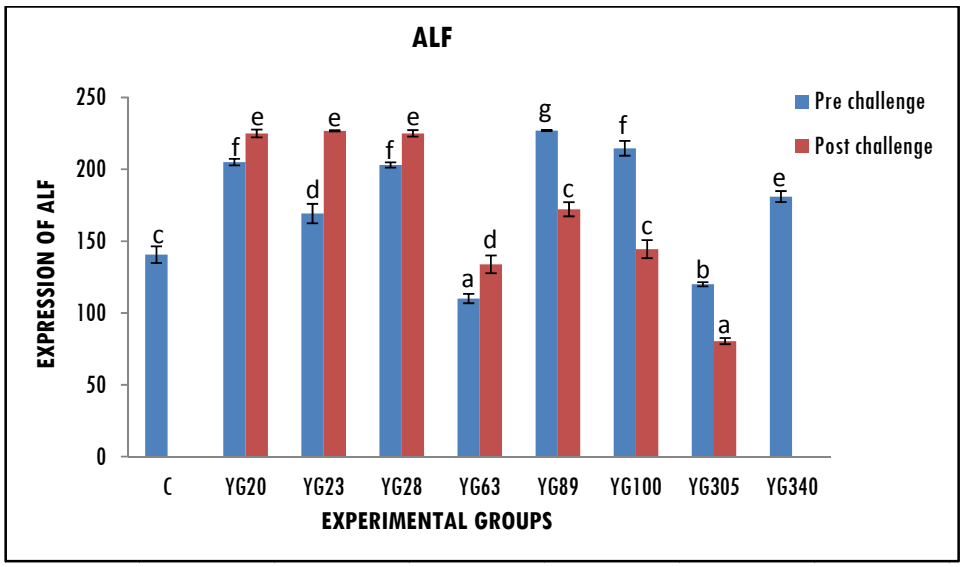


Fig. 2.11 Expression profile of ALF (AMP gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

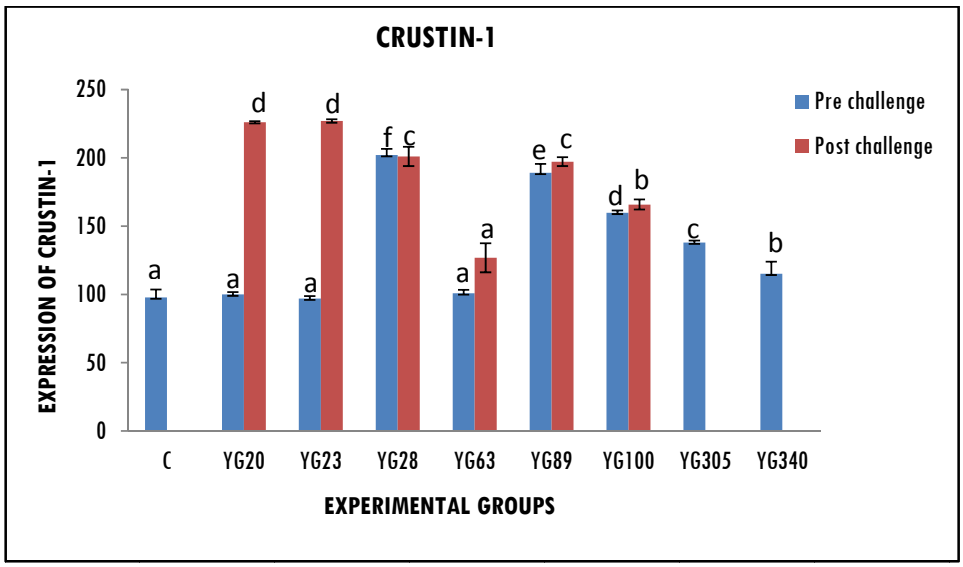


Fig. 2.12 Expression profile of Crustin-1 (AMP gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

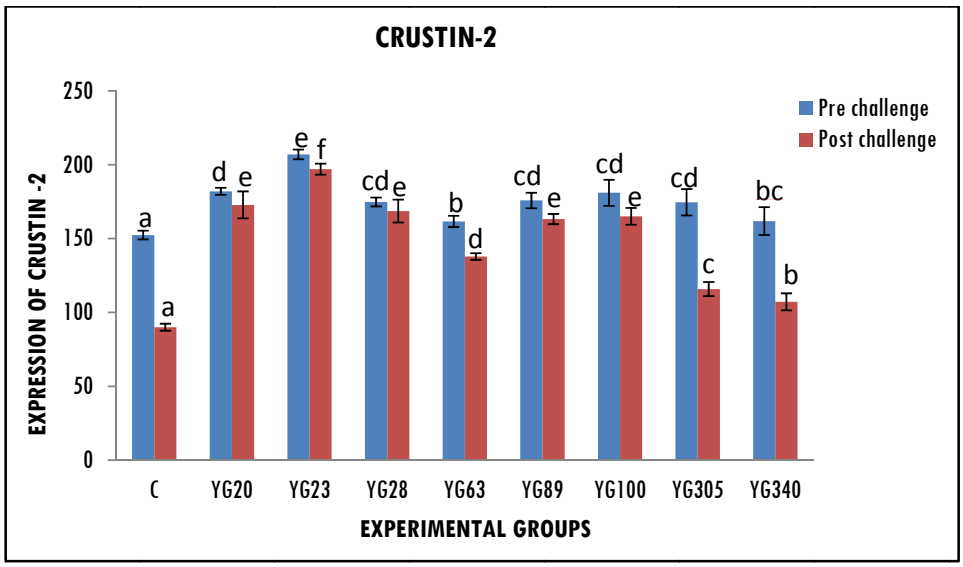


Fig. 2.13 Expression profile of Crustin-2 (AMP gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

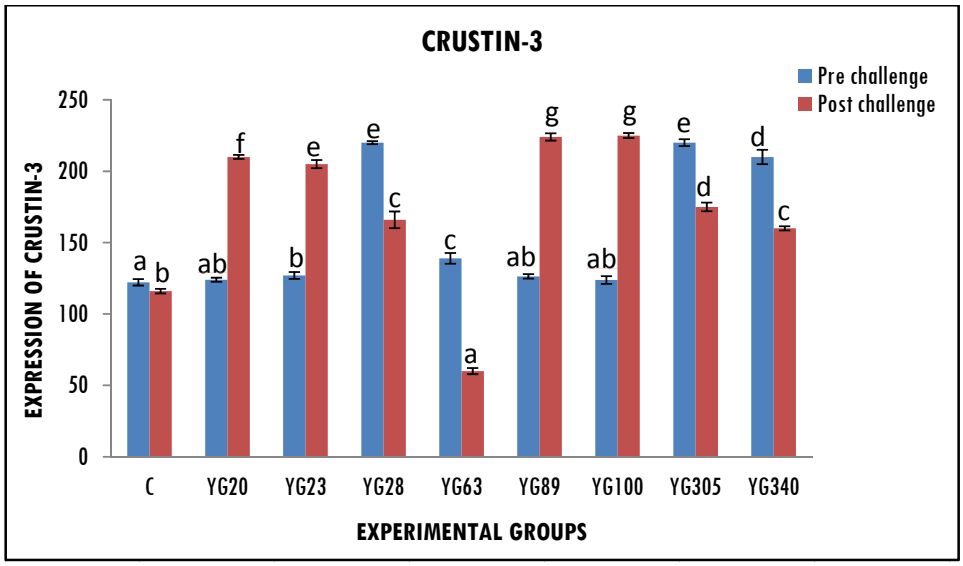


Fig. 2.14 Expression profile of Crustin-3 (AMP gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(Control – Control feed administered group , YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

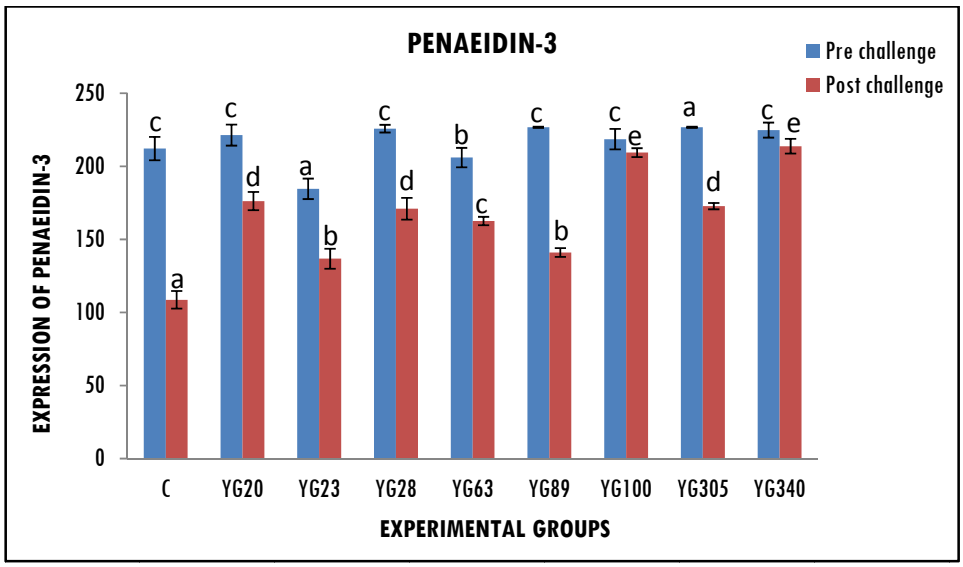


Fig. 2.15 Expression profile of Penaeidin-3 (AMP gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

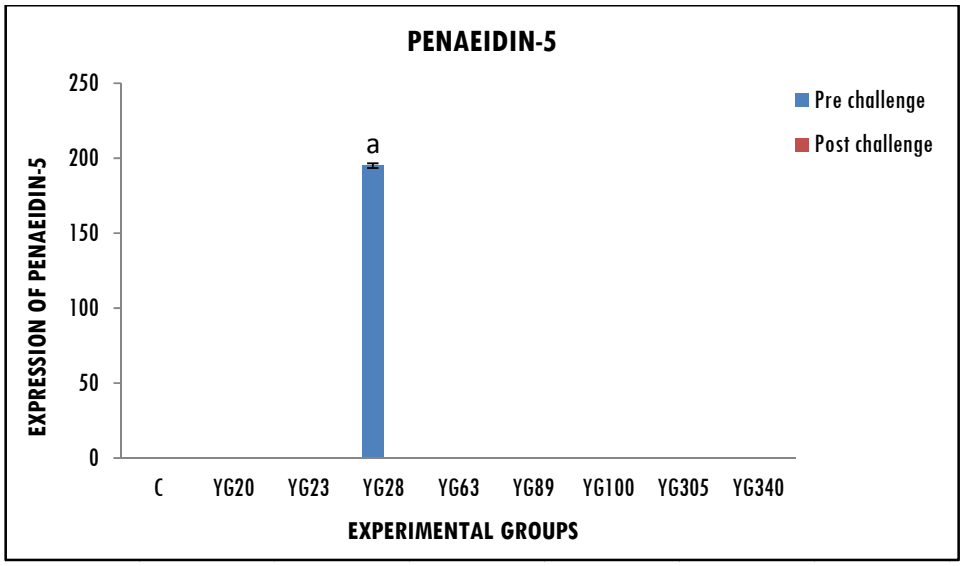


Fig. 2.16 Expression profile of Penaeidin-5 (AMP gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

2.3.6 Expression profile of immune genes in *P. monodon* post larvae in response to various marine yeast glucans and WSSV challenge

Expression profile of the 12 immune related genes other than the AMPs was studied in response to different glucan treatments and WSSV challenge (Fig. 2.17 & Fig. 2.18-2.24). Out of these seven genes were differentially expressed pre- and post-challenge. However, five immune genes viz., glutathione peroxidase, transglutaminase, catalase, caspase and superoxide dismutase were not expressed in the post larval stage both pre- and post-challenge. Furthermore, yeast glucans YG20 and YG23 were found to be convincing in terms of immune gene response in shrimps. Though, cathepsin and proPhenol oxidase were found to be up-regulated in YG20 and YG23 administered shrimps; α-2-macroglobulin was found to be down-regulated in these groups. Generally, all the genes were down-regulated in the control post-challenge WSSV.

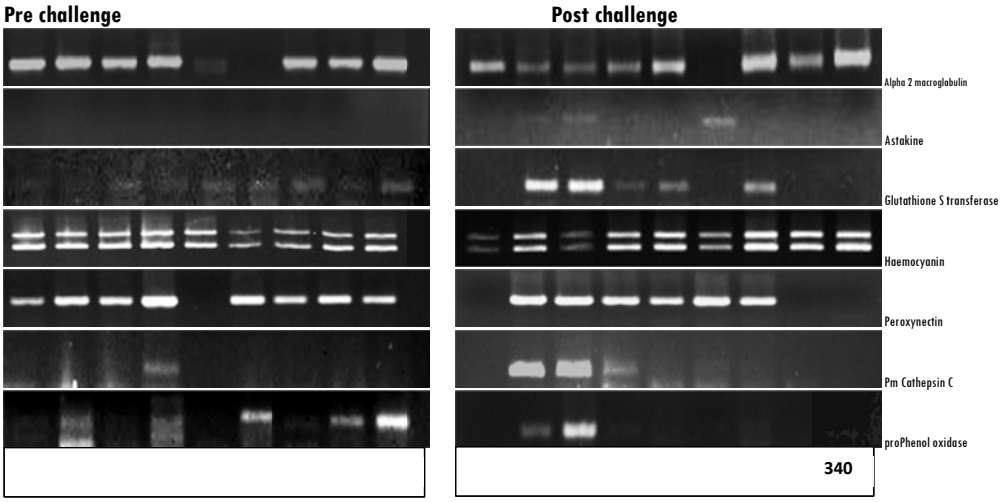


Fig. 2.17 Expression profile of immune genes (α-2-macroglobulin, astakine, glutathione-S-transferase, haemocyanin, peroxinectin, Pm Cathepsin C and proPhenol oxidase) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucan pre- and post-challenge WSSV (Agarose gel electrophoretogram).

(C – Control feed administered group, 20 – Yeast glucan 20 administered group, 23 – Yeast glucan 23 administered group, 28 – Yeast glucan 28 administered group, 63 – Yeast glucan 63 administered group, 89 – Yeast glucan 89 administered group, 100 – Yeast glucan 100 administered group, 305 – Yeast glucan 305 administered group, 340 – Yeast glucan 340 administered group)

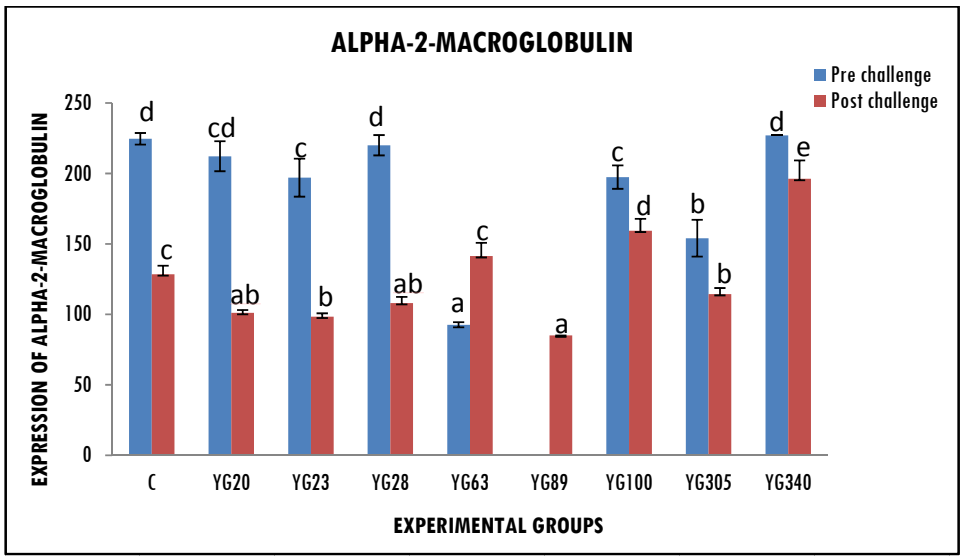


Fig. 2.18 Expression profile of α-2-macroglobulin (immune gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

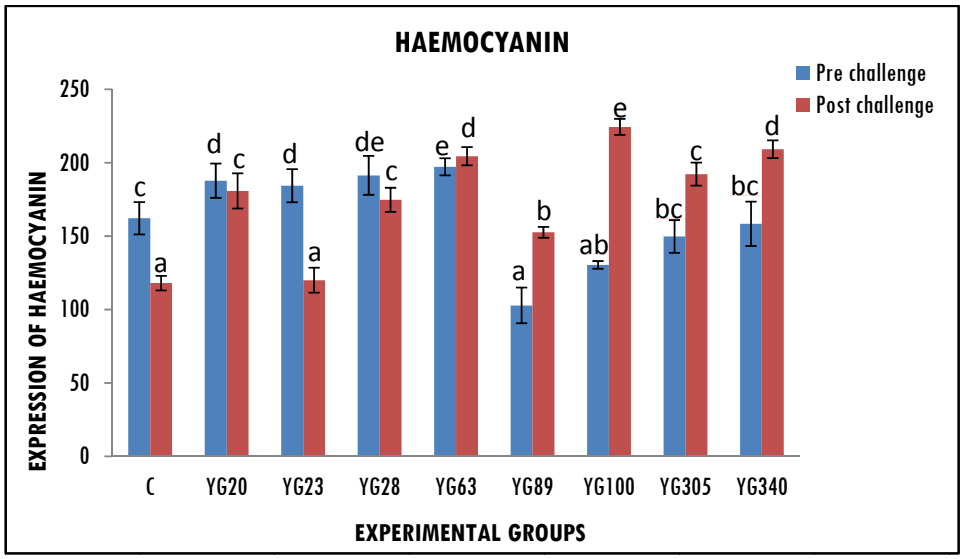


Fig. 2.19 Expression profile of haemocyanin (immune gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

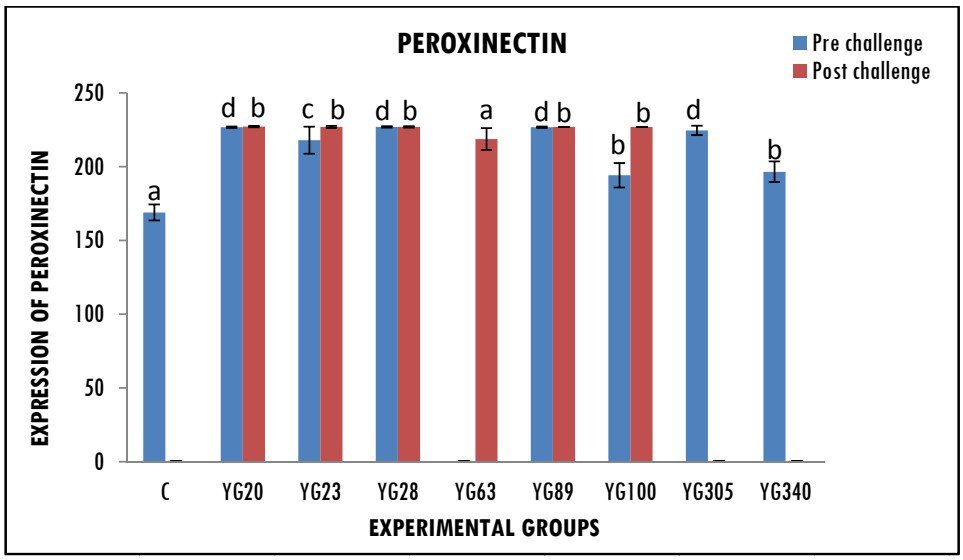


Fig. 2.20 Expression profile of peroxinectin (immune gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

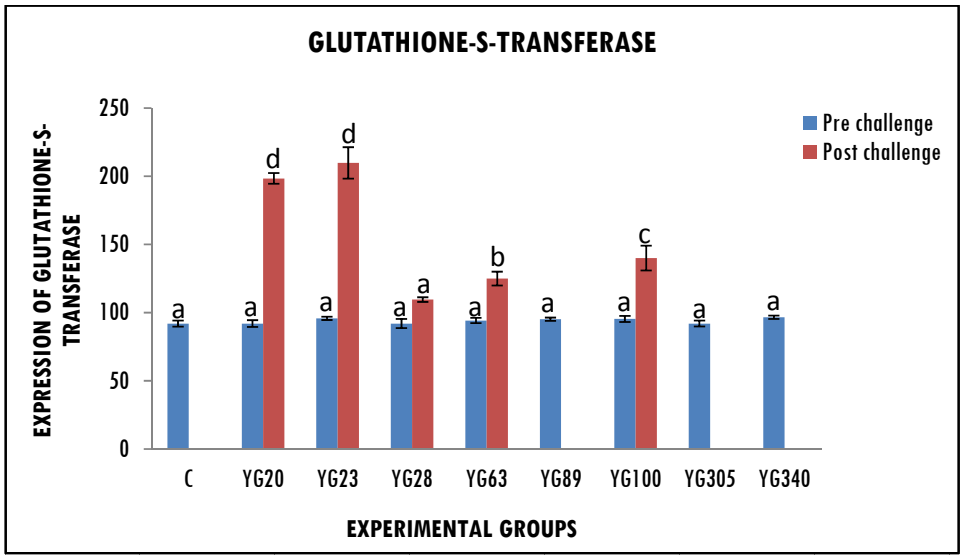


Fig. 2.21 Expression profile of glutathione-S-transferase (immune gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

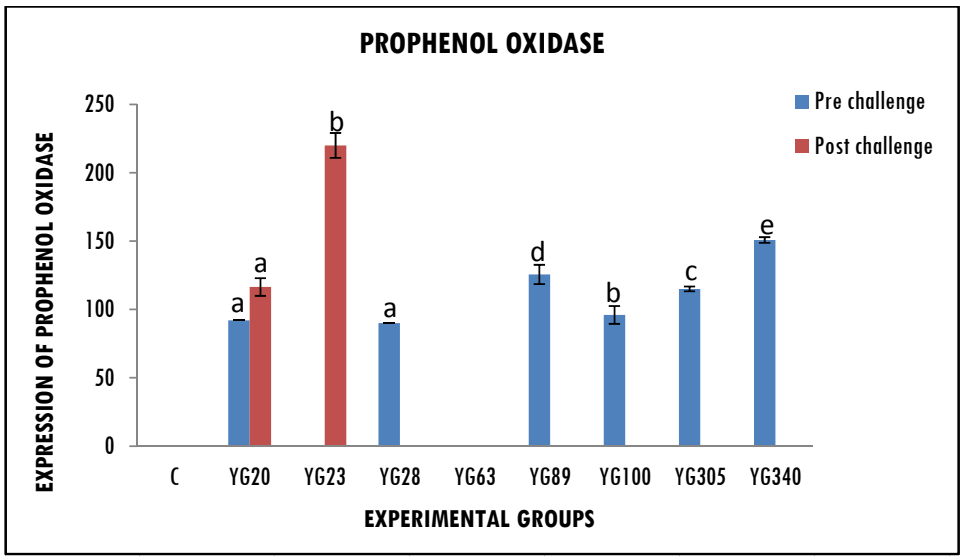


Fig. 2.22 Expression profile of proPhenol oxidase (immune gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

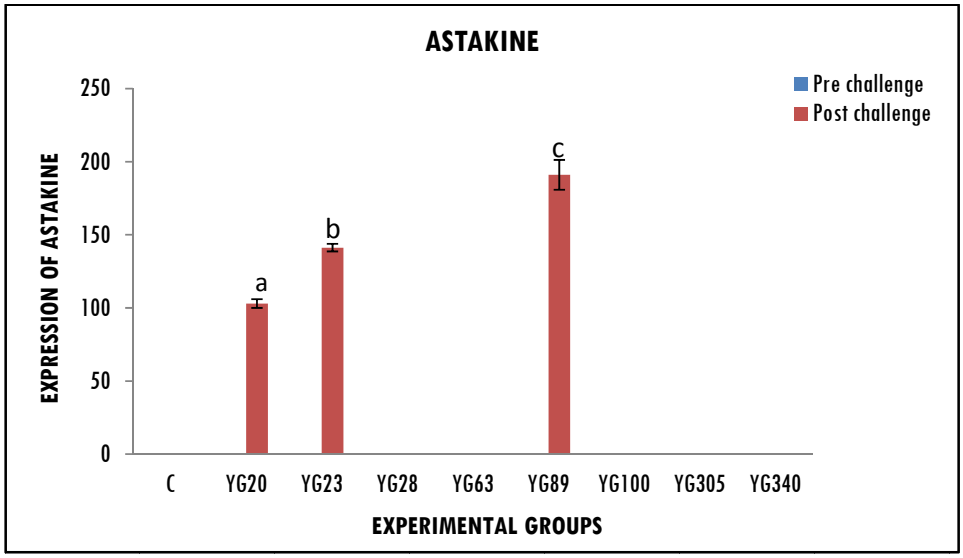


Fig. 2.23 Expression profile of astakine (immune gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

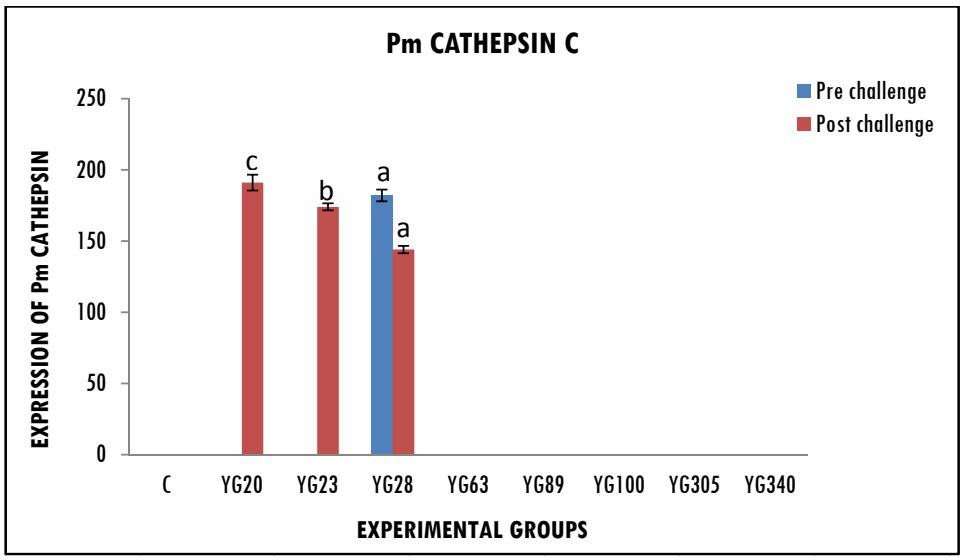


Fig. 2.24 Expression profile of Pm Cathepsin C (immune gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

2.3.7 Expression profile of WSSV genes in *P. monodon* post larvae in response to various marine yeast glucans on challenge with WSSV

The expression of seven WSSV related genes (DNA polymerase, endonuclease, immediate early gene, latency related gene, protein kinase, thymidine kinase and VP28) could be detected in the positive (WSSV challenged) control group of *P. monodon* post larvae and that confirmed WSSV infection in the experimental groups (Fig. 2.25 & 2.26-2.32). The intensity of WSSV infection was found to vary in each marine yeast glucan treated group in terms of the various WSSV genes analysed. However, the intensity of infection was found to be less in the experimental groups when compared to the control group. Among the YG treated groups, the expression of WSSV genes were

comparatively lower in YG23 fed group followed by YG20, YG28, YG89 and YG100. Meanwhile, YG20, YG23 and YG28 were found to be the convincing based on lesser WSSV gene transcripts. Immediate early gene was least expressed in YG23 and YG28 and latency related gene in YG20. Additionally, expression of protein kinase was minimum in YG23 and thymidine kinase and VP28 in YG28 glucan administered groups. Hence, in terms of enhanced immune gene expression and better post-challenge survival, glucans YG20, YG23 and YG28, were identified as potent immunostimulants.

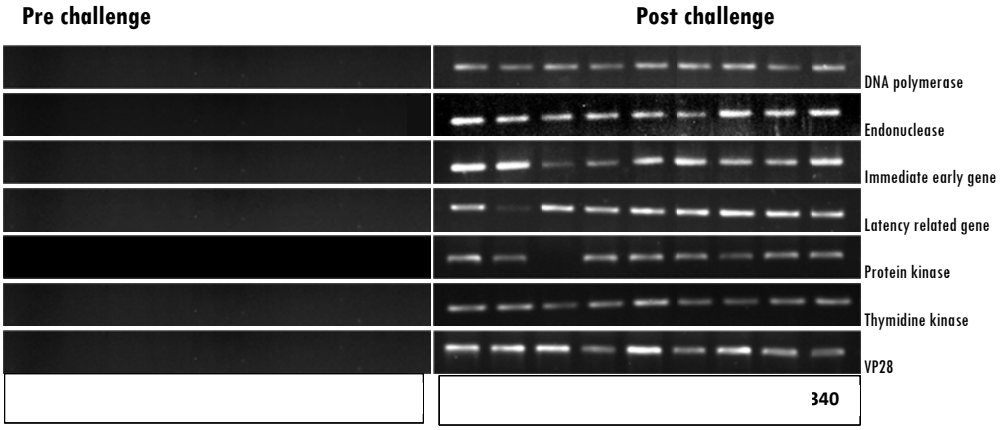


Fig. 2.25 Expression profile of WSSV genes (DNA polymerase, endonuclease, immediate early gene, latency related gene, protein kinase, thymidine kinase and VP28) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucan pre- and post-challenge WSSV (Agarose gel electrophoretogram)

(C – Control feed administered group, 20 – Yeast glucan 20 administered group, 23 – Yeast glucan 23 administered group, 28 – Yeast glucan 28 administered group, 63 – Yeast glucan 63 administered group, 89 – Yeast glucan 89 administered group, 100 – Yeast glucan 100 administered group, 305 – Yeast glucan 305 administered group, 340 – Yeast glucan 340 administered group)

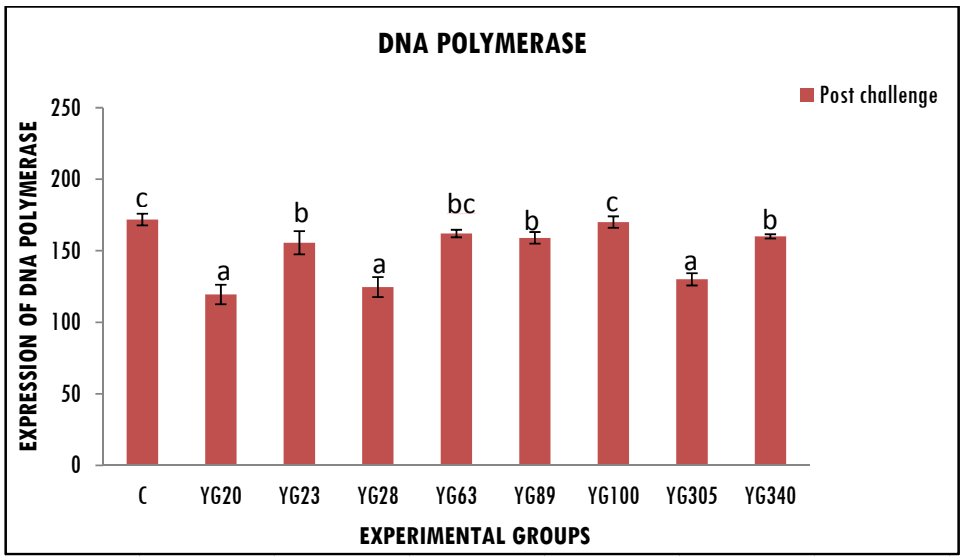


Fig. 2.26 Expression profile of DNA polymerase (WSSV gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

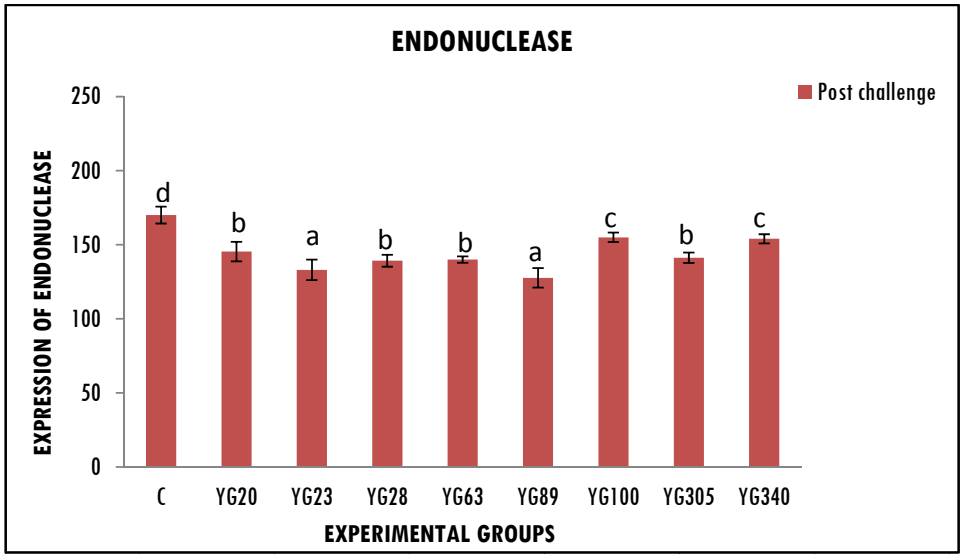


Fig. 2.27 Expression profile of endonuclease (WSSV gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post- challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

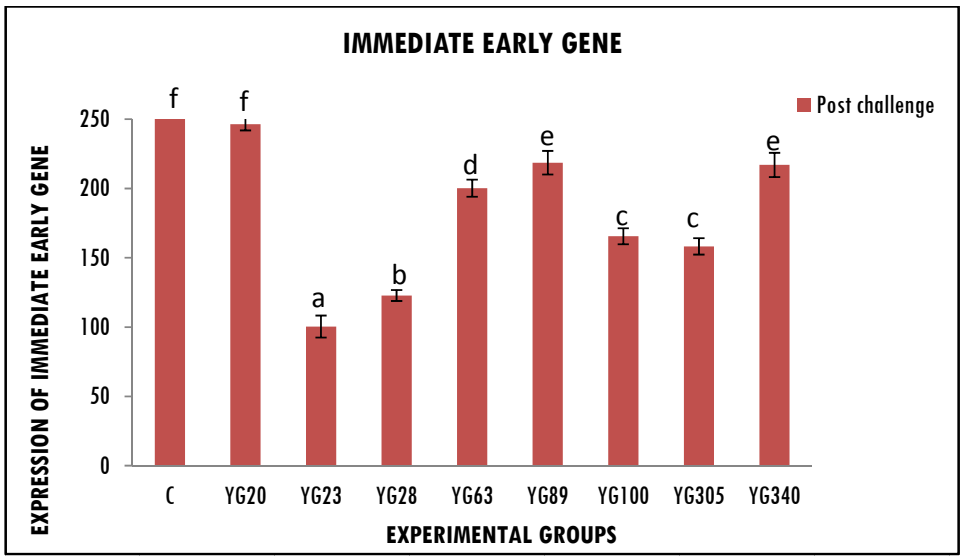


Fig. 2.28 Expression profile of immediate early gene (WSSV gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

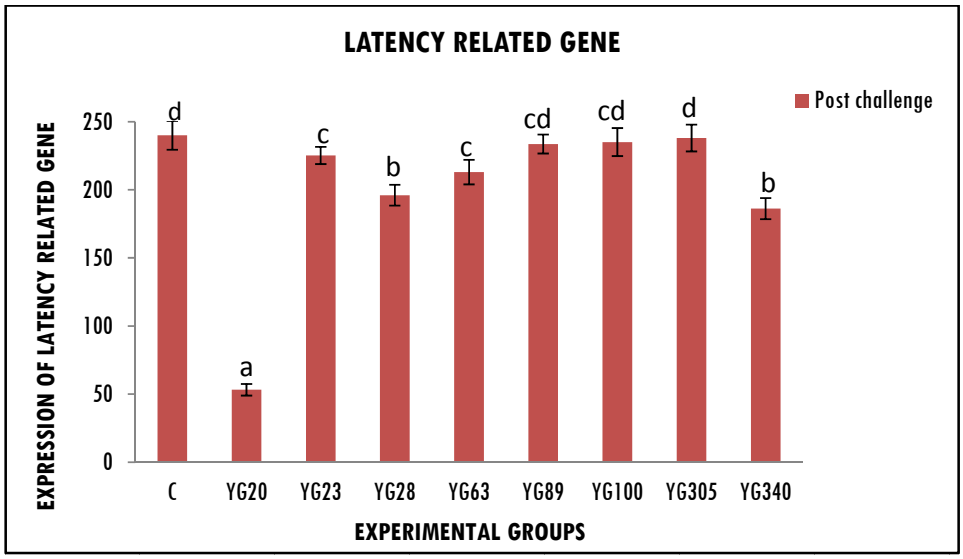


Fig. 2.29 Expression profile of latency related gene (WSSV gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

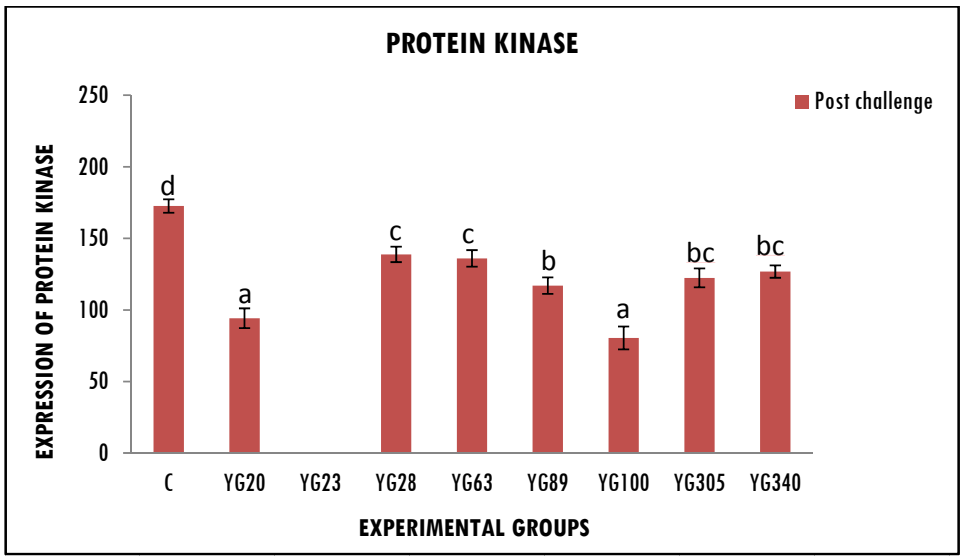


Fig. 2.30 Expression profile of protein kinase gene (WSSV gene) in the post larvae of P. monodon in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

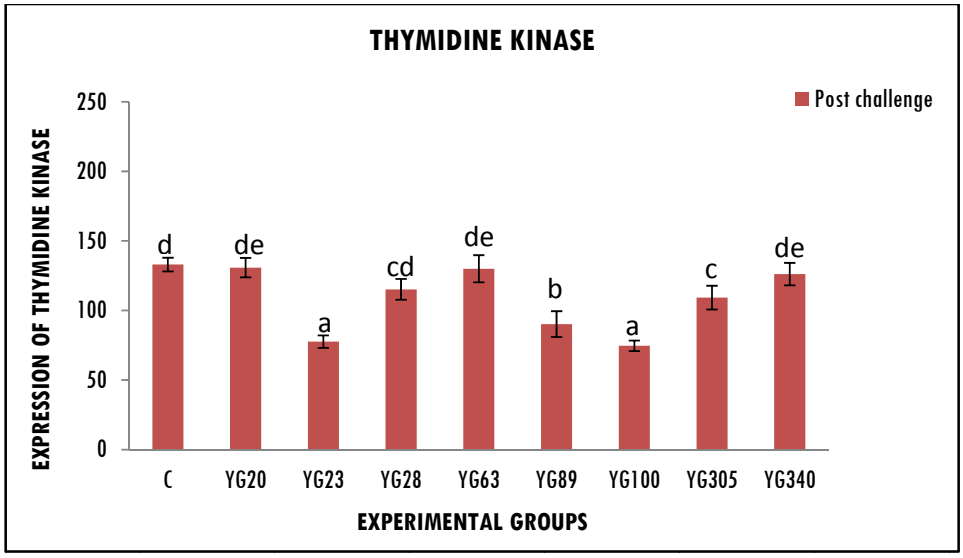


Fig. 2.31 Expression profile of thymidine kinase gene (WSSV gene) in the post larvae of P. monodon in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

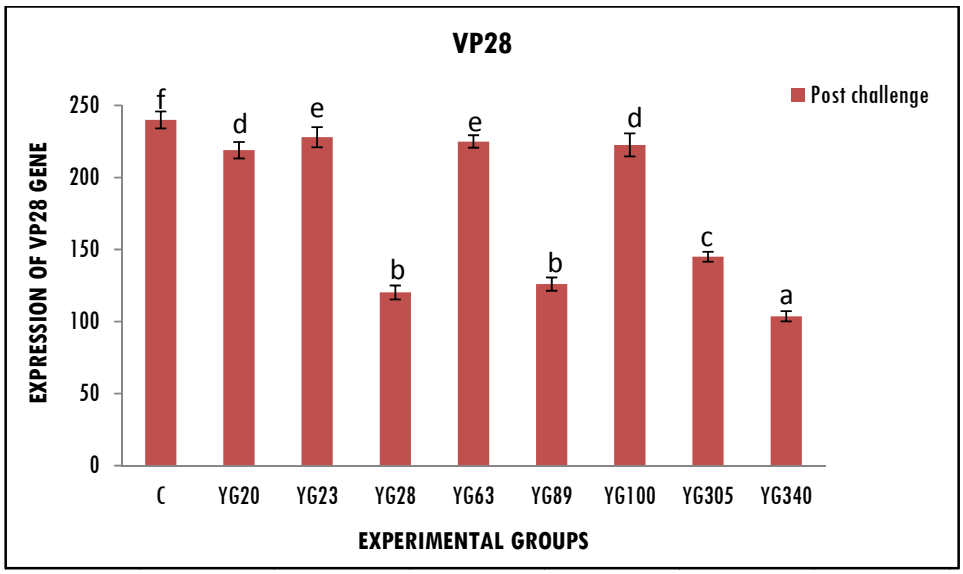


Fig. 2.32 Expression profile of VP28 gene (WSSV gene) in the post larvae of *P. monodon* in response to the administration of various marine yeast glucans pre- and post-challenge WSSV

(C – Control feed administered group, YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG28 – Yeast glucan 28 administered group, YG63 – Yeast glucan 63 administered group, YG89 – Yeast glucan 89 administered group, YG100 – Yeast glucan 100 administered group, YG305 – Yeast glucan 305 administered group, YG340 – Yeast glucan 340 administered group)

2.4 Discussion

Glucans, the cell wall component of yeasts is made up of β-1,3 linked glucose polymer with varying degree of β-1,6 branching. β-Glucan has received great attention due to its bioactive and medicinal properties such as immune-stimulating, anti-inflammatory, antimicrobial, antiviral, antitumor and lowering of cholesterol levels (Stone and Clarke, 1992; Yadomae and Ohno, 1996; Kogan, 2000). β-Glucans defend various bacterial infections such as *Vibrio vulnificus* (Sung et al., 1994), *V. campbellii*, *V. proteolyticus* (Marques et al., 2006), *Aeromonas veronii* (Lin et al., 2011), *V. harveyi* (Amparyup et al., 2012) and viral infections such as white spot syndrome virus infection (WSSV) (Chang et al., 2003; Sukumaran et al., 2010), Taura syndrome virus (TSV) (Song et al., 2003), infectious hypodermal and hematopoietic necrosis virus

(IHHNV) (Yeh et al., 2009) and infectious myonecrosis virus (IMNV) (Nunes, 2010) in shrimps. The aim of the present study is to test the efficacy of marine yeast glucans as immunostimulants/ antiviral agents in *P. monodon* by analysing the expression profile of various immune genes and WSSV genes before and after WSSV challenge.

The impacts of marine yeast glucan as an immunostimulant have been studied by earlier researchers; Antony et al. (2011c) in *P. monodon* and Sajeevan et al. (2009) in *F. indicus*. Sukumaran et al. (2010) recorded the amount of glucan from the different yeast isolates and showed that *Debaryomyces hansenii* had maximum glucan content and minimum in *C. tropicalis*. The present study also showed that glucan yield was maximum in *Debaryomyces fabryi* and *Hortaea werneckii* and minimum in *C. spencermartinsiae*.

The proton NMR study revealed that, the marine yeast glucan isolates are (1-6)-branched (1-3)-β-D-glucan as reported by Ohno et al. (1999) and it shows differences in the level of (1-6)-linkages in the various glucan isolates as reported by Sukumaran et al. (2010). Chang et al. (1999) reported that the administration of β-glucan through the feed enhanced the survival and the resistance of post larvae of *P. monodon* to WSSV infection.

The effective dose (2 mg ml^{-1}) required in animals was found to be much lower than the observed and predicted IC_{50} values of the glucans in HEp-2 cell line which were between 6.07 and 7.86 mg ml^{-1}, ensuring safe handling and application of yeast glucans.

The present study was mainly focused on the comparative efficacy of various yeast glucans as immunostimulants in *P. monodon*. All the glucan treated groups showed significantly higher survival rate; whereas 100% mortality could be observed in the control group on 7^{th} day post-challenge

WSSV. The present study revealed that three glucan extracts viz., YG23, YG20 and YG28 supported better survival (above 60%) than the other groups tested in which survival rate varied between 43 and 58%. Sajeevan and Philip (2009) noticed the potential of β-1,3-glucan from the marine yeast *C. sake* on *F. indicus* post larvae through dip treatment against WSSV infection. Marques et al. (2006) described the protective role of β-glucan in gnotobiotic Artemia against two pathogenic bacteria viz., *V. campbellii* and *V. proteolyticus*. Administration of β-glucan and mannan oligosaccharide exhibited significantly higher survival in *P. monodon* juvenile against WSSV infection (Andrino et al., 2014). These previous reports indicates that improvement of shrimp survival against viral infection due to β-glucan treatment could be attributed to enhanced immune responses similar to that noticed in the present study.

Among the control genes analysed, ELF was found to be a reliable reference gene than β-actin in *P. monodon* for gene expression studies. Similar result was obtained by Antony et al. (2011b) who studied three control genes viz., β-actin, ELF and 18S rRNA and found ELF as the potent one.

The expression profile of AMP genes was completely different in the case of glucan administered groups, in which up-regulation of the AMP genes was observed. It was interesting to notice that all the AMP genes were down-regulated in the control group of shrimps upon WSSV challenge, reflecting a decline in the immunity of the shrimps especially ALF, crustin-1 and penaeidin-5. Similar down-regulation of AMP genes due to WSSV infection has been shown by Antony et al. (2011a). Of the various glucans YG23, YG20 and YG28 were found to be better immunostimulants.

Most of the AMPs and immune genes were up-regulated in the YG23, YG20 and YG28 treated groups at a considerable level when compared to the

control group. The expression of crustin in the post larvae was higher on challenge with WSSV during the glucan treatment when compared to control, suggesting that glucans have the ability to stimulate the immune system. This is supported by Antony et al. (2011c) who suggested the antiviral defense of crustin and the up-regulation of this gene by marine yeast glucans proving the immunostimulant potency of these compounds against WSSV infection in *P. monodon*. A study conducted by Amparyup et al. (2008) in *P. monodon*, reported the remarkable up-regulation of the crustin transcripts following challenge with *V. harveyi*. Supungul et al. (2008) reported down-regulation of the crustin gene on administration of LPS or Gram-negative bacteria in *Litopenaeus vannamei*.

In the case of penaeidin, it could be noticed that penaeidin-3 was differentially expressed before and after challenge. When compared to control group of shrimps, post-challenge WSSV, all the glucan fed groups showed considerable level of immune gene up-regulation. Penaeidin-5 transcripts could not be detected in post-challenge group and only the YG28 fed group responded pre-challenge. Earlier works have also shown reduced penaeidin transcripts in larval stages (Munoz et al., 2003; Jiravanichpaisal et al., 2007). Antony et al. (2011a) reported an up-regulation of penaeidin-3 and penaeidin-5 on administration of probiotic in *P. monodon* on WSSV challenge.

In the present study, ALF was considerably up-regulated in glucan administered groups post-challenge WSSV. Antony et al. (2011b) studied the involvement of ALF in the antiviral defense mechanisms in shrimps especially against WSSV and have reported that these peptide molecules are involved in defense mechanisms in *P. monodon* against WSSV invasion. They noticed up-regulation of ALF in early to late period (on a time course basis) during WSSV infection. The induced ALF transcription in kuruma prawn *Marsupenaeus*

japonicus upon bacterial challenge was noticed by Nagoshi et al. (2006). In addition, a considerable level of up-regulation of ALF was noticed by Antony et al. (2011a) in *P. monodon* both pre and post WSSV challenge on administration of probiotic bacteria. Analysis of the results of AMP gene expression pattern showed that all the tested glucans have the potential to up-regulate the immune genes and within that YG23, YG20 and YG28 could significantly up-regulate the expression under post-challenge condition also.

The study also analysed the immune gene expression profile in different glucan fed *P. monodon* post larvae both pre and post WSSV challenge. The target genes viz., α-2- macroglobulin, astakine, glutathione-S-transferase, haemocyanin, peroxinectin, pm Cathepsin C and proPhenoloxidase were found to be differentially expressed in response to various glucan incorporated feeds. Whereas, glutathione peroxidase, transglutaminase, catalase, caspase and superoxide dismutase could not be detected in either pre- or post-challenge group of shrimps. This shows that the immune system is underdeveloped in the post larvae. Jiravanichpaisal et al. (2007) have commented that in early larval stages of shrimp, it is not usually exposed to microorganisms from the surrounding environment. This is in agreement with our findings that some of the gene transcripts were completely absent when compared to other genes tested. Under challenged conditions they produce AMPs such as ALFs, crustins and penaeidins to defend themselves against the pathogens.

In the present study peroxinectin was found to be up-regulated in all the glucan treated PL pre-challenge when compared to the control. Whereas, in case of post-challenge condition, six glucan fed groups markedly increased the peroxinectin gene transcripts. However, peroxinectin transcripts could not be detected in the control group of shrimps. Peroxinectin is a multifunctional molecule with cell adhesion activity and it is inciting to contemplate that this

protein might be involved in several developmental processes occurring during larval development, such as metamorphosis, moulting, cell proliferation and differentiation (Jiravanichpaisal et al., 2007). Jiravanichpaisal et al. (2007) noted the presence of high amount of peroxinectin transcripts from early larval to post larval stage of *P. monodon*. The cellular immune response such as cell adhesion and peroxidase activity of peroxinectin in the presence of β-1-3 glucan and lipopolysaccharide was demonstrated by Sritunyalucksana et al. (2001). It has been reported that up-regulation of peroxinectin (PE) and LPS- and β-1,3-glucan-binding protein (LGBP) mRNA enhanced the resistance of white shrimp *Litopenaeus vannamei* against WSSV and *Vibrio alginolyticus* when the shrimp fed the *Gynura bicolor* diets (Wu et al., 2015). In our study the glucans could induce the peroxinectin gene post-challenge. This is a positive sign that our experimental glucans have the potential for better enhancement of larval development and immune response against viral infection.

The proPhenoloxidase (proPO) system is considered as a constituent of the immune system in invertebrates (Lai et al., 2005) and is also involved in cell adhesion, encapsulation and phagocytosis processes (Gillespie et al., 1997). ProPO can be activated by cell wall components of microbial origins, such as β-1, 3-glucan, lipopolysaccharide (LPS) and peptidoglycan. In the present study the yeast glucan treated YG23 and YG20 group could up-regulate the expression of proPO in a considerable manner post-challenge and there was no proPO transcript in the other glucan treated groups post-challenge. During the larval stages of shrimp, the lack of a functional proPO system might be a critical factor for the susceptibility of larvae to pathogenic microorganisms. The two glucans YG20 and YG23 could induce the expression of proPO post-challenge WSSV. The study conducted by Luna-Gonzalez et al. (2003) reported the absence of proPO in larval homogenates of the molluscs viz., *Crassostrea gigas*, *Argopecten ventricosus* and

Nodipecten subnodosus. However, Babu et al. (2013) reported the up-regulation of proPO in the marine yeast treated adult *P. monodon* post-challenge. Hence, based on the result of ProPO transcripts, the present study emphasize that among the tested glucans, YG23 and YG20 have potential for the highest up-regulation of the proPO immune pathways post-challenge; an important factor for the effectiveness of the defense mechanism. On the other hand, proPO transcripts could not be detected both pre- and post-challenge control group of shrimps, reinforcing our finding that glucan has the potential to stimulate the immune pathways.

The basal expression of α-2-macroglobulin was found to be higher in the control and most of the experimental groups (except YG63 and YG89). However, the gene was found to be down-regulated upon WSSV challenge. α-2-macroglobulin is a high molecular weight proteinase inhibitor and their down-regulation promotes serine proteinase activity and thereby the proPhenol oxidase system. The yeast glucan YG340 supported maximum up-regulation of α-2-macroglobulin gene post-challenge WSSV. The administration of peptidoglycan as an immunostimulant in kuruma shrimp and *P. monodon* significantly induced α-2-macroglobulin expression (Lin et al., 2007).

Astakine is involved in cell proliferation and differentiation (Soderhall et al., 2005). The expression of astakine was noted at the middle phase of Cray fish embryo development (Zhang et al., 2010b). The presence of astakine was also reported by Jiravanichpaisal et al. (2007) in the larval stage of *P. monodon*. In the present study astakine was expressed in the YG20, YG23 and YG89 groups. So the elevation of these transcripts in the YG20, YG23 and YG89 treated groups indicate that astakine proteins may be essential for cell proliferation and cell differentiation that could be enhanced even in the presence of WSSV infection.

Haemocyanin, a multifunctional molecule serves as an oxygen carrier for arthropods such as crustaceans (Decker and Tuczek, 2000) and participate in multiple roles of immune defense. In the present study, haemocyanin was expressed in all the glucan treated and control pre- and post-challenge groups. When compared to post-challenge control, all the glucan treated groups showed up-regulation of the gene and the glucan YG100 supported maximum up-regulation of haemocyanin. So the involvement of glucan in the up-regulation of haemocyanin gene could enhance a better protection against viral infection and defense responses in *P. monodon*. Zhang et al. (2004) have also proved the transcripts of haemocyanin subunit-L gene, as an immune factor against WSSV infection in *P. monodon*. Jiravanichpaisal et al. (2007) noted an increase in haemocyanin transcript in *P. monodon* at protozoea 3 stage.

Cathepsin C is a lysosomal cysteine protease and is responsible for intracellular protein degradation in invertebrates. Qui et al. (2008) noticed that *P. monodon* cathepsin C (Pm cathepsin C) was up-regulated on LPS stimulation. In the present study YG28 supports the up-regulation of cathepsin C in *P. monodon* pre-challenge and YG20, YG23 and YG28 post-challenge. The up-regulation of cathepsin C in the glucan administered group shows that lysosomal protease based mechanism is in action against the virus invasion.

In case of antioxidant activity involving glutathione-S-transferase, significant difference could not be detected in the expression pattern of shrimps pre-challenge compared to control; but it was notable in YG20, YG23, YG28, YG63 and YG100 post-challenge WSSV. Glutathione peroxidases (GPxs) could not be detected both pre- and post-challenge and control group. Glutathione-S-transferases (GSTs) and glutathione peroxidases (GPxs) are important components to defend cells against reactive oxygen species (ROS). Mathew et al. (2007) reported that WSSV infection induced a significant reduction in the

activities of glutathione-dependent antioxidant enzymes (GPx and GST) in *P. monodon*. The up-regulation of glutathione-S-transferases in the post larvae of glucan administered animals, activating glutathione dependant antioxidant mechanism is noteworthy.

Another antioxidant enzyme, superoxide dismutase was not found to be expressed in any of the groups both pre- and post-challenge. Whereas, in the shrimp, *L. vannamei* (Gomez-Anduro et al., 2012) manganese superoxide dismutase (MnSOD) transcripts were found in response to lipopolysaccharides (LPS) and white spot virus infection. Additionally, caspase involved in apoptosis, transglutaminase for coagulation and catalase in antioxidant activity could not be detected in the glucan administered *P. monodon*. Similarly, a decrease in catalase activity in WSSV infected *P. monodon* was noticed by Rameshthangam and Ramasamy (2006) and Mathew et al. (2007). Based on these results we could assume that since these animals are not exposed to diverse microorganisms during the larval stages, some of the immune genes are not expressed.

On the basis of post-challenge survival and gene expression analysis in post larvae, YG23 and YG20 could be segregated in terms of performance. YG23 and YG20 showed significant up-regulation in immune gene expression i.e., ALF, crustin-1, crustin-2, crustin-3, astakine, glutathione-S-transferase, peroxinectin, Pm cathepsin C and proPhenol oxidase. A recent study by Miest et al. (2016) reported that oral administration of β-glucan (MacroGard®) induced immunomodulatory response such as modulation of genes involved in immunity, digestion and development and also increased survival rate in turbot larvae. The effect of β-glucan on immune gene expression in *Pangasianodon hypophthalmus* in response to a pathogen *Edwardsiella ictaluri* was reported by Sirimanapong et al. (2015). These reports support the present study in which the marine yeast β-

1,3/1,6-glucans could significantly enhance the up-regulation of various immune genes against WSSV infection in *P. monodon* post larvae.

All the seven WSSV genes were found to be expressed within 48 h of challenge in the control group. There are reports regarding the WSSV gene expression during the early period of infection (Liu et al., 2005b; Lan et al., 2006) and the increase in the intensity of infection over the experimental period (Antony et al., 2011b). The presence of protein kinase, thymidine kinase, VP28 and DNA polymerase gene transcripts at early stages have been reported by earlier researchers in *P. monodon* post-WSSV infection (Tsai et al., 2000; Liu et al., 2001; van Hulten et al., 2001; Chen et al., 2002). These results emphasize the fact that WSSV genes express within 48 h.

When compared to the control, the YG treated groups were less infected with WSSV. Antony et al. (2011c) reported the expression of WSSV related genes from the haemocytes of glucan treated group of *P. monodon* post-challenge WSSV and it was less intense than the control group. In the present study it was found that yeast glucan YG23, YG20, YG28 and YG89 could suppress the expression of WSSV related genes at a considerable level. YG100, YG305, YG63 and YG340 also exhibited a differential pattern of down-regulation with respect to each WSSV related gene. This is in agreement with the post challenge survival data, especially in the case of YG23 fed group which showed 70.27% survival. Gene transcripts for protein kinase could not be detected in YG23 post challenge showing antiviral activity induced by the glucan.

From the above results it is apparent that WSSV infection tends to down-regulate the expression of immune related genes. However, administration of glucans generally enhanced the shrimp immune system, even upon WSSV challenge. The study showed that YG23 and YG20 are potent immunostimulants

and confer protection against viral infection. From the present study, we could infer that, marine yeasts possess immunostimulatory properties in *P. monodon* post larvae especially in case of WSSV infection.

In the current study, an effort was made to segregate potent glucans from various marine yeasts based on their immunostimulant property in shrimps in terms of immune gene expression and survival. The study revealed the potential of marine yeast glucans as a source of immunostimulant in *P. monodon* post larvae. All the glucans tested in this experimental study showed above 40% survival in *P. monodon* post larvae. Among these, glucan extracted from *H. werneckii* R23 (YG23) exhibited highest protection in post larvae. At the same time glucan from *C. parapsilosis* R20 (YG20) and *C. spencermartinsiae* R28 (YG28) also conferred better protection against WSSV infection in *P. monodon* post larvae. So these marine yeast glucans (YG) are a potential immunostimulant in *P. monodon* post larvae for protection against WSSV infection. All the above information emphasizes the role of marine yeast glucans as an immunostimulant against WSSV infection. Application of glucans would increase the immunocompetence and confer better protection to shrimps. In case of onset of mortality in shrimp culture systems, the farmers get enough time to plan for an early harvest protecting the crop from incurring a massive loss.

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Chapter 3

Characterization and evaluation of selected glucans as immunostimulants in adult *P. monodon* against WSSV challenge

3.1 Introduction

Penaeid shrimp farming is a worldwide aquaculture practice for decades and the black tiger shrimp *Penaeus monodon,* is one of the best species of penaeid shrimp cultured worldwide. Suitability of *P. monodon* for farming as a candidate species is due to its highest growth rate, high nutritive value and high economic value (Liao and Liu, 1989; Soundarapandian et al., 2009). This giant black tiger shrimp accounts for more than half of the total shrimp aquaculture output. Other cultured penaeid shrimp species include *P. indicus, P. merguiensis, P. vannamei, P. chinensis, P. stylirostris* and *P. japonicus.*

In most of the shrimp-farming countries, aquaculture industry is facing serious threat of numerous infectious diseases prompted by various pathogens (Chang et al., 2003; Song et al., 2003; Yeh et al., 2009; Nunes, 2010; Amparyup et al., 2012) resulting in high economic losses all over the world. Virus assaults and mass mortality have affected severely many of the world's leading shrimp farming countries (Flegel, 1997; Flegel and Alday-Sanz, 1998; Lightner, 1999;

OIE, 2003; Thitamadee et al., 2016). A number of Asian countries including Thailand, Indonesia, Taiwan, Japan, China, Philippines and India also have been accompanied by significant losses due to these viral assaults. Among various shrimp viral pathogens, white spot syndrome virus (WSSV) is highly pathogenic and responsible for huge economic losses to the shrimp culture industry worldwide (Chen, 1995; Nair, 2000; Sanchez-Martinez et al., 2007). Penaeid shrimps are more susceptible to WSSV infection if the stocking density is high and environmental conditions are stressful (Raja et al., 2015). This virus has wide host range including several species of penaeid shrimps, crabs and other related crustaceans (Lightner, 1996; Lo et al., 1996, Wongteerasupaya et al., 1996; Sanchez-Martinez et al., 2007; Gopalakrishnan et al., 2011). Several carriers have been reported in the transmission of this virus which includes penaeid shrimps, crabs, lobsters, copepods and insect larvae (Thitamadee et al., 2016).

To overcome the disease problems, practice of applying antibiotics and other chemicals have been practiced for years in this industry whereas the undesirable risks reduced its usage. Research is being focused on other prophylactic measures to overcome this serious issue. Appropriate control measures such as general husbandry practices as well as proper management of farms with the application of immunostimulants (Sajeevan et al., 2009; Sukumaran et al., 2010; Antony et al., 2011c; Chen et al., 2016), probiotics (Antony et al., 2011a; Leyva-Madrigal et al., 2011; Luis-Villasenor et al., 2013; Miandare et al., 2016) and bioremediators (Antony and Philip, 2006) have been shown to protect the industry from the assault of diseases at least to a certain extent.

Application of immunostimulants has been accepted special interest in the aquaculture industry to boost up the immune system of shrimps as an alternative. The immune system of crustaceans is mainly non-specific and depends on phagocytosis, encapsulation, agglutination and the pro-phenoloxidase cascade

which enhances the innate defense and helps to protect the host against a broad spectrum of pathogen (Smith and Soderhall, 1983; Sakai, 1999). Based on the crustacean immune systems, they have the ability to detect and act in response against the cell wall components of microorganisms such as β-glucan, PG and LPS in place of pathogens. These cell wall components activate the cellular immune responses of host organism by binding to specific binding proteins present in serum as recognition proteins (Ji et al., 2009). Recently, Zhang et al. (2016) identified a peptidoglycan recognition receptor protein (SmPGRP2) in turbot and an up-regulation of SmPGRP2 was noticed in classical immune tissues after challenge with Gram-positive bacteria and Gram-negative bacteria, which shows the recognition of peptidoglycan of the bacterial cell wall and the decisive role in host immune defense against pathogen infection. In another study it has been reported that combination of β-1,3/1,6-glucan and vitamin C stimulated the non-specific immune response of white shrimp *Litopenaeus vannamei* (Wu et al., 2016). Variety of immunostimulants are available in market and used in the commercial aquaculture (Dalmo, 2008; Ringo et al., 2012), even though its mode of action is not yet fully elaborated. Extensive research is needed in this area to understand the specific mechanism of action and for the commercialization of these compounds.

Although varieties of immunostimulants are available, glucans are the most popular immunostimulants used in aquaculture. Yeasts are good candidates as a source of glucan and the organism itself can also act as good immunostimulant, have been reported by earlier studies (Sung et al., 1994; Chang et al., 1999). Apart from terrestrial yeasts, marine yeasts also exhibit good immunostimulation properties even though not many studies have been undertaken in this area (Reviewed in chapter 2). The halotolerant properties of marine yeast make it as a suitable candidate in aquaculture feed preparations.

The cell wall of yeast encompasses 15 to 25% of the total dry weight of cell. The composition of yeast cell wall usually consist of glucan which is a major component of cell wall (~50-60% of which 20% is alkali soluble), followed by mannan (30%), protein (10-15%), lipid (8-9%) and chitin (1-2 %). The composition of the cell wall is subject to considerable variation according to yeast strain, cell age and growth conditions. Yeast cell wall consists of a complex layered structural arrangement with an outer mannoprotein layer and an inner layer of β-1,3 and β-1,6 glucan complexed with chitin. The inner layer gives shape and strength to the cell. The major cell wall polysaccharide β-glucan component is extracted from the insoluble cell wall after yeast cell digestion. Most commonly applied method for glucan extraction is the alkali-acid hydrolysis method of Hassid (1941). Later it is redefined by Williams et al. (1991) according to which more than 97% pure form of glucan could be obtained (Lowman and Williams, 2001). The residue obtained by this method is alkali insoluble β-1,3-glucan. It has been reported that β-glucan with high molecular weight is insoluble even in high concentration of alkali (up to 10 M) (Hayen et al., 2001; Bahl et al., 2009).

Various chemolytic methods such as partial chemical or enzymatic hydrolysis, methylation analysis, periodate oxidation and Smith degradation have been carried out to determine the yeast glucan structure (Bell and Northcote, 1950; Manners and Patterson, 1966). NMR spectroscopy is an effective tool for the structural analysis of various glucans. Later on, structural elucidation of the yeast glucan is being done with NMR Spectroscopy (Kim et al., 2000; Lowman et al., 2003; Sukumaran et al., 2010). Lowman and coworkers (2011), have elucidated the structure of glucan isolated from yeast *C. glabrata* based on extensive ^{1}H and ^{13}C 1D and homonuclear (COSY, NOESY and TOCSY) and heteronuclear (HSQC, HSQC-NOESY, HSQC-TOCSY and HMBC) 2D NMR studies. The

isolated glucan contains a (1→3)-β-D-glucan polymer backbone with (1→6)-β-D-glucan-containing side chains composed of multiple (1→6)-β-linked glucosyl repeat units. These methods can provide additional information about the sequence and ratios of the monosaccharides present, the type of bonds (α- or β-) between individual sugars, side chain length, the branching points and branching frequency.

Previous reports point out that the physicochemical properties of glucans such as primary structure, polymer size, surface charge, solution conformation and side chain branching are the possible determinants for recognition and interaction with pattern recognition receptors (PRRs) in the innate immune system of host (Muller et al., 1996; Mueller et al., 2000; Adams et al., 2008). Controversial reports exist regarding the structural-activity relationship of glucan as immunostimulants, such as triple helix and single helix in enhancing the biological activities (Ohno et al., 1996; Zhang et al., 2005; Wang and Zhang, 2009). Similarly, molecular weight, degree of structural complexity and solubility has also been subjected for the relationship study concerning β-glucans and its immunomodulation activity (Cleary et al., 1999; Ishibashi et al., 2001; Fang et al., 2012). Ability of β-1,3/1,6-glucans to stimulate innate immune cells is subjected to its branched structure and side chain length. Products of β-1,3/1,6-glucans with only one single glucose molecule in their side chain, have lower macrophage stimulating activity than β-1,3/1,6 yeast cell wall glucan (Raa et al., 2015). Sukumaran et al. (2010) have been reported that β-(1-3)-glucan isolated from marine yeast *C. tropicalis* (S186) with a higher molecular weight and a lower degree of branching acts as better immunostimulant than a comparatively lower molecular weight and higher branching glucans in *P. monodon* post larvae during a comparative study including glucan from Baker's yeast (*Saccharomyces cerevisiae* S36).

Even though immunostimulants are being used in aquaculture for years, little is known about their effects at the molecular level in the host immunity. Among the previous studies using various immunostimulants, a response of differential pattern of gene expression has been exhibited in the experimental animals. Oral administration of peptidoglycan induced expression of serine proteinase, a proPhenoloxidase (proPO) activating factor (Rattanachai et al., 2005) and crustin, an antimicrobial peptide (Rattanachai et al., 2004) in kuruma shrimp *M. japonicus*. In another study, the mRNA expression of LPS/β-1,3-glucan binding protein (LGBP), peroxinectin, cytosolic SOD, penaeidin-5 and a single WAP domain protein have been significantly increased in *P. monodon* by the feeding of sodium alginate incorporated diet (Liu et al., 2006a). In kuruma shrimp *M. japonicus*, oral administration of peptidoglycan lead to the significant increase in expression of several biodefense-related genes such as prophenoloxidase, 2 types of proPhenoloxidase-activating factor, Masquerade protein, TGase, lysozyme, α-2-macroglobulin and penaeidin by quantitative real-time RT-PCR (Aoki and Hirono, 2005). In contrast to these reports, *L. vannamei* when injected with LPS exhibited a down-regulation of antimicrobial peptide genes (AMPs) including PEN2, PEN3, PEN4 and crustin, whereas serine proteinase and proPO did not show a significant change in mRNA levels after injection (Okumura, 2007).

Although β-glucans have been shown to enhance the non-specific defense mechanisms in shrimps, knowledge regarding how it works at molecular level is limited. Gene expression analysis by quantitative real-time PCR (qPCR) gives insight into the quantification of gene expression and enables the detection of mRNA at very low copy numbers. Wang et al. (2008) have been reported a differential time-series expression of immune-related genes of *L. vannamei* in response to dietary inclusion of β-1,3-glucan from *Schizophyllum commune* by

quantitative real-time PCR. A differential pattern of gene expression could be noticed among the genes that include penaeidin 3, lysozyme, cytosolic manganese superoxide dismutase, β-glucan binding protein-high density lipoprotein and lipopolysaccharide/β-glucan binding protein, whereas haemocyanin, crustin, prophenoloxidase and transglutaminase did not respond to the glucan treatment. So far, the effect of marine yeast β-glucan on the expression of various biodefense genes and their quantification by qPCR in response to WSSV infection are not yet available. Owing to the extensive application of immunostimulants especially β-glucan in shrimp aquaculture, β-glucan from different sources and its mechanism of action at molecular level against WSSV infection is relevant.

Therefore the present study was focused on the immunostimulatory effect of β-glucan from three marine yeasts viz., *Candida parapsilosis* R20, *Hortaea werneckii* R23 and *Debaryomyces fabryi* R100 in adult *P. monodon*. Effect of these glucans on Post-challenge survival of *P. monodon* against WSSV infection was analyzed. The immunostimulatory potential of these three glucans were assessed by analysing the expression profile of biodefense genes by quantitative real-time qPCR before and after challenge with WSSV in adult *P. monodon*. Expression of VP28 gene of WSSV was also analyzed to confirm and to quantify the intensity of viral infection in the experimental animals.

3.2 Materials and Methods

3.2.1 Microorganisms used as source of glucans

Three marine yeasts (*Candida parapsilosis* R20, *Hortaea werneckii* R23 and *Debaryomyces fabryi* R100) maintained in the Microbiology Laboratory of the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, India were used in the present study.

3.2.2 Glucan extraction

Lawn cultures of yeasts were prepared using swab inoculation on Malt Extract Agar plates (malt extract, 20 g; mycological peptone, 5 g; agar, 20 g; 20‰ seawater, 1000 ml; pH 6) and the biomass was harvested using sterile seawater. The harvested cells were then separated by centrifugation at 10,000 rpm for 20 min at 4°C in a refrigerated centrifuge (Kubota, Japan) and dried under vacuum. Glucan was extracted from the dried yeast biomass following the method of Williams et al. (1991) with some modifications.

3.2.3 Structural characterization of the cell wall glucan

For structural characterization of yeast glucan, 1D and 2D NMR spectra were collected using a Bruker Avance III 600 NMR spectrometer with a DCH cryoprobe in 5-mm OD NMR tubes. Each sample was dissolved in DMSO-d6 with gentle heating as needed. YG100 contained insolubles that were removed by settling overnight so that only the supernatant was examined. A few drops of trifluoroacetic acid-d were added to each solution in the NMR tube to shift the exchangeable proton resonances downfield below about 7 ppm. Spectral data collection parameters for 1D NMR experiments were the following: scans = 256 using 30° pulses, dummy scans = 2, FID size = 65,536, sweep width = 20.6 ppm with the pulse frequency centered at 6.175 ppm, acquisition time = 2.66 sec, relaxation delay = 1.0 sec, probe temperature = 72°C (345°K), and exponential apodization with a line broadening of 0.30 Hz. Spectral data collection parameters for the 2D COSY NMR experiments were the following: size of the FID = f1: 128, f2: 2048, size of the real spectrum = f1: 1024, f2: 1024, dummy scans = 8, scans = 16, sweep width = 8.1 ppm with the pulse frequency centered

at 2.758 ppm, relaxation delay = 1.4 sec, probe temperature = 72°C (345°K), and sine apodization in both dimensions. NMR spectra were processed with TopSpin version 3.2 on a MacBook Pro running OSX 10.9.5.

3.2.4 Preparation of glucan incorporated feed

Three experimental feeds and a control feed (without glucan) were used for the study (Fig. 3.1). The experimental feeds were prepared by incorporating 0.2% homogenized glucan to a standard shrimp diet as per Sajeevan et al. (2009). The experimental glucan diet prepared from different marine yeast strains are referred as YG20 (*C. parapsilosis* R20), YG23 (*H. werneckii* R23) and YG100 (*D. fabryi* R100). The feed preparations were stored at -20°C prior to use.

3.2.5 Experimental animals and rearing conditions

Healthy adult *P. monodon* of 20-25 g body weight (Fig. 3.2) and PCR-negative for WSSV were collected from a local shrimp farm in Kodungalloor, Thrissur, India. They were transferred to aquarium tanks of 500 L capacity containing 15‰ seawater and acclimatized for two weeks under laboratory conditions. Aeration was continuously provided in all the tanks during the experimental period. They were fed standard shrimp diet twice daily at a rate of 10-15% of body weight and the unused feed and faecal matter were siphoned out daily just before feeding. Water quality was maintained by 25% water exchange every second day just before feeding.

Fig. 3.1 Experimental diets used in the study

(YG20 = Yeast glucan YG20 incorporated feed; YG23 = Yeast glucan YG23 incorporated feed; YG100 = Yeast glucan YG100 incorporated feed; CF = Control feed without glucan)

Fig. 3.2 Experimental animal- Adult black tiger Shrimp, *Penaeus monodon*

3.2.6 Virus inoculum preparation

A collective sample of gills and soft parts of cephalothorax (500 mg) from a freshly infected *P. monodon* was macerated in 10 ml cold PBS (NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; KH_2PO_4, 0.2 g; double distilled water, 100 ml) with glass wool to a homogenous slurry using mortar and pestle in an ice bath. The slurry was centrifuged at 8000 x g in a refrigerated centrifuge at 4°C and the supernatant was subjected to filter sterilization using a 0.22 µ pore size PVDF membrane filter. The preparation was streaked on ZoBell's agar plates

and incubated at 28 ± 2°C for 72 h to confirm the absence of bacterial contamination. Viral infectivity titre was determined as the extractable virus and expressed as LD_{50} in shrimps following Reed and Muench (1938). The virus stock thus prepared for the experiment was stored in 500 µl aliquots at −80°C.

3.2.7 Determination of WSSV titer

The virus titer was determined through quantitative Real-time PCR method (Sudheer et al., 2015) targeting WSSV VP28 gene. Briefly, the PCR mix (25 µl) contained 12.5 µl Power SYBR Green master mix (Applied Biosystems, USA, California), 1 µl each of forward and reverse primer (586F-GGGAACATTCAAGGTGTGGA-3' and 586R-GGTGAAGGAGGAGGTG TTGG5'), 1 µl template DNA and 9.5 µl MilliQ water. The PCR was done at an initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 68°C for 45 sec. The control used was serial dilutions of plasmid containing cloned VP28 gene insert. All reactions were done in triplicates and data analysed using StepOne™ Software v2.2.2 (Applied Biosystems). The viral titer of the inoculum was 1×10^{14} DNA copies/ml. Viability of WSSV in suspension was tested by injecting 10 µl to a batch of apparently healthy shrimps and mortality was confirmed over a period of 3 to 7 days.

3.2.8 Experimental design and WSSV challenge

The experimental animals were randomly divided into four groups of 20 animals each. Triplicate tanks were maintained for each group. Three experimental groups were maintained on glucan incorporated diets once in seven days based on the previous studies by Sajeevan et al. (2009) and rest of the days, the animals were fed the control diet. The fourth group was fed the control diet alone. All four experimental groups including the control group were fed twice daily. Six animals each from all the groups were sampled after 45 days. Only

those in the intermoult stage were sampled during the experiment. All experimental groups were injected intramuscularly (Sixth abdominal segment) with WSSV on the 46th day and thereafter maintained on their respective feeding schedules for the following days. After 24 h, six animals each from all the groups were sampled. At the same time the percentage survival of the *P. monodon* of all four groups were also monitored till all animals die in control group post-challenge WSSV.

3.2.9 Haemolymph collection

Haemolymph was collected humanely from the rostral sinus using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase-free 10% sodium citrate, pH 7.0) and was suspended in TRI reagent (Sigma) for total RNA isolation. Haemolymph collected aseptically from shrimps before WSSV injection was maintained as pre-challenge samples and samples after WSSV injection as post-challenge respectively for gene expression studies.

3.2.10 Gene expression analysis

3.2.10.1 Total RNA isolation and reverse transcription

Total RNA was extracted from the haemolymph using TRI Reagent (Sigma) following the manufacturer's protocol. The first strand cDNA was generated in a 20 µl reaction volume containing 5 µg total RNA, 1x RT buffer, 2 mM dNTP, 2 µM oligo d(T_{20}), 20 U of RNase inhibitor and 100 U of M-MLV reverse transcriptase. The reaction was conducted at 42°C for 1 h followed by an inactivation step at 85°C for 15 min.

3.2.10.2 Quantitative Real-time PCR analysis of gene expression

Expression of the ten immune related genes i.e., Anti-lipopolysacharide factor (ALF), crustin, penaeidin, proPhenoloxidase activating enzyme (PPA), phagocytosis activating protein (PAP), survivin, lysozyme-like (lys-like), ferritin, lectin, haemocyanin, and VP28 (WSSV gene) as well as one internal control, i.e., elongation factor (EF-1α), were measured by quantitative real-time PCR (qPCR). Amplification was performed using the respective primers of the target genes (Table 3.1). The qPCR was carried out in the StepOnePlus real-time PCR system (Applied Biosystem). The reaction was performed in triplicate in a 20 µl reaction volume containing 10 µl of 2x SYBR Green PCR Master mix, 0.5 µl (each) gene specific forward and reverse primers, 1 µl of cDNA and 8µl PCR-grade water. The thermal profile for qPCR was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

Gene expression analysis and fold change were determined using the comparative threshold cycle (Ct) method. All target mRNA levels were normalized by the mRNA levels of internal control EF-1α. The results were expressed as the ratio of reference gene to target gene by using the following formula: ΔCt = Ct (Target genes) - Ct (EF-1α). To determine the relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct$ (Treated) - ΔCt (Control). Thus, the expression levels were reported as fold differences relative to the control.

3.2.11 Statistical analysis

In order to determine significant differences between the various experimental groups, the results were analyzed using One Way Analysis of Variance (ANOVA) and Duncan's multiple comparison of the means using SPSS 22.0 for windows. Significant differences were considered at $p < 0.05$.

Table 3.1 Primers used for the study

Target gene	Sequence (5'-3')	Reference
I.Control gene		
EF-1α	F- ttccgactccaagaacgacc R- gagcagtgtggcaatcaagc	Leelatanawit et al., 2012
II. Immune genes		
ALF3	F-tctcatctctcaacaggaggccaa R-ggtagagcttccattgccaactgc	Soonthornchai et al., 2010
Crustin	F-tccctggaggtcaattgagtg R-agtcgaacatgcaggcctatcc	Nayak etal., 2011
Penaeidin	F-acagtcgtatttgtcccagcaggt R-aacaccaaccacacacagacccat	Nayak et al., 2011
Ferritin	F-tgaagcctccattaacaagc R-gagatgaggatcgttgttgc	Nayak et al., 2011
Haemocyanin	F-aagtgctcggaatcttcggtaa R-cctgcctcgatctttgcaa	Somboonwiwat et al., 2010
Lectin	F-agtgctggacgagtgcttctatct R-ttgagagcatagacgttcctgggt	Nayak et al., 2011
Lysozyme- like	F-gcggcggacagtctcg R-ttccgatctgatgtctgatgatatc	de la Vega et al., 2007
PAP	F- attgcatcatccaccatg R-gggactttgtcatcttca	Ponprateep et al., 2009
PPA	F-ctggggcaccatctactacggc R-ctgtcaccctggcacgaatcct	Ponprateep et al., 2009
Survivin	F-gggaggagcacaaaaaccat R-acaagaacagaggagtgaa	Ponprateep et al., 2009
III. WSSV gene		
VP28	F-agttggcacctttgtgtgtggta R-tttccaccggcggtagct	Chen et al., 2011

3.3 Results

3.3.1 Glucans from marine yeast isolates

The 1D proton NMR spectral data of the glucans from marine yeasts R20 and R100 revealed that the major component of these isolates was (1-6)-branched (1-3)-β-D-glucan (Fig. 3.3). The (1-6)-linked branching frequency

(Br Freq) for R100 is about 2.6 times more frequent and the (1-6)-linked side chain length (SC Length) is about 25% shorter than for R20 (Table 3.2). 1D NMR spectra of R20 and R100 are compared in Fig. 3.4 around 4.38 ppm spectral regions. The resonance at 4.38 ppm is a multiplet resonance for R20 (blue spectrum) suggesting a linear structure (Lowman et al., 2011) and is more nearly a triplet resonance for R100 (red spectrum) suggesting a cyclic structure (Lowman et al., 2014).

In order to further confirm the structures for R20 and R100, 2D COSY NMR spectra were examined. Figure 3.5 showed an overlap of the COSY spectral data for R20 (blue spectrum) and R100 (red spectrum). Cross peaks for the anomeric protons of the non-reducing termini of the (1-6)-linked side chain (SCNRT) and anomeric protons of the branch point glucosyl repeat units (Br) of the glucans from R20 and R100 are present while the cross peak for the non-reducing terminus of the (1-3)-linked backbone (NRT) in R100 is not present. This result indicates that the glucan isolated from R100 is a cyclic glucan while the glucan isolated from R20 is a linear glucan. Chemical shift assignments for R20 are shown in Table 3.3. Comparison of the 1D and 2D COSY NMR spectra for R20 and R100 indicates that the chemical shifts and thus the proton assignments for both R20 and R100 are the same except for the absence of a resonance assignable to NRT in R100. Chemical shift assignments for H1 and H2 in Br, NRT, and SCNRT for R20 and R100 are compared with previously assigned linear and cyclic glucan chemical shifts for the same protons in Table 3.4. All evidence supports R20 as a linear branched, (1-3, 1-6)-β-D-glucan and supports R100 as a cyclic branched, (1-3, 1-6)-β-D-glucan.

While the major component in the glucan isolated from marine yeast R23 appears to be a (1-3, 1-6)-β-D-glucan, it was not possible to determine either the structure of the glucan, that is, linear versus cyclic, or the branching

frequency or side chain length reliably from the 1D NMR spectrum due to the overlapping multiplet resonances at the 4.38 ppm spectral region. Results for branching frequency and side chain length for the R23 glucan (Table 3. 2) should be taken as an estimate only. In addition, the COSY 2D NMR spectral region around 4.38 ppm for R23 (blue spectrum) compared to R20 (red spectrum) in Fig. 3.6 does not show any cross peaks from R23 that correspond with Br, SCNRT and NRT of R20.

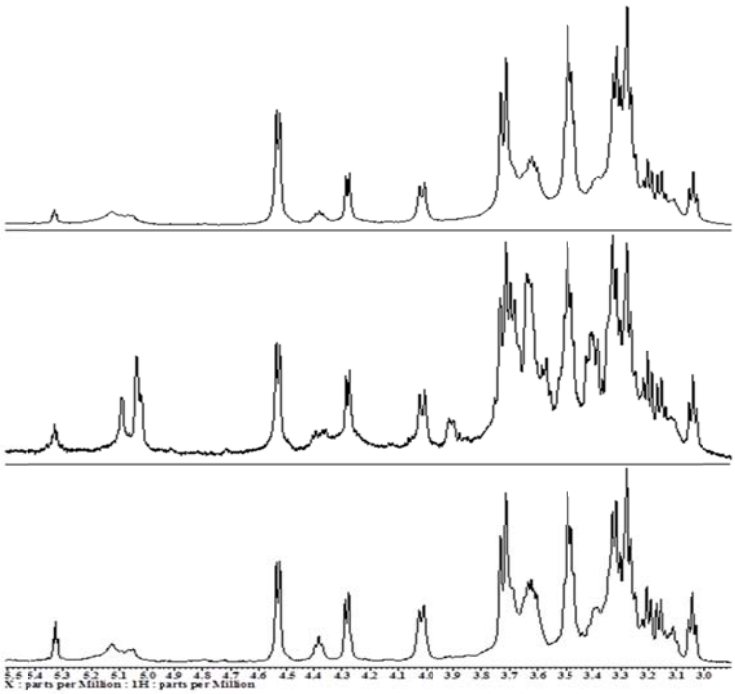

Fig. 3.3 The Proton NMR spectra of glucans isolated from *Candida parapsilosis* (R20, top), *Hortaea werneckii* (R23, middle) and *Debaryomyces fabryi* (R100, bottom)

Table 3.2 Side chain length (SC Length) and branching frequency (Br Freq) for each glucan

Sample	SC Length	Br Freq
R20	5.9	15.2
R23	5.6	9.7
R100	4.4	5.8

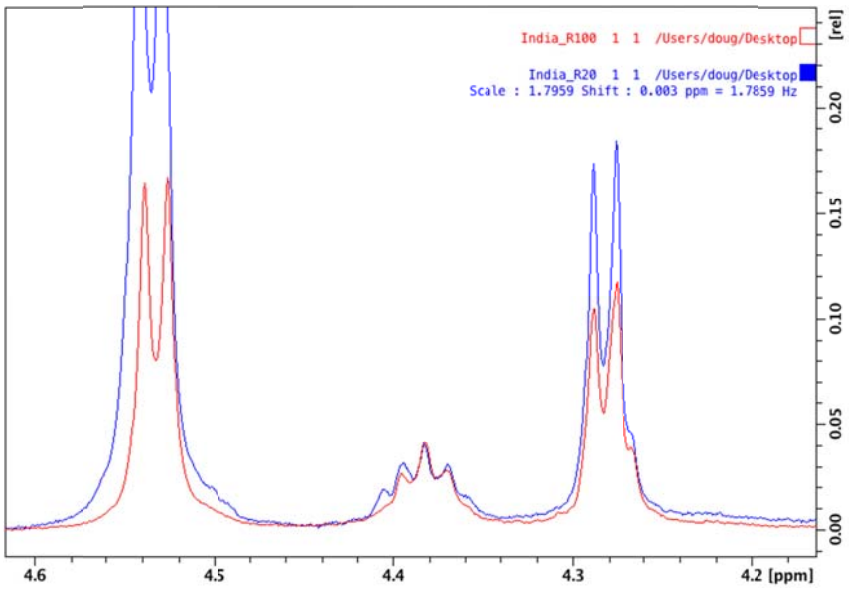

Fig. 3.4 Stacked plot of the 1D spectra for R20 (blue) and R100 (red) around the 4.38 ppm spectral region

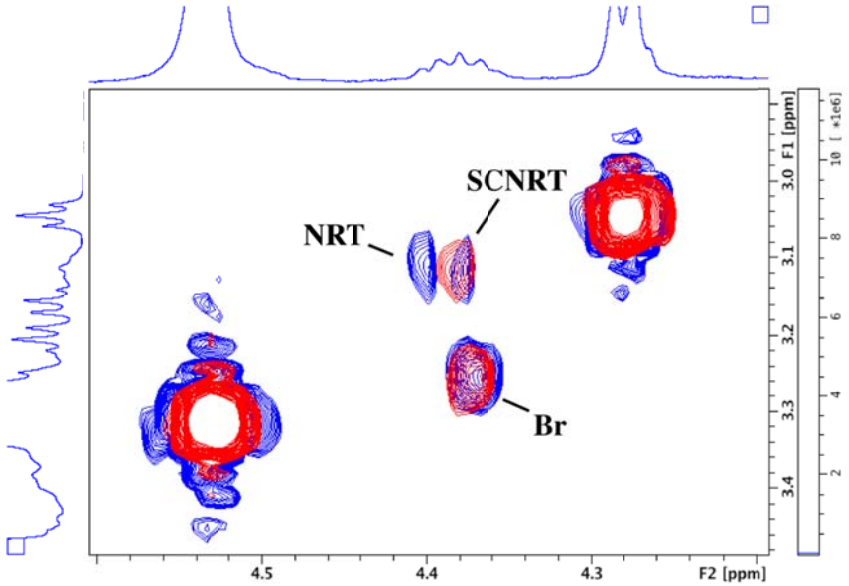

Fig. 3.5 Overlay plot of the COSY 2D NMR spectral region around 4.38 ppm for R20 (blue spectrum) and R100 (red spectrum). Overlapping cross peaks for Br and SCNRT are present for both R20 and R100 while the cross peak for NRT in R100 is absent

Table 3.3 Proton NMR assignments, in ppm, for the assignable protons of R20

Proton Assignment	(1→3)-β-Linked Backbone Chain	Br	SC (1→6)-β-Linked Side Chain	SCNRT (1→6)	NRT (1→3)
H1	4.534	4.369	4.279	4.375	4.401
H2	3.314	3.255	3.046	3.117	3.109
H3	3.493	3.409	3.209	3.235	3.224
H4	3.259	-----[a]	3.162	-----[a]	-----[a]
H5	3.301	-----[a]	3.323	-----[a]	-----[a]
H6	3.722	-----[a]	4.022	-----[a]	-----[a]
H6'	3.490	-----[a]	3.620	-----[a]	-----[a]

[a] **Not observed due to resonance overlap**

Table 3.4 Proton NMR assignments, in ppm, for H1 and H2 of Br, SCNRT, and NRT in R20 and R100

Repeat Unit	H1, H2, ppm	
	R20	R100
Br	4.37, 3.25	4.37, 3.26
	4.36, 3.24 [a]	*4.37, 3.27* [b]
SCNRT	4.37, 3.12	4.36, 3.11
	4.37, 3.10	*4.39, 3.13*
NRT	4.40, 3.11	-----[c]
	4.39, 3.09	-----[c]

[a] Chemical shifts in italics are from a linear glucan (Lowman et al, 2011)
[b] Chemical shifts in italics are from a cyclic glucan (Lowman et al, 2014)
[c] Resonances not observed

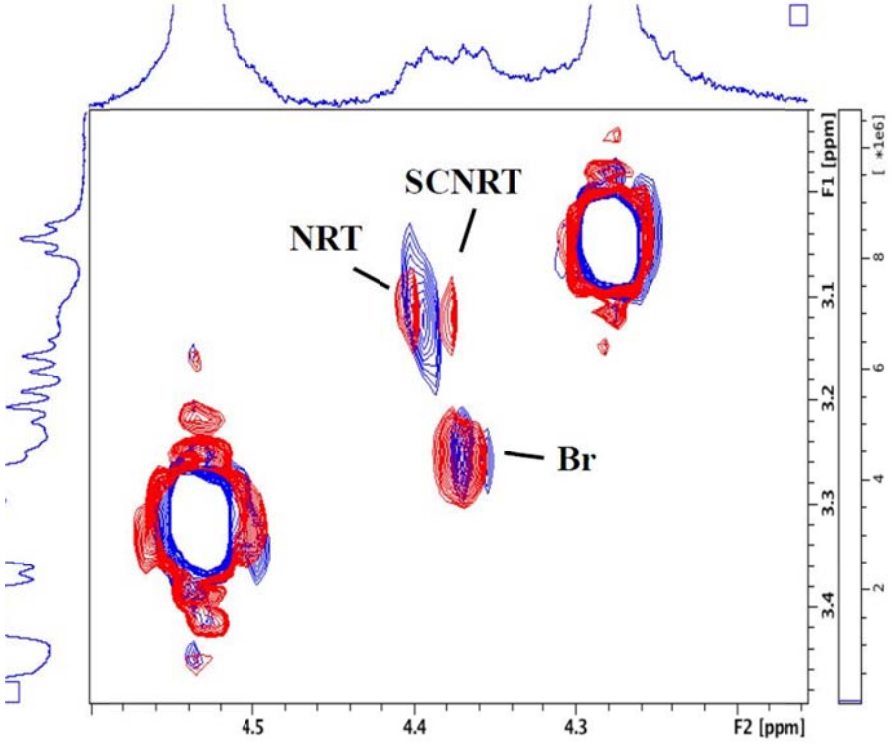

Fig. 3.6 Overlay plot of the COSY 2D NMR spectral region around 4.38 ppm for R20 (red spectrum) and R23 (blue spectrum). Cross peaks for Br, SCNRT, and NRT in R20 do not overlap with any of the cross peaks of R23 in this region

3.3.2 Comparison of the immunostimulatory efficacy of the yeast glucans

The protective effect of these marine yeast glucans against WSSV revealed that they have potential immunostimulatory activity. It was found that the marine yeast glucan administered group developed higher post-challenge survival against WSSV infection. Among the various treated group of shrimps, *D. fabryi* R100 (YG100) glucan fed group exhibited the highest post-challenge survival (60.41 %), followed by YG20 from *C. parapsilosis* R20 (54.83 %) and YG23 from *H. werneckii* R23 (46.87 %) (Fig. 3.7).

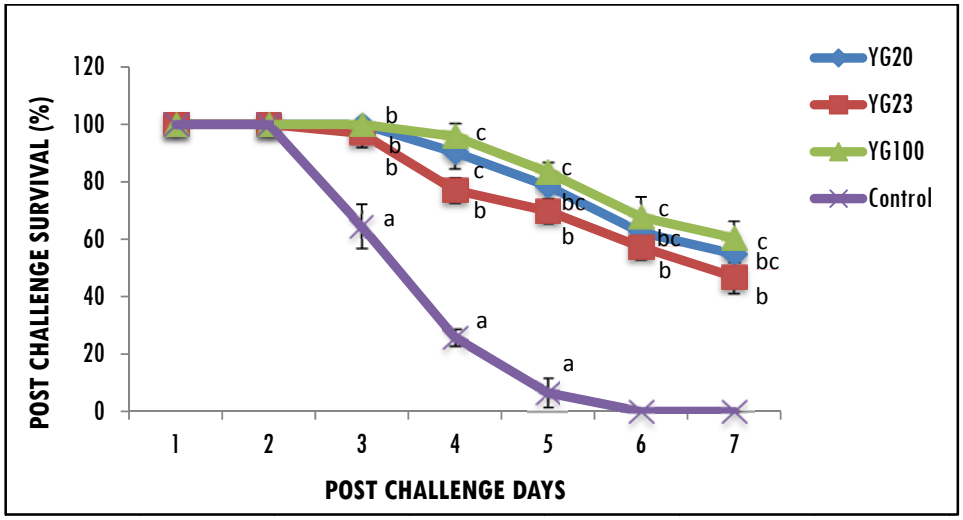

Fig. 3.7 Post challenge survival of *P. monodon* when fed with different marine yeast glucan incorporated diets and challenged with WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG100 – Yeast glucan 100 administered group, Control – Control feed administered group)

3.3.3 Expression of immune genes in haemocytes of *P. monodon* in response to marine yeast glucan and WSSV challenge

Relative expression of the genes in response to marine yeast glucans were analysed by quantitative real-time PCR in adult *P. monodon*. The genes were found to be differentially expressed in response to the dietary inclusion of β-1, 3-glucan (Fig. 3.8-3.18). While considering the AMP genes (ALF3, crustin and penaeidin), all three glucan treated groups were found to up-regulate the expression significantly when compared to the control. Among the various glucan treated groups, YG20 was found to be more effective in terms of AMP gene expression. Expression of crustin was lesser on YG20, YG23 and YG100 administration than ALF3 and penaeidin genes.

Marine yeast glucan treated shrimps demonstrated a differential mRNA expression pattern upon WSSV challenge. The AMP genes (ALF3, crustin and

penaeidin) were found to be up-regulated in all three YG20, YG23 and YG100 supplemented groups. In the case of penaeidin, the three YG treated groups were found to be significantly up-regulated on WSSV challenge. While all three marine yeast glucans up-regulated the AMP genes, YG100 exhibited the highest level of up-regulation. Transcripts of crustin were found to be lower in the YG23 fed group of shrimps post-challenge WSSV than the YG100 and YG20 fed groups.

In case of immune genes other than the AMPs, dietary intake of all three marine yeast β-1,3-glucans significantly enhanced the expression of PPA and PAP. Among the glucans tested, yeast glucan, YG20 was found to enhance the expression of the immune genes considerably, followed by YG100 and YG23 pre-challenge. Enhanced up-regulation of haemocyanin was also noticed in all three glucan treated groups. Also, lysozyme and ferritin was found to be up-regulated in all three glucan administered groups with YG20 administered shrimps exhibiting the highest level of expression compared to that of YG100 and YG23 fed groups. Transcripts of lectin could be detected in all glucan treated experimental groups and was higher in YG100 fed group. A complete down-regulation of survivin was noticed in the YG20, YG23 and YG100 glucan fed groups compared to control.

Even upon WSSV infection, the mRNA transcript of PPA and PAP was found to be significantly up-regulated in all the three YG treated groups. YG20, YG23 and YG100 also significantly up-regulated the expression of immune genes i.e., haemocyanin, lysozyme and ferritin post-challenge WSSV. White spot syndrome virus challenge enhanced the mRNA expression of survivin in all three YG treated groups. Enhancement of the lectin mRNA transcript also could be noticed in case of YG100, YG20 and YG23 treated groups. In all the three glucan treated groups, VP28 transcripts were lower compared to control i.e., 2.76 fold lower in YG100, followed by YG20 (2.62 fold) and YG23 (1.47 fold).

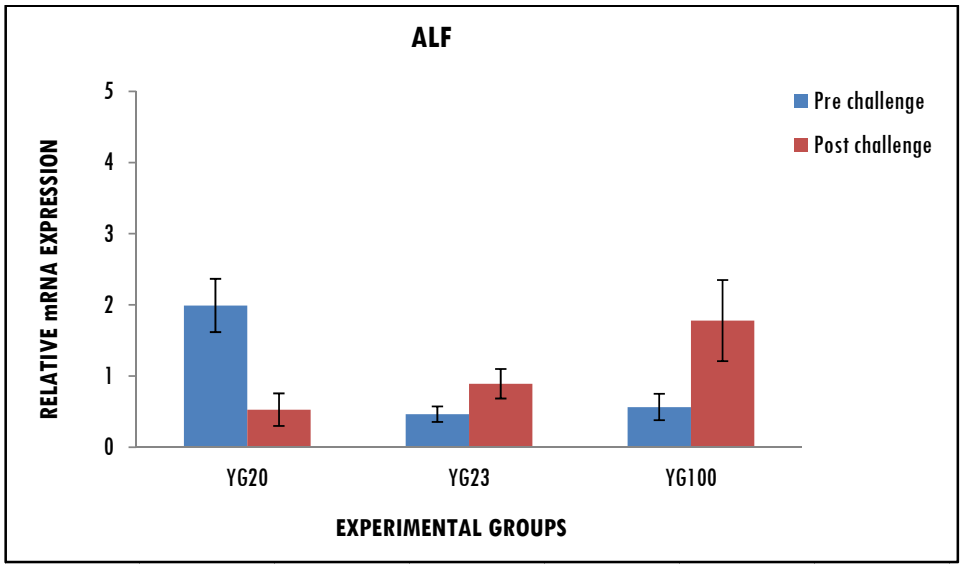

Fig. 3.8 Expression profile of ALF (AMP gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

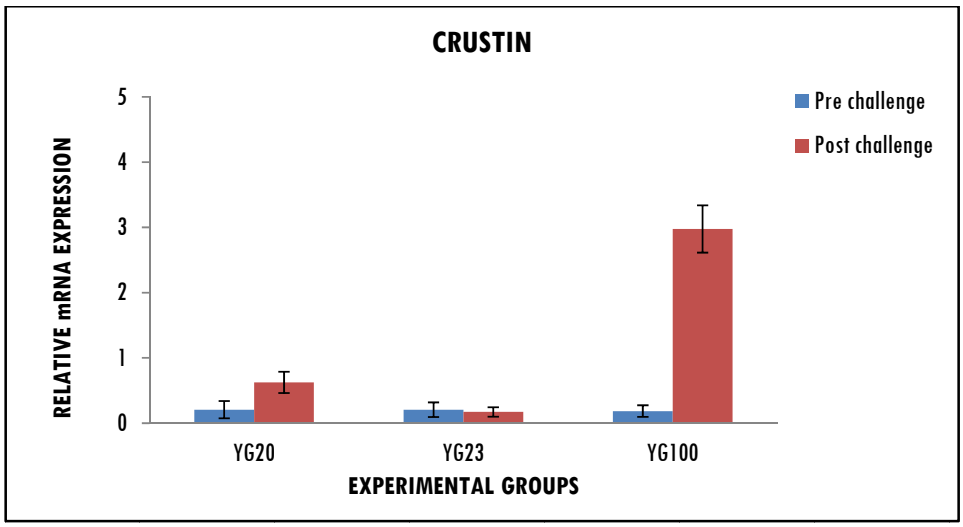

Fig. 3.9 Expression profile of crustin (AMP gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG100 – Yeast glucan 100 administered group)

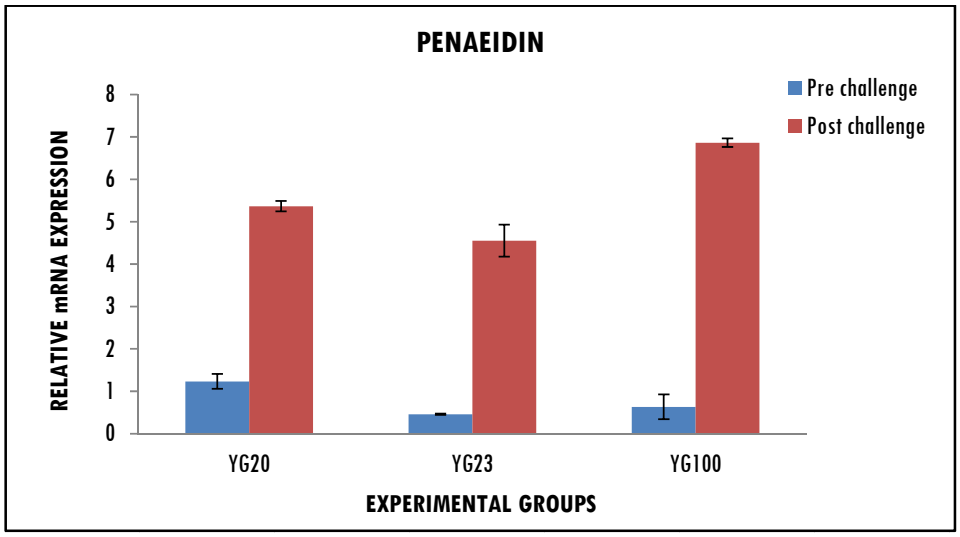

Fig. 3.10 Expression profile of penaeidin (AMP gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

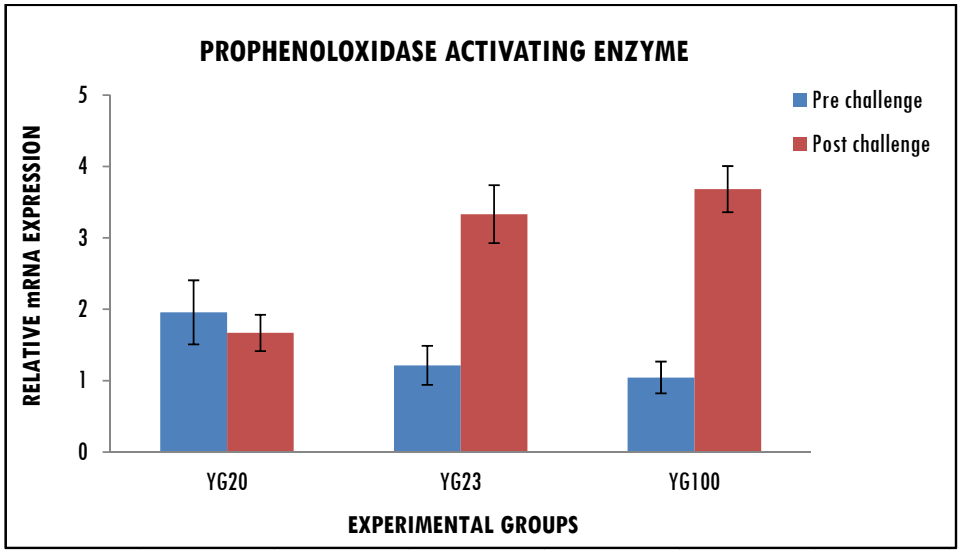

Fig. 3.11 Expression profile of proPhenoloxidase activating enzyme (biodefense gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG100 – Yeast glucan 100 administered group)

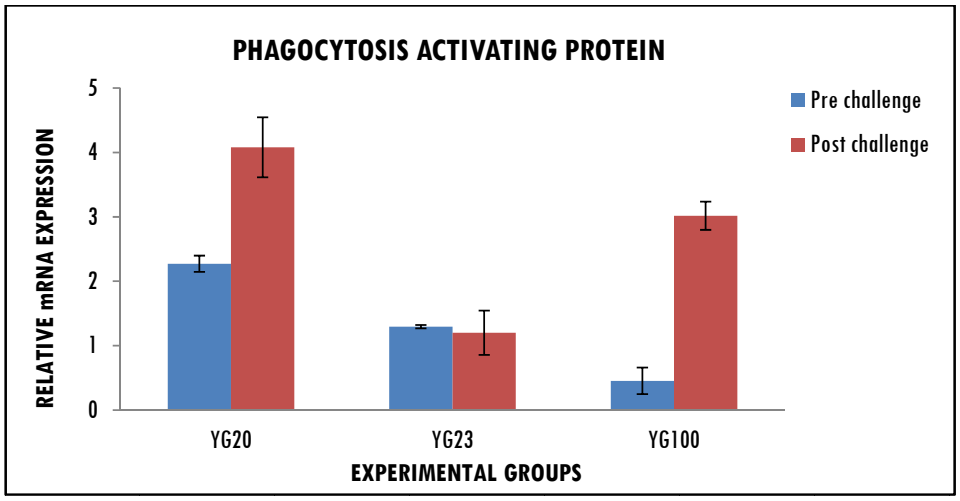

Fig. 3.12 Expression profile of phagocytosis activating protein (biodefense gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

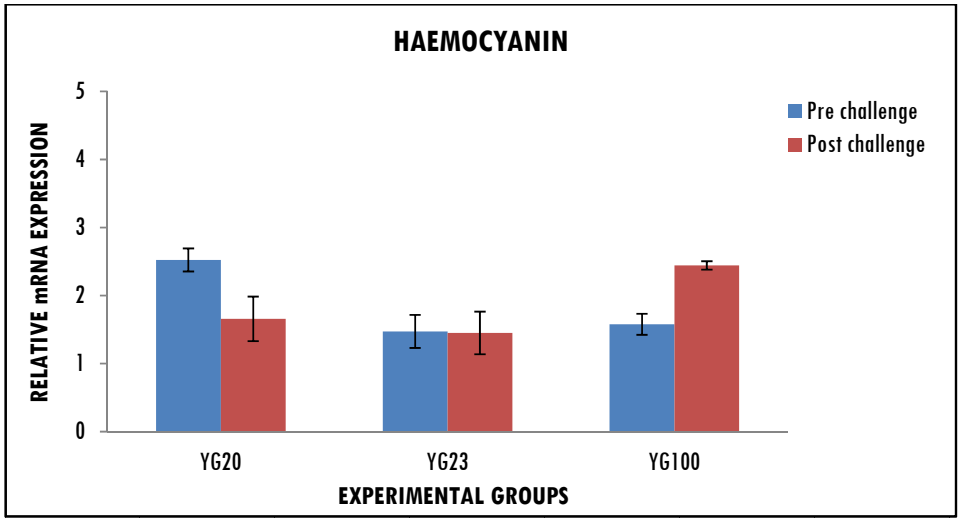

Fig. 3.13 Expression profile of haemocyanin (biodefense gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG100 – Yeast glucan 100 administered group)

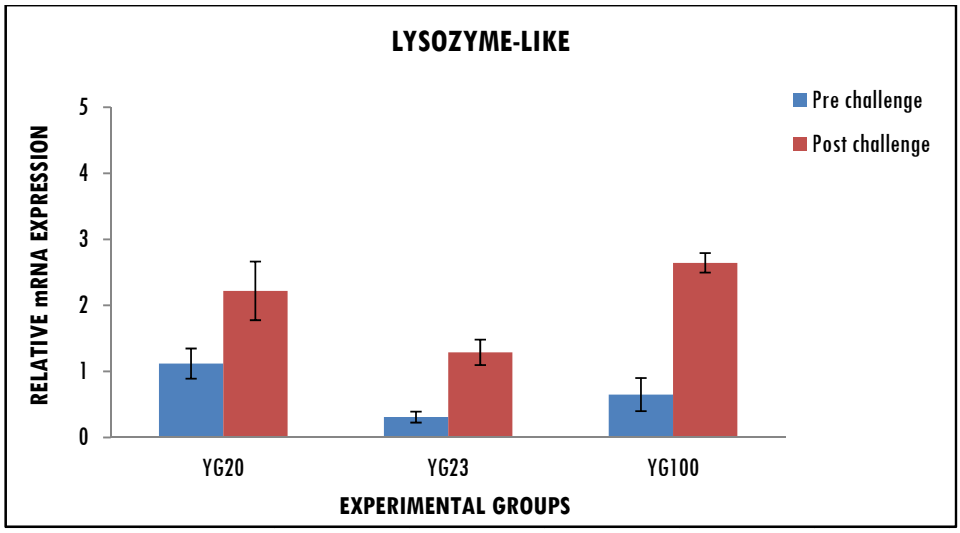

Fig. 3.14 Expression profile of lysozyme-like (biodefense gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

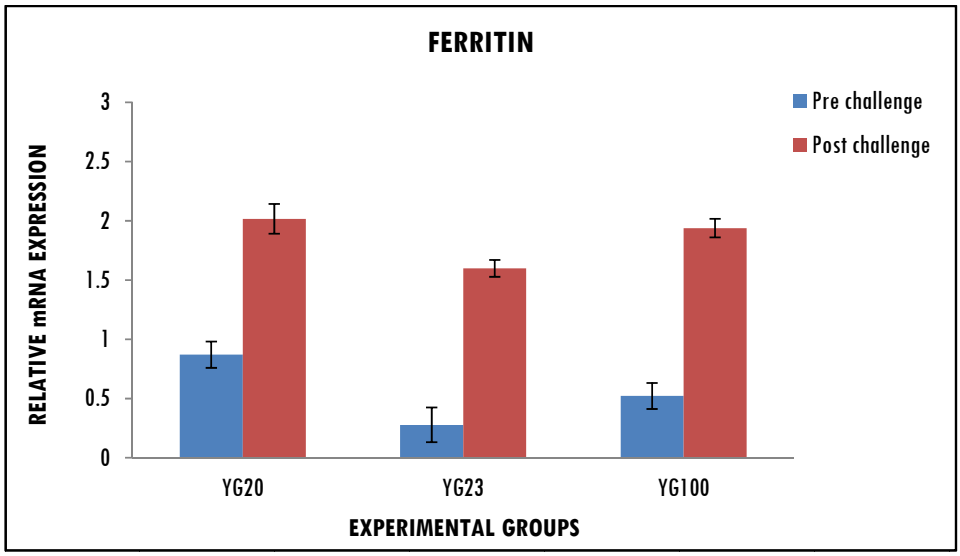

Fig. 3.15 Expression profile of ferritin (biodefense gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG100 – Yeast glucan 100 administered group)

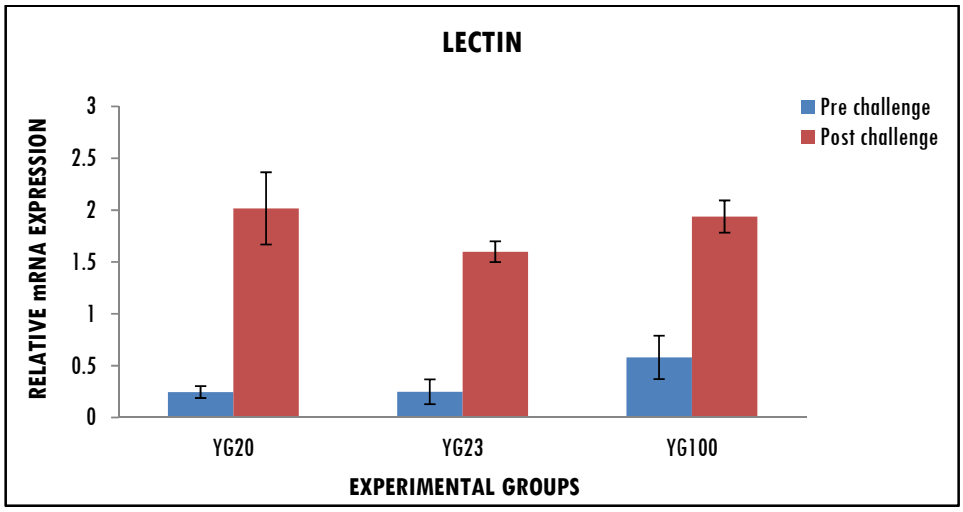

Fig. 3.16 Expression profile of lectin (biodefense gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post-challenge WSSV

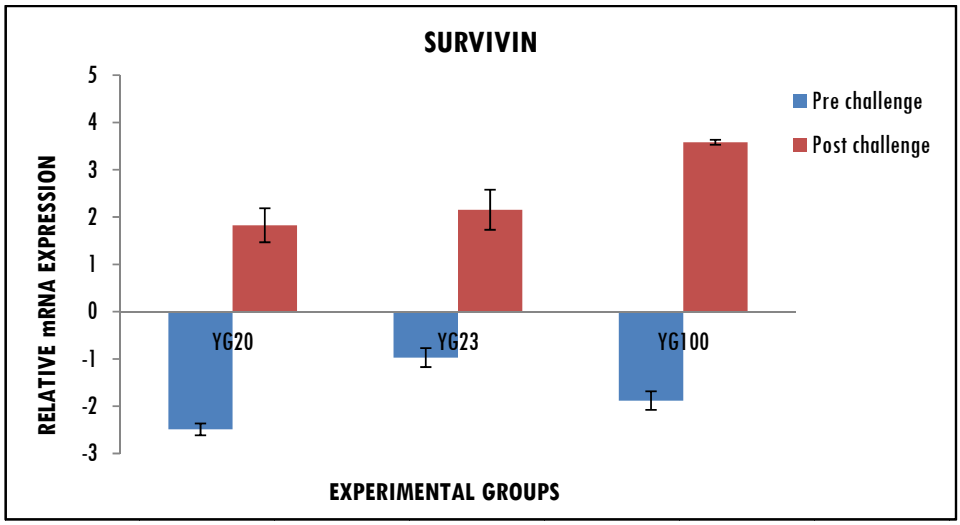

Fig. 3.17 Expression profile of survivin (biodefense gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans pre- and post- challenge WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG100 – Yeast glucan 100 administered group)

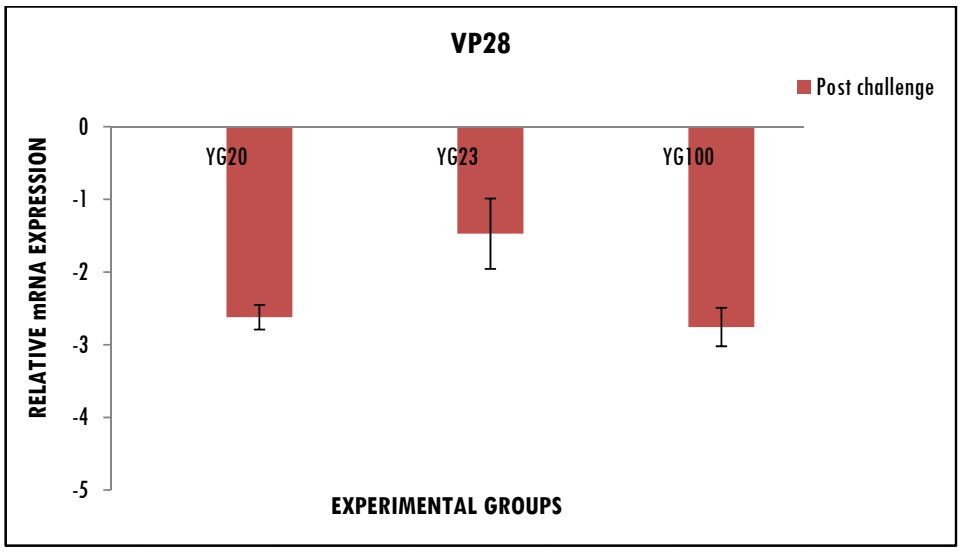

Fig. 3.18 Expression profile of VP28 (WSSV gene) in the haemocytes of adult *P. monodon* in response to the administration of marine yeast glucans and challenge with WSSV

(YG20 – Yeast glucan 20 administered group, YG23 – Yeast glucan 23 administered group, YG100 – Yeast glucan 100 administered group)

3.4 Discussion

Several commercial immunostimulants are being applied in shrimp culture system to induce protection or disease resistance against various pathogens, even though the exact mechanism is not yet completely known. Effective scientific interventions are prerequisite for the health modulators in shrimp culture for disease control. Even though few studies have been conducted on the enhancement of immune response related to the application of glucan in shrimp culture, immune gene expression studies are limited. Reports regarding quantitative gene expression studies on the gene expression of biodefense genes with respect to the application of marine yeast glucans and also in the course of WSSV infection is hardly to notice.

β-glucans, the integral cell wall constituent of yeasts, consisting of a (1,3)-β-linked backbone with (1,6)-β-linked side chains are recognised for

their immune-modulating effects (Bohn and BeMiller, 1995). Protection of shrimps from various diseases by the application of β-1,3-glucans has been demonstrated by earlier researchers (Song et al., 2003; Chang et al., 2003; Sukumaran et al., 2010; Nunes, 2010; Lin et al., 2011; Amparyup et al., 2012). Based on the previous experimental study in *P. monodon* post larvae in chapter 2, three potent marine yeast glucans were selected for further analysis. Hence the present study was undertaken to assess the potential of selected marine yeast glucans as immunostimulants in adult *P. monodon* by analysing the biodefense gene expression profile employing quantitative real-time PCR pre and post WSSV challenge.

The proton NMR study revealed that, glucan isolates from the three marine yeasts are (1-3)-β-D-glucan with (1-6)-branching as stated by Ohno et al. (1999). Previous study reported by Sukumaran et al. (2010) and Wilson et al. (2015) also stated that glucans extracted from different marine yeasts were (1-3)-β-D-glucan with varying degrees of (1-6)- linked branching.

In previous NMR reports, overlapping multiplet resonances at 4.38 ppm were assigned to anomeric protons on the branch point glucosyl repeat unit (Br), non-reducing terminus of the (1-6)-linked side chain (SCNRT), and the non-reducing terminus of the (1-3)-linked backbone (NRT) of linear (1-3, 1-6)-β-D-glucans isolated from *Candida glabrata* yeast (Lowman et al., 2011) and cyclic (1-3,1-6)-β-D-glucans isolated from *Candida albicans* hyphae (Lowman et al., 2014) using heteronuclear single quantum correlation (HSQC) 2D NMR spectra. In the present study, we discovered that assignments from the COSY spectrum can be used to accomplish the linear versus cyclic structural analysis.

All the experimental glucans were found to significantly enhance the survival rate of the experimental animal above 40%, whereas the control groups exhibited 100% mortality on 6th day post-challenge. In a previous study by Wilson et al. (2015), glucan administered post larvae of *P. monodon* was found to exhibit more than 50% survival upon oral challenge with WSSV. In the present experiment also, marine yeast glucan YG100 from *D. fabryi* R100 was found to possess potent immunostimulatory property in the adult tiger shrimps on challenge with WSSV *via* intramuscular injection. Sajeevan et al. (2009) reported that Indian white shrimp *Fenneropenaeus indicus* fed marine yeast glucan once in seven days showed significant resistance to WSSV challenge. The immunostimulatory effects of marine yeast β-(1-3)-glucan incorporated diet conferring better survival in *F. indicus* have been recorded by Subramanian and Philip (2013). The potential effect of β-glucan against various bacterial pathogens such as *Vibrio vulnificus* (Sung et al., 1994), *V. campbellii* and *V. proteolyticus* (Marques et al., 2006) and *Aeromonas veronii* (Lin et al., 2011) were also noticed. Similarly, the present study also proved the efficacy of marine yeast glucans in enhancing the immunity of adult *P. monodon* to combat WSSV infection.

Among the different genes tested, the relative expression of ALF was found to be significantly up-regulated in YG20 pre-challenge followed by YG100 and YG23 treated groups. The anti-lipopolysaccharide factor (ALFs) is an antimicrobial peptide (AMP) found in crustaceans, which exhibit a potent antimicrobial activity against a broad range of microorganisms (Rosa et al., 2013). Also the expression of ALF was found to be up-regulated with the administration of probiotic bacteria both pre- and post-challenge WSSV (Antony et al., 2011a). In the present study also, yeast glucan YG100, YG23 and YG20 diet fed groups showed significant up-regulation on WSSV

challenge. Antony et al. (2011b) proposed the possible role of ALF in antiviral defence of *P. monodon*. Liu et al. (2006b) proved that ALF has an antiviral effect towards WSSV in a study conducted in crayfish *Pacifastacus leniusculus.* Wilson et al. (2015) also noticed a considerable up-regulation of ALF in the glucan administered post larvae of *P. monodon* groups both pre- and post-challenge WSSV. The present study once again confirms the potential of marine yeast glucans to up-regulate the AMP gene ALF, confirming the possible role of ALF in antiviral defence.

In the case of crustin, glucan administration resulted in a similar pattern of gene up-regulation in YG20, YG23 and YG100 administered groups pre challenge WSSV. A significant up-regulation of the gene could be noticed in the YG100 treated group (2.97 fold) followed by YG20 and YG23 under WSSV challenge conditions. Antony et al. (2011c) suggested the immunostimulant potency of marine yeast glucans against WSSV infection in adult *P. monodon* through the up-regulation of crustin genes and thereby antiviral defence. Fagutao et al. (2008) reported an increase in the expression of crustin gene upon administration of peptidoglycan, derived from *Bifidobacterium thermopilum* in kuruma shrimp, *Marsupenaeus japonicus.* Wilson et al. (2015) found that marine yeast glucan administration could significantly up-regulate the various crustins (crustin-1, crustin-2 and crustin-3) and also noticed a down-regulation in control shrimps fed the normal diet. From the present study and previous reports, it is evident that, marine yeast glucan has the potential to up-regulate crustin both pre- and post-challenge WSSV.

Penaeidins are one of the important and unique AMP families in penaeid shrimps. The dietary intake of marine yeast glucan enhanced the up-regulation of penaeidin both pre- and post-challenge WSSV. mRNA transcript of penaeidin was found to increase 6.9 fold in YG100 treated groups post-

challenge WSSV followed by YG20 (5.4 fold) and YG23 (4.6 fold) when compared to that of the control group. Similarly, an up-regulation of penaeidin gene could be noticed upon glucan administration. Interestingly, the gene was found to be swiftly up-regulated upon WSSV challenge. Wang et al. (2007) noticed the highest level expression of PEN3 in the white shrimp *L. vannamei* by qPCR. Antony et al. (2011a) also noticed an over expression of penaeidin-3 in WSSV challenged *P. monodon* and suggested that these penaeidins might possess antiviral activity. Wilson et al. (2015) noticed a significant up-regulation of penaeidin-3 post-challenge WSSV compared to pre-challenge, when *P. monodon* post larvae were maintained on marine yeast glucan diet. All these results endorse the role of marine yeast glucans as an immunostimulant in *P. monodon* by the up-regulation of an important group of immune genes *viz.* the AMPs and thereby participating in antiviral defence mechanisms.

Activation of proPhenoloxidase (proPO) by a proteolytic cascade initiated by the proPO-activating enzyme (PPA) resulting in melanization and elimination of pathogens is one of the important innate immune responses in arthropods (Jearaphunt et al., 2014). Cerenius and Soderhall (2004) reported that the proPO system is elicited by the presence of minute amounts of compounds of microbial origin, such as β-1,3-glucans, lipopolysaccharides and peptidoglycans, which ensures that the system will become active in the presence of potential pathogens. In the present study PPA gene expression was also evaluated both pre- and post-challenge WSSV. Interestingly, dietary marine yeast glucan (YG) could up-regulate the PPA under both pre- and post-challenge. During pre-challenge, YG20 showed two fold increase in the up-regulation of PPA followed by YG23 (1.2 fold) and YG100 (1 fold) compared to that of the control. PPA was found to be significantly up-regulated under post-challenge conditions when compared to the glucan treated groups pre-

challenge. YG100 enhanced the expression up to 3.7 fold followed by YG23 (3.3 fold) and YG20 (1.7 fold) in post-challenge WSSV.

Yeh et al. (2009) noticed a down-regulation of proPO gene expression in white shrimp, *L. vannamei s*hrimps co-infected with WSSV and infectious hypodermal and hematopoietic necrosis virus (IHHNV). Similarly, shrimps fed β-glucan diets (BG) showed a significantly higher proPO concentration than the BG free group and after WSSV challenge, the proPO concentration gradually increased (Chang et al., 2003). Takahashi et al. (2000) noticed an increase of proPO on oral administration of cell wall constituent, LPS in viral challenged shrimps. Jearaphunt et al. (2014) studied the function of proPO fragments in innate immunity in response to bacterial infection in crayfish. After incubation with the proPO-ppA fragment, heavy agglutination could be noticed for both Gram-negative and Gram-positive bacteria. The study reported that recombinant proteins proPO-ppA had antimicrobial roles that has been proven by an extreme decrease in the number of *Escherichia coli in vitro*. While considering all the above information, authors suggest that the increased mRNA transcripts of PPA in WSSV infected shrimps might have resulted from the potential of YG to up-regulate the gene at the time of viral infection.

Phagocytosis activating protein (PAP) gene is able to activate phagocytosis of shrimp haemocytes. Oral administration of an immunostimulant such as inactivated *V. harveyi* (IVH) exhibited significant expression of the PAP gene and enabled protection against WSSV in *P. monodon* (Deachamag et al., 2006). In this study, it was found that YG could enhance the up-regulation of PAP gene in the haemolymph of *P. monodon* both pre- and post-challenge WSSV. Among the pre-challenge groups YG20 treated group exhibited highest expression followed by YG23 and YG100. During post-challenge, the highest expression was noticed in YG20 treated groups (4.0 fold) followed by YG100

(3.0 fold) and YG23 compared to the control. In a recent study by Khimmakthong et al. (2013) in *L. vannamei*, oral immunisation of chitosan-PAP-phMGFP nanoparticles significantly increased PAP expression against WSSV, YHV and *V. harveyi*. Khimmakthong et al. (2011) showed that PAP-phMGFP immunisation in *L. vannamei* resulted in the highest expression of PAP gene and survival.

Haemocyanin, the respiratory protein of arthropods and molluscs also function against various pathogenic invasions (Destoumieux-Garzon et al., 2001; Lee et al., 2002; Lei et al., 2008). Haemocyanin has potent immune responses and multiple functions such as antiviral (Nesterova et al., 2011), anticancer (Lammers et al., 2012), antiparasitic (Guo et al., 2011) and therapeutic potential (Zanjani et al., 2014). It may be an effective non-specific innate immune defence molecule (Decker and Jaenicke, 2004; Lei et al., 2008; Coates and Nairn, 2014). In the present study, the gene expression levels of haemocyanin in *P. monodon* treated with the polysaccharide YG before and after WSSV challenge were higher than that of the control group. Increased level of haemocyanin mRNA transcripts could be detected in the case of YG20 treated groups (2.5 fold increase) which was followed by YG100 (1.6 folds) and YG23 (1.5 folds). At the time of WSSV infection also marine yeast glucans could significantly up-regulate the haemocyanin gene especially in YG100 (2.4 folds) followed by YG20 (1.7 folds) and YG23 compared to that of the control. So these findings reveal the importance of marine yeast glucan as an immunostimulant in shrimp antiviral defence and thus an active involvement related to shrimp innate immunity. The impact of marine yeast glucan in WSSV infected *P. monodon* post larvae has been recently reported by Wilson et al. (2015) and the present information in terms of immune gene expression also emphasize marine yeast glucan as a potential immunostimulant. Wang et al. (2013a) observed higher haemocyanin

gene expression, relative to the control group, in haemocytes and gills (but not hepatopancreas) of crayfish *Cherax quadricarinatus* injected with polysaccharides.

Lysozyme is considered to be an essential component of the innate immune system which can hydrolyse bacterial cell walls and act as a non-specific innate immunity molecule that defends against microbial infections (Ji et al., 2009; Tanekhy and Fall, 2015). In *Fenneropenaeus chinensis,* oral administration of algal polysaccharide, extracted from *Sargassum fusiforme* (SFPS) enhanced the lysozyme activity and improved the resistance to vibriosis efficiently (Huang et al., 2006). Yao et al. (2008) reported a moderately increased lysozyme-like activity in the haemocytes of *F. chinensis* after laminarin injection. Wang et al. (2008) reported that β-glucan administration in *L. vannamei* significantly increased the mRNA transcript of lysozyme. In the present study, all three marine yeast glucans enhanced the up-regulation of lysozyme-like gene pre-challenge when compared to control at a level of 1.11 fold in YG20 treated group followed by YG100 (0.64 fold) and YG23 (0.3 fold). These results suggest that the lysozyme-like expression level in *P. monodon* was enhanced on YG administration. The lysozyme-like mRNA transcript level in shrimps post-challenge WSSV up-regulated considerably i.e., 2.64 fold in YG100 treated group followed by YG20 (2.21 fold) and YG23 (1.28 fold). So the up-regulation of lysozyme-like gene in shrimps post-challenge WSSV endorses the efficiency of marine yeast glucan as a promising immunostimulant in shrimps.

C-type lectin plays an important role in the innate immunity of invertebrates by mediating the recognition of pathogens to host cells and clearing micro invaders (Luo et al., 2006). In the present study also, the administration of yeast glucans up-regulated the lectin pre-challenge with 0.57 fold increase in YG100. Whereas in post-challenge, an up-regulation at a level

of 1.94 fold in YG100 treated group followed by YG20 and YG23, could be noticed when compared to control. In *M. japonicus* upon WSSV challenge, both LdlrLec1 and LdlrLec2 mRNA were apparently up-regulated (Xu et al., 2014). Zhao et al. (2009) noticed an increase in the expression of lectin genes upon WSSV challenge in shrimps suggesting their key role in antiviral responses.

Ferritin, primary iron storage proteins in living cells, is necessary for maintenance of iron homeostasis. It also displays functional roles in biological processes including development (Levenson and Fitch, 2000), neuronal differentiation (VanLandingham et al., 2003) and detoxification (Munro et al., 1978). In invertebrates, ferritin is considered to be an essential element in the innate immune system (Pan et al., 2005; Ruan et al., 2010). Zhang et al. (2006) reported that ferritin is supposed to play a key role in host defence responses in animals and may be involved in the response against viral infection. The exhaustion of cellular iron can lead to the inhibition of ribonucleotide reductase, preventing new DNA synthesis and hence inhibiting cell proliferation (Nyholm et al., 1993). From the present results, it is evident that immunostimulant YG administration in penaeid shrimp could up-regulate the ferritin gene pre-challenge compared to control. While considering the mRNA transcript of this gene, YG20 could produce more transcripts (0.87 fold higher) followed by YG100 (0.52 fold) and YG23 (0.27 fold) treated groups compared to control. This observation suggests that the administration of glucan up-regulated ferritin compared to the control. The present study also documented that glucan incorporated diet supported higher ferritin mRNA transcripts post-challenge WSSV compared to the control. Among the three glucans higher transcript levels could be detected in the YG20 treated group (2.01 fold increase) followed by YG100 (1.93 fold) and YG23 (1.59 fold) post-challenge.

A recent work by Ye et al. (2015) stated that ferritin injection could protect shrimp *L. vannamei* from WSSV by inhibition of virus replication. They also noticed that shrimps injected with ferritin protein exhibited a lower mortality rate than shrimps without ferritin protein. Ruan et al. (2010) reported that ferritin injection significantly increased the survival of *L. vannamei* challenged with WSSV and noticed an increase in THC and O_2^- levels as well as SOD and PO activities in the ferritin injected shrimps. These reports suggest that ferritin could enhance the host's nonspecific immune system. Based on the work that has been done using glucan and other immunostimulants to study ferritin gene expression, it is possible to suggest that YG can up-regulate the ferritin gene during viral attack and is also a part of the protection mechanism for the shrimps among various other immune genes involved in the enhancement of their host defence mechanism.

Survivin is a member of the inhibitor of apoptosis (IAP) family. The function of this protein is known to inhibit caspase activation and thereby leading to negative regulation of apoptosis or programmed cell death. LvIAP1 and LvIAP3 that was cloned and characterised from *L. vannamei*, have been noticed for its host defence against WSSV infection by dsLvIAP mediated gene silencing in *L. vannamei* (Wang et al., 2013b). Li et al. (2012) identified a member of the inhibitor of apoptosis protein (IAP) family termed baculoviral IAP repeat-containing BIRC7 from channel catfish (*Ictalurus punctatus*). The gene expression study revealed that *CcBIRC7* gene expression was significantly up-regulated in the pathogen infected channel catfish and suggested that this protein might play a potential role in channel catfish innate immune system against bacterial and virus infections. In the present study also the survivin gene was found to be up-regulated in shrimps post-challenge WSSV, maximum in YG100 treated group (3.58 fold) followed by 2.15 fold

increase in YG23 and 1.82 fold in YG20 compared to the control. Each of the three glucans could enhance the up-regulation of survivin gene with YG100 providing the best enhancement. When compared to the shrimps post-challenge WSSV, the pre-challenge group exhibited a down-regulation in the entire YG treated group. Information on the expression of survivin gene in invertebrates while using an immunostimulant is limited. However, the present data suggest that glucan could enhance up-regulation of this gene at the time of WSSV infection and may be involved in antiviral defence of shrimps.

VP28 is a major envelope protein of WSSV, which is essential for the attachment and penetration of WSSV into shrimp cells. WSSV- related gene VP28 was also analysed to detect the presence and intensity of viral infection after glucan treatment in the experimental shrimps post-challenge. The intramuscular injection is believed to be a more effective method of WSSV challenge when compared to the oral route (Namikoshi et al., 2004). Here the intensity of infection will be more and the growth of the infection will occur at a faster rate as well as the exact dosage can also be validated. In the present study, it was found that glucan could reduce the intensity of infection in all the experimental groups when compared to the control group. Wilson et al. (2015) reported that marine yeast glucan treated post larvae of *P. monodon* were found to be less infected than the control group. Antony et al. (2011c) also reported the impact of marine yeast glucans on WSSV infection in *P. monodon.* The mRNA transcript of VP28 was least in the YG100 treated group followed by YG20 and YG23. This is also in agreement with the post-challenge survival data in which YG100 glucan treated group exhibited higher survival followed by YG20 and YG23. Marine yeast glucan treated groups were less infected than the control group indicating the efficacy of marine yeast glucan as an effective immunostimulant.

From the present study, it is evident that marine yeast glucan has the potential to protect the shrimps against WSSV infection through the up-regulation of immune genes. This study also revealed the difference in structural arrangement of glucans extracted from three different yeast isolates. The proton 1D and 2D COSY NMR study revealed that the R20 marine yeast glucan is a linear (1-3, 1-6)-β-D-glucan while R100 marine yeast glucan is a cyclic (1-3, 1-6)-β-D-glucan. The glucan isolate from marine yeast R23 could not be characterized as either linear or cyclic (1-3, 1-6)-β-D-glucan unequivocally. The cyclic glucan YG100 conferred more protection against WSSV infection by the up-regulation of biodefense genes at the time of viral challenge, emphasizing the importance of structure based effectiveness of glucan while used as an immunostimulant. The frequency and nature of side chains affect the ability of glucans in binding to surface receptors on the target cells influencing the efficacy of the glucan as an immunostimulant (Ringo et al., 2012). All three marine yeast glucans supported more than 40% survival in *P. monodon* upon WSSV challenge while also up-regulating the immune genes in shrimps both pre- and post-challenge. So these three marine yeast glucans (YG100, YG20 and YG23) are potential immunostimulant in black tiger shrimp *P. monodon* for protection against WSSV infection.

Marine yeast glucans play crucial roles in enhancing the shrimp innate immunity and supporting better survival against microbial invasions. These findings will facilitate the development of effective prophylactic approaches such as the use of immunostimulants for disease prevention in the aquaculture industry. Thus the marine yeast glucans YG100, YG20 and YG23 could be suggested to the aquaculture feed manufacturing industries for incorporation in their aquatic feed formulation and to help the farmers to get a better yield.

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Chapter 4

Characterization and evaluation of the bioactivity of melanin from a marine black yeast, *Hortaea werneckii*

4.1 Introduction

In nature, pigments ensue across several taxonomical groups, from bacteria to plants and animals. The presence of pigments has endowed organisms with the ability to develop new and diversified strategies of survival. Owing to the amazing chemistry of marine organisms, many species exhibit a wide range of colours, many of which exhibit several biological properties and constitute an evolutionary adaptation. One of the untapped sources in the marine environment are various types of microbial community like bacteria, yeasts, mold and algae and they are presently explored for pigments. The advancement in cultivation, extraction and the emergence of sophisticated technology has made its use further possible (Nigam and Luke, 2016). Microbial pigments exhibit a wide range of hues from bright coloured carotenoids, quinones, prodigiosion, phenazines, indigoidine, violaceins, to the dark pigment melanins.

Yeast cultures are considered as a commendable source of microbial pigments due to their unicellular nature and high growth rate. Among the

microbial pigment producers, yeast has gained a substantial position due to its wide use across several industries, from foodstuffs to cosmetics and pharmaceuticals. Yeasts are distributed in almost every part of the aquatic environment including marine and other hyper saline environments and a few of the marine yeasts are good source of microbial pigments. β-carotene, Torulene, Astaxanthin, Canthaxanthin are some of the bioactive pigments explored from yeasts. They also synthesize a dark pigment melanin, which offers the dark-coloured fungi a competitive advantage under harsh environmental conditions. Even though presence of melanin have been reported from some of the yeast species, characterization of the melanin pigments and its application is least investigated.

Natural melanin pigments are found in dark colours ranging from brown to black with high molecular weight formed by oxidative polymerization of phenolic and/or indolic compounds which have their presence in animals, plants and in most of the microorganisms (Plonka and Grabacka, 2006). These complex polymers are amorphous in nature, negatively charged, hydrophobic and soluble in neither aqueous nor organic solvents. They are resistant to concentrated acids and are susceptible to bleaching by oxidizing agents (Butler and Day, 1998; Liu and Simon, 2003; Nosanchuk and Casadevall, 2006).

Pronounced diversity of functions have been attributed to melanins. Some of the chemical properties of melanins are: it can act as redox polymers, radical scavengers, ion chelating agent, semiconductor materials with high capacitance useful for nanotechnological devices and ability to bind a range of biomolecules, and organic agents (drugs, antibiotics and xenobiotics) (Hill, 1992; Lee et al., 2007; Bothma et al., 2008; Solano, 2014). Furthermore, melanin is also used for sexual attraction and for sexual differentiation between male and female in many of the avian species (McGraw, 2008). The ink used by octopus,

cuttlefish and other cephalopods consist of melanin as a defense mechanism against marine predators. The colour variation of skin, hair and eyes also arise from different types of melanins besides conferring protection from sunlight (Hill, 1992).

Melanins have variety of biological functions such as photoprotection (against UV and visible light) (Paramonov et al., 2002), radio protective (Dadachova et al., 2007a), free radical scavenging (Rozanowska et al., 1999), antioxidant (Tu et al., 2009), antitumor, anti-inflammatory (El-Obeid et al., 2006) and also act as an immunostimulant (Sava et al., 2001). Melanins interact readily with free radicals and other species because of the existence of unpaired electrons in their molecules and this property make them a strong candidate to scavenge reactive oxygen species and act as a natural antioxidant.

Melanin production is one of the most universal, but at the same time mysterious adaptations of living organisms to the variable conditions of the Earth. Melanins are enigmatic pigments that are produced by a wide variety of microorganisms including several species of bacteria and fungi (Selvakumar et al., 2008). The ability of many pigments to stabilize reactive oxygen species (ROS) may be inherently linked to the ability of these compounds to confer colour sensorium. It was suggested that most pigments evolved initially as a mechanism to combat environmental ROS, but over time, these compounds were adapted to serve divergent functions (Liu and Nizet, 2009). In last few decades, there has been an increasing trend towards replacement of synthetic colourants with natural pigments because of the strong consumer demand for more natural products. It is therefore, essential to explore various natural sources of pigments and their potential. In this context, the microbial melanins are less studied with regard to their characteristics and functional potential.

4.1.1 Sources of melanin

Plant/vegetable melanin: Various plant/vegetable sources exhibited melanin production such as tea leaves, grapes, chestnut, sunflower seeds, black beans etc. Melanins were also detected from a number of botanical sources and the biological properties were also reviewed (Zherebin et al., 1982; Sava et al., 2001; Mohagheghpour et al., 2000; Pugh et al., 2005; Pasco et al., 2005). Catechol is the most common precursor for melanin formation in plants and so named catechol-melanin, a sub type of allomelanin. Catechol oxidase is the enzymatic system involved in the melanin synthesis. Besides normal catechol, some vegetables use different catecholic acids such as protocatechuic, caffeic, gallic or chlorogenic acids as a precursor (Mayer, 1986; Yao et al., 2012; Solano, 2014).

Animal Melanin: Melanins are very common among most of the animals such as insects, fish, amphibians, reptiles and humans. It is mainly seen in hair, skin, eye, inner ear, brain, bird feathers and exoskeleton of insects (Sugumaran, 2002; McGraw et al., 2008; Simon and Peles, 2010). Eumelanin and pheomelanin are the two large groups of animal melanins (Roulin et al., 2013). L-tyrosine, L-Dopa, 5-cys-dopa, dopamine, 5-S-cys-dopamine and N-acetyl dopamine are the main precursors of animal melanins (Solano, 2014).

Sepia melanin is a distinct type of melanin found in the ink of cuttle fish and is usually eumelanin. In most of the studies, sepia melanin has been used as an excellent model to understand the structure and properties of eumelanin (Magarelli et al., 2010).

Synthetic melanin: chemical or enzymatic oxidation of tyrosine or 3, 4-dihydroxy-L-phenylalanine (L-DOPA) is a method for the production of synthetic melanin (Kim et al., 2012). Synthetic soluble melanin can be produced

by combining dopachrome and an appropriate enzyme, or by incubating 5, 6-dihydroxyindole-2-carboxylic acid alone or with 5, 6-dihydroxyindole, or with 3-amino-tyrosine (Pawelek et al., 1995). These synthetic melanins are often used as a standard for most of the biophysical studies.

Microbial melanin: Melanin, the enigmatic pigment are produced by wide variety of microorganisms including several species of yeasts, fungi and bacteria (Dong and Yao, 2012; Kejzar et al., 2013; Tarangini and Mishra, 2014). Most of the fungi may synthesize melanin from endogenous substrate via 1, 8-dihydroxynaphthalene (DHN) intermediate and some fungi produce melanin from L-3,4-dihydroxyphenylalanine (DOPA). The occurrence of melanin in bacteria is common and formed through various melanin biosynthesis pathways (Plonka and Grabacka, 2006).

Herbal melanin, extracted from *Nigella sativa* L. was characterized by electron spin resonance (ESR), infra-red (IR), ultraviolet-visible (UV-VIS) and nuclear magnetic resonance (NMR). Herbal melanin modulates tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) production suggesting for treatment of diseases associated with imbalanced cytokine production and for enhancing cancer and other immunotherapies (El-Obeid et al., 2006). Melanins extracted from black tea (BT-melanin), black soybean (BS-melanin) and black-bone silky fowl (SF-melanin) were compared with synthetic melanin (SY-melanin). Melanins from these three sources exhibited similar physicochemical properties and they have the capacity for antioxidation and photoprotection from UV irradiation (Hsieh and Lien, 2012). Melanin extracted from the muscles of Taihe Black-bone silky fowl (TBSF) exhibited strong antioxidant activity by *in vitro* evaluation suggesting their potential use as a natural antioxidant in the food, cosmetic and

pharmaceutical industries (Tu et al., 2009). These studies show the potential of melanin as a bioactive compound and also for various industrial applications.

4.1.2 Fungus as a source of melanin

Many fungal species including yeasts produce melanin and it act as a multifunctional defensive macromolecule against various environmental stresses (Eisenman and Casadevall, 2012). *Aspergillus* sp, *Lachnum* sp, *Cryptococcus* sp, *Penicillium marneffei, Paracoccidioides brasiliensis, Pestalotiopsis microspora, Saccharomyces neoformans var. nigricans, Yarrowia lipolytica, Hortaea werneckii, Aureobasidium pullulans, Cladosporium cladosporioides, C. carrionii, C. bantianum, Phaeotheca triangularis, Trimmatostroma salinum, Cladophialophora carrionii, C. bantiana, Exophiala jeanselmei* and *E. mansonii* are some of the fungi identified to produce melanin (Taylor et al., 1987; Yurlova et al., 2008; Ye et al., 2014; Yu et al., 2015). Most of the melanin producing microorganisms are from the terrestrial sources and little is known from the marine environment.

Study reports regarding marine fungi that produce melanin are scanty. An obligate marine fungus *Cirrenalia pygmea* showed melanin production ability in its mycelium and conidia (Ravishankar et al., 1995). DHN melanin present in *C. pygmea* gives protection from the bursting due to hasty changes in the external osmotic shock. The ascomycetous black yeasts *Hortaea werneckii*, *Phaeotheca triangularis* and *Trimmatostroma salinum* were isolated from hypersaline water of a crystallization pond in a solar saltern at the eastern coast of the Adriatic Sea. The pigment synthesized by these three fungi was reported as DHN-melanin under saline and non-saline growth conditions (Kogej et al., 2004). Tricyclazole was found to be a specific inhibitor of DHN melanin synthesis (Butler et al., 2004).

Recently, marine black yeast *Hortaea werneckii* from slope sediments of Bay of Bengal was isolated and identified by Internal Transcribed Spacer (ITS) sequencing. The pigment production was noticed and identified to be DHN type by two-dimensional NMR spectroscopy analysis. The inhibitory activity of melanin against different human and fish pathogens also was reported (Kutty et al., 2013b).

In another study, *H. werneckii* isolated from the solar salterns water samples near Pondicherry, were produced a diffusible dark pigment and was identified as melanin. The favourable condition for the high yield of melanin was found to be temperature 30ºC, salinity 15‰, pH 7.0 and incubation period of 168 hrs. The effective carbon source was glucose and nitrogen source was peptone for melanin production. They also reported rice bran as a cheaper substrate for melanin production i.e., 5.60g/L melanin. As well, it exhibited antibacterial activity against potential pathogens like *Salmonella typhi*, *Vibrio parahaemolyticus* and *Klebsiella pneumonia* (Rani et al., 2013).

A novel strain of *Aspergillus bridgeri* isolated from rhizosphere soil was able to produce melanin and is identified as a member of DHN melanin family based on chemical analysis and UV-visible, FTIR and EPR spectra. Significant free radical scavenging activity of the identified melanin was reported (Kumar et al., 2011). Although most of the Ascomycetes produce DHN-melanin, some reports suggest that *Aspergillus nidulans* does not produce this type of melanin. The pigment extracted from highly melanized strains (MEL1 and MEL2) of *A. nidulans* showed that the pigment produced by MEL1 and MEL2 mutants possesses similar physical and chemical properties of DOPA-melanin (Goncalves et al., 2012).

Most of the fungal melanin polymers are derived from the phenolic compounds including tyrosine via 3,4-dihydroxyphenylalanine (DOPA) in various fungi and other microorganisms, γ-glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol in *Basidiomycetes*, and 1,8-dihydroxynaphthalene (DHN) in *Ascomycetes* and related *Deuteromycetes* (Bell and Wheeler, 1986). The most prevalent fungal melanin appears to be DHN melanin, produced by a polyketide pathway and named after the end product of the pathway 1,8-dihydroxynaphthalene (DHN), which is polymerized to form the melanin product and provides the dark-coloured fungi a competitive advantage under harsh environmental conditions.

4.1.3 Enzymes for microbial melanin synthesis

The key enzymes involved in melanogenesis are Tyrosinase, Laccases, Polyketide synthases, *p*-hydroxyphenylpyruvate hydroxylase and 4-hydroxyphenylacetic acid hydroxylase. Tyrosinase is a copper-containing monooxygenase known to be the key enzyme in melanin biosynthesis. It catalyzes the ortho-hydroxylation of tyrosine (monophenol) to 3,4-dihydroxyphenylalanine or DOPA (o-diphenol) and the oxidation of DOPA to dopaquinone (o-quinone). This o-quinone can then be transformed into melanin pigments through a series of enzymatic and nonenzymatic reaction. In fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin form tyrosine (Baurin et al., 2002). *Streptomyces glaucescens* and the fungi *Neurospora crassa* and *Agaricus bisporus* are the best-characterized tyrosinases derived microbial melanin sources. Tyrosinase is also used to produce synthetic melanin which offers protection against radiation and used as cation exchangers, drug carriers, antioxidants, antiviral agents or immunogens (Zaidi et al., 2014).

Laccases are blue multicopper oxidases, which catalyze the monoelectronic oxidation of a broad spectrum of substrates such as ortho- and para-diphenols, polyphenols, aminophenols and aromatic or aliphatic amines. Laccase activity has been demonstrated in many fungal species belonging to ascomycetes and basidiomycetes. Regardless of the organisms or the type of melanin (DHN or L-DOPA), laccases are the required and conserved factors for melanin biosynthesis (Upadhyay et al., 2013, Viswanath et al., 2014).

Polyketide synthases (PKS) belong to an old family of multidomain proteins related to the animal fatty acid synthases and involved in DHN melanin synthesis (Kroken et al., 2003). DHN-melanin biosynthesis starts with a polyketide synthase (PKS) using acetate as a precursor. Up-regulation of polyketide synthase gene (PKS1) in *Bipolaris oryzae* in the melanin biosynthesis pathway was reported (Moriwaki et al., 2004).

Pyomelanin or homogentisinic acid (HGA) based melanin synthesis is carried out by the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) which catalyzes the reaction from 4-hydroxyphenylpyruvate to HGA. The latter then auto-oxidizes to form benzoquinone acetic acid and then self-polymerizes to produce the pigment pyomelanin. Biosynthesis of pyomelanin mediated by HPPD in *A. fumigatus* and *Aeromonas media* has been reported (Schmaler-Ripcke et al., 2009; Wang et al., 2015).

Another enzyme, 4-hydroxyphenylacetic acid hydroxylase (HPA) catalyzes the formation of melanin pigment acting on 4-hydroxyphenylacetic acid and hydroxylation of L-tyrosine has also been reported (Gibello et al., 1997; Sajjan et al., 2010).

4.1.4 Types of melanin

Melanins are mainly classified into three main types based on colour and structural classes: a) Eeumelanins b) Pheomelanins c) Allomelanins.

Eumelanins are black or brown coloured pigment produced by the oxidation of tyrosine and/or phenylalanine to o-dihydroxyphenylalanine (DOPA) and dopaquionone by the action of tyrosinases which further undergoes cyclization to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole- 2-carboxylic acid (DHICA) (Langfelder et al., 2003).

Pheomelanins are red or yellow coloured pigments. These are produced in course of oxidation of tyrosine and/or phenylalanine to dihydroxyphenylalanine (DOPA) as the same pathway in eumelanin synthesis. Cysteinylation of the end product DOPA form cysteinyl DOPA which gets further polymerized to form pheomelanin. These are sulphur containing compounds by the presence of cysteine (Nappi and Ottaviani, 2000; Gomez-Marin and Sanchez, 2010).

Allomelanins are the most heterogeneous group of melanins found in many plants and fungi. Using acetate as a precursor, polyketide synthase (PKS) starts DHN-melanin biosynthesis. Hydroxynaphthalene reductase converts 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone. Dehydration of scytalone forms 1,3,8-trihydroxynaphthalene (1,3,8-THN), which is then converted to 1,8-DHN after an additional reduction and dehydration. Finally, oxidative polymerization of 1,8-DHN gives the DHN-melanin (Moriwaki et al., 2004).

4.1.5 Characterization of melanin

Even if melanin is found throughout nature, little is known about the chemical structure. This is because of the lack of ability of present biochemical

and biophysical techniques to provide a complete chemical structure, because these complex polymers are amorphous, insoluble and not agreeable to either solution or crystallographic structural studies. Characterization of melanin is mainly based on various spectroscopic studies such as UV-Visible spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy (NMR), energy dispersive spectroscopy and electron spin resonance (ESR) spectroscopy. Scanning electron microscope (SEM) is used to understand the morphology of the purified pigment (Suryanarayanan et al., 2004; Ye et al., 2014).

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying the types of chemical bonds (functional groups) and therefore, can be used to elucidate some components present in small size samples (Tu et al., 2009). Earlier study reports have revealed that melanin from various sources exhibit some common physicochemical properties and also differ in type from species to species (Tu et al., 2009; Pal et al., 2014).

4.1.6 Functions of melanin

Most of the functions of melanin are related to protection from the harsh external environmental conditions. Because of this ability of the polymer, it acts as "fungal armor". Protection from UV light and ionizing radiation, function as electron acceptor, antioxidant and a factor of virulence are the main functions of melanins particularly attributed to microbial organisms.

4.1.6.1 Melanin as a radioprotectant

An interesting property of melanin is that it can shield organisms from ionizing radiation. Melanin has a stable free radical population and the radioprotective properties are due to scavenging of free radicals generated by radiation. Melanized fungi isolated from radioactively contaminated soils at the Nevada Test Site and areas around the damaged Chernobyl nuclear

reactors suggest the ability of melanized fungi to survive in the presence of high levels of radiation and these organisms could be used for absorption and decontamination (Dighton et al., 2008). Investigational evidence suggests that fungal melanin can convert the energy of radiation to metabolically useful reducing power (Dadachova et al., 2007b).

An extracellular naturally occurring melanin pigment that was isolated from the fungus *Gliocephalotrichum simplex* was examined in BALB/C mice for the radioprotective effect. The pigment exhibited very effective radioprotective activity against whole body irradiation (WBI) in terms of inhibition of radiation-induced hematopoietic damages as evidenced by improvement in spleen parameters for instance index, endogenous colony forming units, total cellularity and maintenance of circulatory white blood cells and platelet counts (Kunwar et al., 2012).

Recently, melanins (LEM404-a) extracted from *Lachnum* YM404 exhibited strong anti-UV radiation activity for *E. coli, S. aureus* and *S. cerevisiae.* Anti-radiation experiments in mice showed that LEM404-a significantly improved superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in experimental groups while malondialdehyde (MDA) content decreased significantly. The remarkable resistance of LEM404-a to UV radiation indicate its strong anti-ultraviolet radiation activity (Ye et al., 2014). *Lysobacter oligotrophicus* isolated from Antarctica produces Lo-melanin and increased the melanin production by ultraviolet (UV) exposure. The survival rate of *E. coli* was increased by the addition of Lo-melanin to the medium than that of medium without melanin under UV-irradiated condition (Kimura et al., 2015). Melanins produced by *Bacillus thuringiensis* strains in the presence of tyrosine at 42°C have UV-protection properties, so the sunlight mediated degradation of *B. thuringiensis* preparation decreased and helped to increase the toxicity of these

biopesticides in the fields making it ecologically safer and efficient (Ruan et al., 2004).

4.1.6.2 Melanin as a factor of virulence

The ability to produce melanin in pathogenic fungi is an important factor of virulence. In *C. neoformans,* melanin was found to protect the parasite from ROS and RNS (reactive nitrogen species) produced during the oxygen burst by activated host macrophages (Wang et al., 1995). The DHN-melanin present in *Exophiala dermatitidis*, protect against strong oxidants (hypochlorite, permanganate) and free radicals produced in the host and actively neutralize the oxidants (Schnitzler et al., 1999). Melanized strains of *E. dermatitidis* are more resistant to the action of neutrophils than the amelanotic strains (Schnitzler et al., 1999). Similarly, melanotic strains of *Sporothrix schenckii* are more resistant to UV, NO and H_2O_2 than non-pigmented cells (Romero-Martinez et al., 2000). The oxidative burst capacity of macrophages was reduced by melanized *Fonsecaea pedrosoi* cells (Cunha et al., 2010). All these reports suggest that melanin produced by these fungal cells act as a potential mechanism of virulence against the oxidative stress and as a shield against immunological host response.

4.1.6.3 Melanin as antioxidants

Increase in oxidative stress formed by reactive oxygen species (ROS) shows an important role in the development of various diseases. ROS is a term which contains all highly reactive, oxygen-containing molecules, including free radicals. Types of ROS include the hydroxyl radical, hydrogen peroxide, superoxide anion radical, nitric oxide radical, singlet oxygen, hypochlorite radical and various lipid peroxides. These molecules can react with nucleic acids, membrane lipids, proteins and enzymes. Incorporation of antioxidant compounds in food and food products can reduce the disease risk due to the

ROS. Currently, pigments from natural sources have been proved to have antioxidant potential apart from its conventional usage as a colouring agent.

An antioxidant is a molecule stable enough to contribute an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage (Halliwell, 1995). These antioxidants can delay or inhibit cellular damage mainly through their free radical scavenging property and thus providing protection from harmful effects of infectious and degenerative diseases. Melanins interact readily with free radicals and reactive oxygen species because of the existence of unpaired electrons in their molecules and this property make them a strong candidate to scavenge reactive oxygen species and act as a natural antioxidant. Various synthetic antioxidants are in use in various food and cosmetic industries. Due to the unsafe effect of some synthetic antioxidants, search for effective, ecofriendly natural antioxidant compounds with minimal side effects has been increased in recent years.

Melanin extracted from *A. nidulans* (MEL1) exhibited potential antioxidant activity by scavenging the oxidants such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). It may be considered a promising material for the cosmetic industry for the formulation of creams that protect the skin against possible oxidative damage (Goncalves and Pombeiro, 2005). da Silva et al. (2009) reported that resistance to chemically generated Nitric oxide (NO), ROS, hypochlorite and H_2O_2 were more in melanized *Paracoccidioides brasiliensis* than non melanized yeast cells. DHN melanin from *A. bridgeri* showed potential free radical scavenging activity by DPPH method and may find potential application as a natural antioxidant (Kumar et al., 2011).

Published reports regarding the antioxidant and cytotoxic potential of marine yeast melanin is very rare. The antioxidant potential of the crude extract

of *Streptomyces* MS-60 isolated from the coastal region of the Bay of Bengal, India was evaluated by 1, 1-diphenyl-pircrylhydrazyl (DPPH) free radical scavenging assay and cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay on HT-29 (Human colon cancer cell) lines *in vitro*. The extract exhibited strong antioxidant activity and cytotoxicity (Zarina and Nanda, 2014).

Recently, marine actinobacterium *Streptomyces* sp. MVCS6 producing melanin were isolated from marine sediment of Versova coast (Mumbai, India). The antioxidant potential of melanin was evaluated by DPPH radical-scavenging, NO-scavenging and lipid peroxidation assays and displayed a noticeable percentage of inhibition with increasing concentration. The cytotoxic activity of melanin was determined using vero cell lines and cervical cancer cell lines (HeLa) by the MTT assay method. The cervical cancer cell line showed a dose dependent response and the cell proliferation capacity significantly decreased with increasing dose of melanin (Sivaperumal et al., 2014).

For the biological applications of any compound, evaluation of cytotoxicity effects is important. The *in vivo* animal models have always played an important role in the safety evaluation of such agents. Due to ethical considerations and limitations of animal models in relation to human metabolism, alternative methods such as *in vitro* testing using cell lines have been developed. This will help to evaluate the cytotoxic potential of compounds and may give an indication of *in vivo* effects. Various cytotoxicity assays are being used to evaluate the cytotoxic effect of the compounds which use different parameters associated with cell death and proliferation (Cook and Mitchell, 1989; Weyermann et al., 2005; Priyaja et al., 2016).

Currently, synthetic antioxidants were seen to be exceedingly popular for scavenging purposes but are often perceived as undesirable or harmful. In order to overcome this problem, search for effective natural antioxidants is in upsurge. Interest in melanin has been prompted because of their biological origin and activity profile. Besides a few studies regarding the biodiversity of black yeasts from the marine environment, a comprehensive report concerning the structural and functional characterization of melanins from *H. werneckii* has been partial. In this perspective the present work is focussed on the extraction and physicochemical characterization of melanin from a marine black yeast *H. werneckii.* The potential effect of melanin as an antioxidant was performed by *in vitro* free radical scavenging assays. The photoprotective effect of melanin was also analyzed. Biocompatibility of the extracted black yeast melanin of marine origin was carried out by *in vitro* cytotoxicity assays.

4.2 Materials and Methods

4.2.1 Microorganism used for melanin production

Black yeast, *Hortaea werneckii* R23 isolated from the Arabian Sea and maintained at 4°C in the Microbiology Laboratory of Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology (CUSAT), India was used for the present study.

4.2.2 Extraction of melanin

The black yeast, *H. werneckii* R23 was sub-cultured onto malt extract agar plates, incubated at 28±2°C for five days and harvested with sterile saline (30‰). The cell suspensions were centrifuged at 10,000 rpm for 30 min in a refrigerated centrifuge to get the yeast biomass. From the harvested yeast biomass, melanin was extracted in accordance with the protocol of Gadd (1982). Briefly, 1N NaOH was added to the harvested yeast biomass and

autoclaved for 20 min at 121°C. After that it was centrifuged at 8000 rpm for 10 min and the supernatant containing melanin was separated. The supernatant solution was then precipitated by adding concentrated HCl until the pH is reduced to 2. This solution was again centrifuged at 10,000 rpm for 10 min and the pellet was repeatedly washed with distilled water to get the purified melanin pigment. This was dried in a lyophilizer and stored at -20°C till use and designated as R23 melanin.

4.2.3 Characterization of black yeast melanin

4.2.3.1 Solubility

Melanin R23 (0.1g) was added to 10 ml of various solvents viz., water, aqueous acid, organic solvents (acetic acid, acetone, chloroform, ethanol, methanol, petroleum ether, hexane) and dilute alkali (sodium hydroxide, aqueous ammonia), stirred for 1 hour and filtered. Absorbance of the solutions was recorded at 400 nm using TU-1901 UV-VIS double beam Spectrophotometer (Hitachi, Japan) to measure the solubility of melanin.

4.2.3.2 UV-VIS spectra of black yeast melanin

Crude melanin (0.05mg) was dissolved in 1 ml alkaline distilled water (pH 9) prepared by adding 25% of 0.1 ml aqueous ammonia to 10 ml distilled water. The solution was scanned for absorption spectra using UV-VIS Spectrophotometer (Hitachi, Japan) between 230-600 nm wavelengths.

4.2.3.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

Proton one dimensional (1D) Nuclear magnetic resonance (NMR) spectra of R23 melanin and synthetic melanin (Sigma-Aldrich, USA) were carried out. Melanin samples were dissolved in 1M NaOD in D_2O by vortexing and then transferred to 5-mm NMR tubes. The 1M NaOD in D_2O was prepared by diluting

0.25 mL NaOD (40% NaOD in D_2O) with 3.75 mL D_2O. Proton 1D NMR spectra were collected on a Bruker Avance III 600 NMR Spectrometer using a CH cryoprobe operating at 298°K (25°C) and 345°K (72°C). Spectral data were collected and processed as follows: 256 scans, 2 pre-scans, 65,536 points, 20.6 ppm sweep width centred at 6.18 ppm, exponential apodization with 0.2 (298°K) and 1.0 (345°K) Hz broadening, and 1 sec pulse delay. Chemical shift reference is sodium trimethyl silylpropionate-2, 2, 3, 3-d_4 (TMSP-d_4) at 0.0 ppm. Spectra were processed using the JEOL DELTA software package (version 5.0.3) on a MacBook Pro operating system (version 10.9.4).

4.2.3.4 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is an important non-destructive technique, providing information on functional groups and structural characteristics of unknown compounds in small size samples. FTIR spectrum of R23 melanin was recorded at 4,000-400 cm^{-1} using a FTIR spectrum 100 (Perkin Elmer FTIR spectrophotometer, USA) at the Inter University Center for Marine Biotechnology, CUSAT. The spectra of R23 melanin was compared with that of sepia and synthetic melanin standards.

4.2.3.5 Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

Inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis is used to determine the elemental composition of R23 melanin by atomic emission spectrum. The wavelength at which emission occurs identifies the element, while the intensity of this emission is indicative of the concentration of the element within the sample. Synthetic melanin was used as standard. The analysis was carried out using an ICP-AES Thermo Electron

IRIS INTREPID II XSP DUO at Sophisticated Test and Instrumentation Centre (STIC), CUSAT, India.

4.2.3.6 Scanning Electron Microscope – Energy Dispersive Spectrometer analysis (SEM-EDS)

SEM-EDS analysis of R23 melanin was performed to detect the topography and qualitative elemental composition of the compound. The presence of different elements and surface topography emit different quantity of electrons, due to which the contrast in a SEM micrograph is obtained. SEM observations and elemental analysis were performed on gold-coated samples that had been previously air-dried on glass slides using an analytical SEM (JEOL JSM-6390LV) equipped with EDS (JEOL JED-2300) at STIC, CUSAT. Synthetic and sepia melanin standards were used for comparison.

4.2.3.7 Elemental analysis of R23 melanin (CHNS analysis)

Elemental analysis of R23 melanin was performed to determine the percentage of elements such as carbon, hydrogen, nitrogen and sulphur over a wide range of sample matrices and concentrations with Elementar Vario EL III (Elementar Analysis systems, Inc, Germany) at STIC, CUSAT. Synthetic and sepia melanin standards were used for comparison.

4.2.4 Assays for antioxidant activity

The antioxidant activity of extracted marine yeast melanin (R23 melanin) was assessed using the following assays:

- ABTS (2,2-azinobis-3-ethyl-benzothiozoline- 6-sulphonic acid) radical scavenging assay
- DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging assay
- Lipid peroxidation inhibition assay

4.2.4.1 ABTS radical scavenging assay

Free radical scavenging activity of R23 melanin was measured in accordance with Miller and Rice Evans (1997) with slight modifications. ABTS radical cation ($ABTS^{+}$) was produced by reacting 8 mM ABTS solution with 3 mM potassium persulphate and the mixture was allowed to stand for 12-16 h in dark at room temperature (RT) for the accomplishment of free radical generation. The ($ABTS^{+}$) solution was diluted with phosphate buffer (pH 7.4) in order to obtain an absorbance of 0.8±0.01 at 734 nm. 50 µl of different concentrations (5-100 µg ml^{-1}) of melanin solution were mixed with 200 µl of diluted ($ABTS^{+}$) solution and incubated at 30°C. The absorbance was read at 734 nm after 10 min using Microplate Reader (TECAN Infinite Tm, Austria). 1mM Trolox was used as the standard. The capability of R23 melanin to scavenge ABTS radical was calculated as the following equation:

$$I\% = \frac{(Ac - As)}{Ac} \times 100$$

Where,

***Ac** is the Absorbance of fully oxidized control*

***As** is the absorbance of ABTS treated with different concentrations of melanin*

4.2.4.2 DPPH radical scavenging assay

DPPH scavenging activity of R23 melanin was determined according to the method described by Chen et al. (2008) based on the capacity of the antioxidant to donate hydrogen or the radical scavenging ability. Melanin (50 µl) of different concentrations (10-100 µg ml^{-1}), was thoroughly mixed with 100 µl of freshly prepared DPPH solution (0.1mM in 95% ethanol) and 100 µl of 95% ethanol. The mixture was shaken vigorously and left to stand for 30 min in the dark. Absorbance was measured at 517 nm using Microplate Reader

(TECAN Infinite Tm, Austria). Trolox was used as standard. The ability to scavenge the DPPH radical was calculated using the following equation:

$$I\% = \left[1 - \frac{Ai - Aj}{Ac}\right] \times 100\%$$

Where,

Ac *is the absorbance of DPPH solution without sample (100 µl DPPH + 100 µl 95% ethanol+ 50 µl aqueous ammonia),*

Ai *is the absorbance of the test sample mixed with DPPH solution (50 µl sample + 100µl DPPH + 100 µl 95% ethanol) and*

Aj *is the absorbance of the sample without DPPH solution (50 µl sample + 200 µl 95% ethanol).*

Radical scavenging potential was expressed as the IC_{50} value, which represents the effective concentration of the test sample to achieve 50% inhibition.

4.2.4.3 Lipid peroxidation inhibition assay

A modified thiobarbituric acid reactive substances (TBARS) assay was done based on the method of Dasgupta and De (2004) to measure the lipid peroxide formed using egg yolk homogenate as lipid rich media. Briefly, 0.5 ml of 10% v/v egg homogenate and R23 melanin of different concentrations (75-750 µg ml^{-1}) were added to a test tube and made up to 1 ml with distilled water; 0.05 ml of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and the mixture was incubated at 37°C for 30 min. Then 0.5 ml of 20% trichloro acetic acid and 0.5 ml of 0.8% (w/v) thiobarbituric acid in 50% glacial acetic acid was added to quench the reaction. The resulting mixture was vortexed and heated at 95°C for 60 min. After cooling, 2.0 ml of butanol was added to each

tube and the mixture centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation (I %) by melanin was calculated by the following equation:

$$I\% = \left(1 - \frac{E}{C}\right) \times 100$$

Where,

***C** is the absorbance of the fully oxidized control and*

***E** is the absorbance in the presence of melanin.*

Butylated hydroxy toluene (BHT) was used as positive control.

4.2.5 Biocompatibility and cytotoxicity of R23 melanin

Biocompatibility and cytotoxicity study of melanin was performed in human HEp-2 (Human larynx epithelial cells). Biocompatibility and nontoxicity of yeast melanin (R23 M) was evaluated by assays viz., mitochondrial dehydrogenase activity by reduction of XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) and the assay for protein synthesis using sulforhodamine B dye (SRB).

4.2.5.1 2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay

For the above sequential assays, ~1×10^6 HEp-2 cells were inoculated into each well of a 96 well tissue culture plate containing MEM (minimal essential medium) supplemented with 10% fetal bovine serum (FBS) and incubated for 12 h at 37°C. After incubation, the cells were copiously washed with phosphate buffered saline (PBS), and the medium was exchanged with MEM containing different concentrations of R23 melanin. Following incubation for 24 h at 37°C, the wells were observed under Inverted phase

contrast microscope (Leica, Germany) and sequential cytotoxicity assays were performed following manufacturer's instruction (Cytotox-PAN I, Xenometrix, Germany). For XTT assay, melanin treated cells were treated with 50µl pre-warmed XTT at 37°C for 4 h, mixed the formazan formed in each well and the absorbance was measured as a function of reduction of XTT to soluble formazan by healthy cells at 480 nm in a microplate reader (TECAN Infinite Tm, Austria) with a reference wave length at 690 nm and the percentage inhibition of viable cells were calculated.

$$Percentage\ inhibition\ of\ viable\ cells = 100 - \left(\frac{Ac}{Ao} \times 100\right)$$

Where,

***Ac** is the average absorbance of cells at a particular concentration of melanin,*

***Ao** is the average absorbance of control cells without melanin*

4.2.5.2 Sulforhodamine B (SRB) assay

For SRB assay, XTT solution from each well was discarded and the cells remaining attached to the bottom of the wells were washed with 300 µl wash solution and added 250 µl fixing solution, incubated the plate for 1 h at 4ºC. The cells were washed and added 50 µl labeling solution, incubated for 15 min at room temperature. Washed 2 times with 400 µl rinsing solution and air dried the cells. Dissolved the air dried cells with 200 µl solubilization solution, incubated for 1 h at room temperature and the absorbance was read at 540 nm (reference filter 690 nm) as a measure of protein synthesis. The percentage inhibition of viable cells at each concentration of melanin was calculated (Vichai and Kirtikara, 2006).

$$Percentage\ inhibition\ of\ viable\ cells = 100 - \left(\frac{Ac}{Ao} \times 100\right)$$

Where,

***Ac** is the average absorbance of cells at a particular concentration of melanin,*

***Ao** is the average absorbance of control cells without melanin*

4.2.6 Phototoxicity assay of R23 melanin

R23 melanin with varying concentrations of 50, 100, 200 µg ml^{-1} were treated with HEp-2 cells prepared in 96 well microplate in MEM (minimal essential medium). The cells were exposed to 4.9W UV-C irradiation at a wavelength of 254 nm (TUV 15W/G15T8, Philips, Holland) for 10 min by keeping in an ice chest. Cells were washed copiously with phosphate buffered saline and reactive oxygen species (ROS) were analyzed by nitroblue tetrazolium (NBT) reduction assay (Song and Hsiech, 1994). Briefly, NBT solution (2 mg ml^{-1}) in 0.05 mol l^{-1} Tris HCl buffer (pH 7.6) was added to the melanin exposed cells and incubated for 1 h at 10°C. The samples were then centrifuged at 5000 rpm for 5 min, discarded the supernatant and quenched the reaction by adding 500 µl absolute methanol. Following incubation for 10 minutes, the insoluble formazan residue was fixed by rinsing thrice with 500 µl of 50% methanol. The formazan residue was solubilized with 120 µl of 2M KOH and 140 µl dimethyl sulphoxide (DMSO) and absorbance was read at 620 nm in a microplate reader (TECAN Infinite, Tm Austria). Control cells (HEp-2) were also maintained without melanin under UV exposure and without UV exposure. Synthetic melanin (50 µg ml^{-1}) was used as positive control.

4.2.7 Statistical analysis

The data of all the experiments were recorded as mean ±SD and were analyzed with SPSS (version 22.0 for Windows, SPSS Inc.). The IC_{50} was calculated by probit analysis using SPSS.

4.3 Results

4.3.1 Production and extraction of melanin from black yeast

Yeast inoculated on malt extract agar exhibited melanin producing black colonies after 74 hours of incubation (Fig. 4.1a and 1b). Melanin extracted from *H. werneckii* R23 was brownish black in colour (Fig. 4.1c). The yield of melanin was 0.0122 g dry weight/g wet weight of black yeasts.

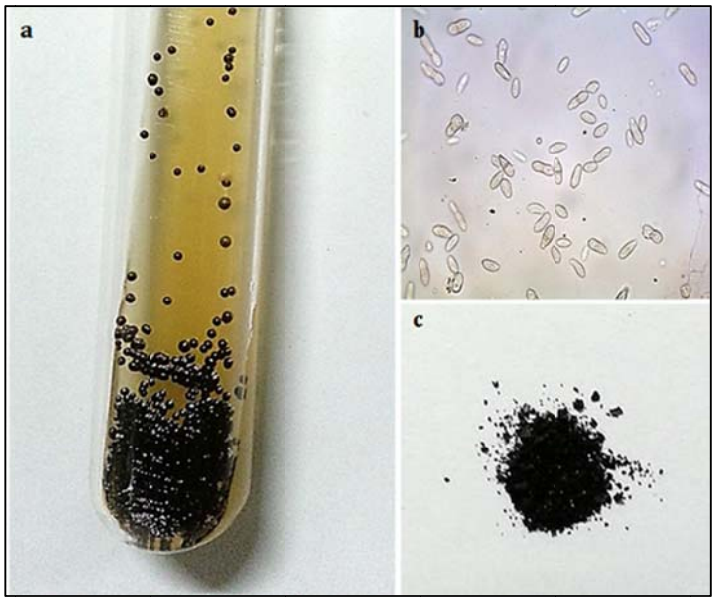


Fig. 4.1 (a) Colony morphology of black yeast *Hortaea werneckii* R23 on malt extract agar (b) Micromorphology of *Hortaea werneckii* R23 (c) Melanin extracted from black yeast *Hortaea werneckii* R23 (lyophilized)

4.3.2 Physicochemical characterization of black yeast melanin

4.3.2.1 Solubility of black yeast melanin

The extracted melanin was insoluble in water and most of the organic solvents tested, viz., acetic acid, acetone, chloroform, ethanol, methanol, petroleum ether and hexane. R23 melanin was soluble only in sodium hydroxide and aqueous ammonia (25%). Moreover, it exhibited precipitation in acidic aqueous solution (0.1 M HCl) below pH 3. This solubility result also indicates the characteristic of typical melanin.

4.3.2.2 UV-VIS spectra of R23 melanin

R23 melanin exhibited an absorption maximum at a wavelength 230 nm in the UV region and an additional absorption peak at 275nm. The absorption spectrum of R23 melanin revealed that absorption peak was at UV region and declined towards the visible region which is a characteristic property of typical melanin (Fig. 4.2).

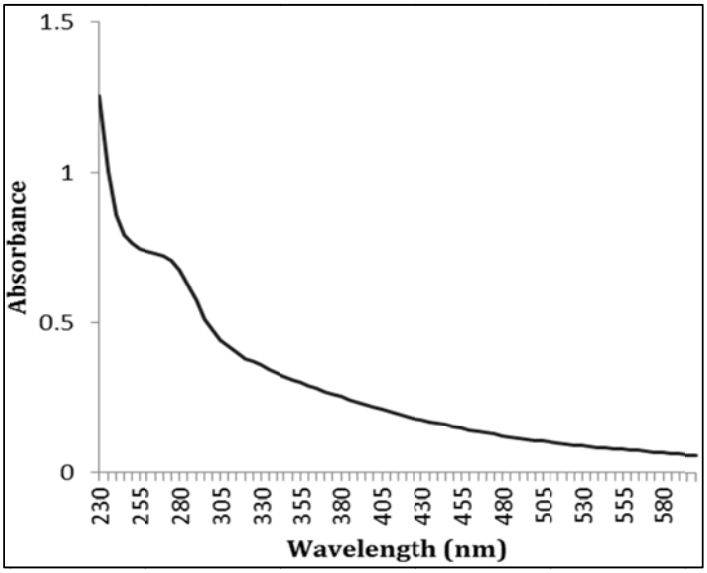


Fig. 4.2 UV–VIS spectra of R23 melanin

4.3.2.3 Fourier Transform Infrared Spectroscopy (FTIR)

Peak assignments of R23 melanin is OH or NH stretching at 3283.34 cm^{-1}, CH_2 asymmetrical stretching (2919.08 cm^{-1}), CH_2 symmetrical stretching (2850.83 cm^{-1}), C = C stretching or C=O stretching (1631.3 cm^{-1}), NH bending (1532.48 cm^{-1}), CH_2CH_3 bending (1456.46 cm^{-1}), CN stretching (1411.12 cm^{-1}), phenolic COH stretching (1228.12 cm^{-1}) and CO stretching (1043.23 cm^{-1}). The peak at 1707.9 cm^{-1}, suggest the free carboxylic group. Additionally, the two peaks at 1532.48 and 1411.12 cm^{-1} strongly imply a pyrrole or indole NH group. These FTIR features have a close similarity to the typical structure of melanin. The most noticeable differences between R23 melanin with sepia melanin and synthetic melanin are the peaks at 2919.08, 2850.83 and 1456.46 cm^{-1}, which also implies the presence of a considerable amount of aliphatic groups in the R23 melanin structure (Fig. 4.3)

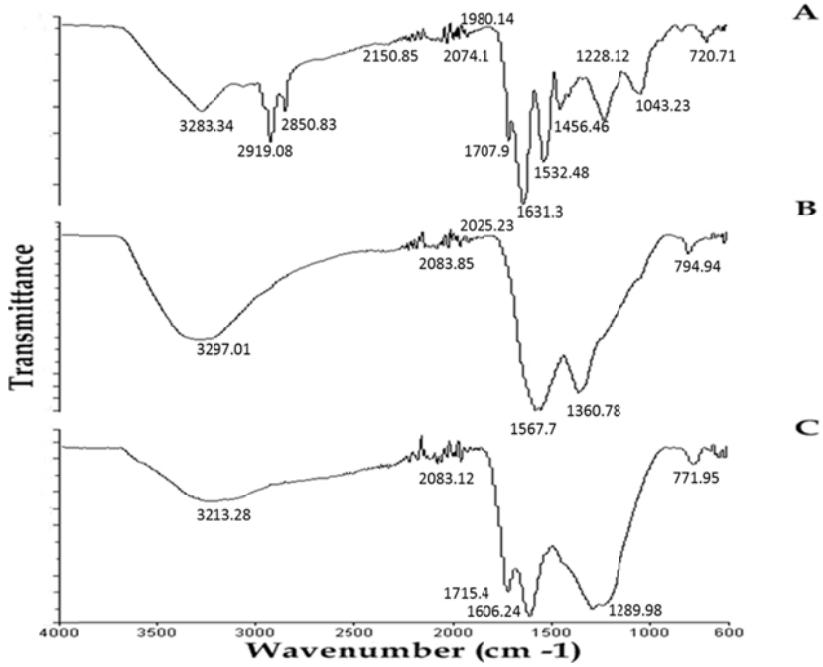


Fig. 4.3 FTIR spectra analysis of (A) R23 melanin, (B) synthetic melanin and (C) sepia melanin

4.3.2.4 Nuclear Magnetic Resonance (NMR) spectra of melanin

The proton NMR spectra of R23 melanin and standard synthetic melanin are shown (Fig. 4.4 and Fig. 4.5). The resonance in the aliphatic region of R23 melanin is almost similar to the standard synthetic melanin and some more additional peaks could be detected in the 3.0 to 4.0 ppm. Similarly the resonance in the aromatic region also exhibit close similarity compared to synthetic melanin. These resonances are similar to resonances in the aromatic region with indole or pyrrole structural units previously reported for melanin isolated from human hair.

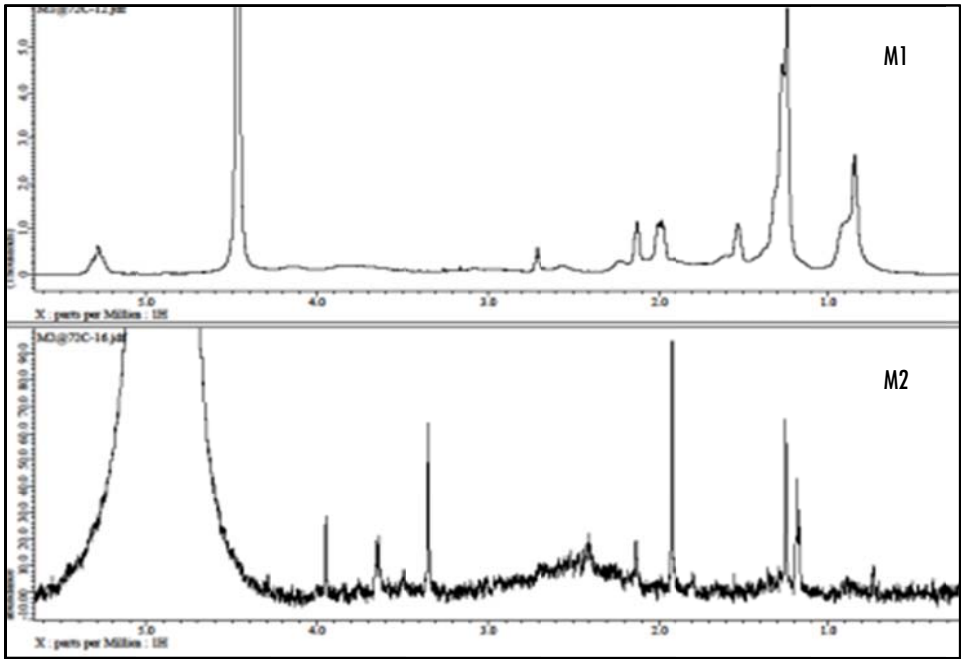


Fig. 4.4 Comparison of the aliphatic proton of Proton NMR spectra spectral regions taken at 600 MHz of synthetic melanin (MI) and *H. werneckii* Melanin (M2) plotted between about 1.0 and 5.0 ppm.

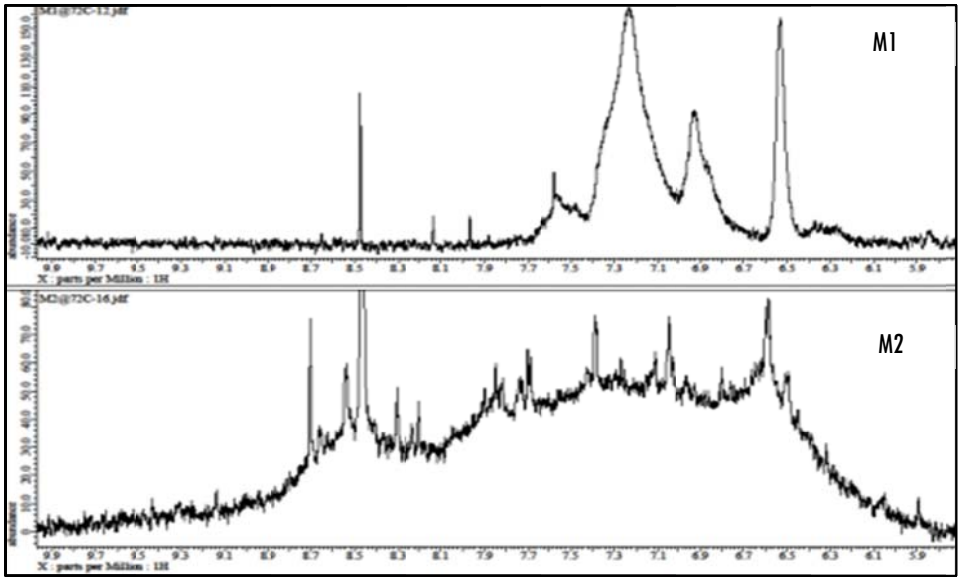


Fig. 4.5 Comparison of the aromatic proton of Proton NMR spectra spectral regions taken at 600 MHz of synthetic melanin (MI) and *H. werneckii* Melanin (M2) plotted between about 5.9 and 9.9 ppm.

4.3.2.5 Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

Different metal ions were detected in R23 melanin by ICP-AES. It includes Na (I), Ca (II), Mg (II), K (I) and Fe (III). Table 4.1 lists the amount of metal ions present in R23 melanin and synthetic melanin. When compared to synthetic melanin, an elevated percentage of sodium, calcium and magnesium ions were detected in R23 melanin. Melanin is linked with many metal ions that are subject to various functional groups. As stated in the proposed molecular structures of melanin, the pigment contains phenolic, hydroxyl (OH), carboxyl (COOH) and amine (NH) groups as potential functional binding groups for metal ions.

Table 4.1 ICP-AES analysis of melanin samples

Sl. No.	Sample Name	Ca	Fe	K	Mg	Na	Unit
1	Yeast Melanin	5.573	0.334	0.527	1.860	6.539	%
2	Synthetic Melanin	0.226	0.612	0.024	0.032	0.035	%
	Detection Limit	0.01	0.01	0.01	0.01	0.01	ppm

4.3.2.6 Elemental analysis of melanin (CHNS analysis)

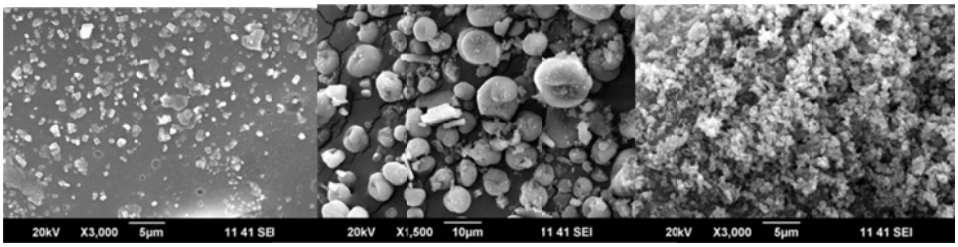
The elemental analysis detected the amounts of carbon, nitrogen, hydrogen and sulphur in the melanin samples (Table 4.2). The percentage elemental composition of R23 melanin showed higher percentages of hydrogen, nitrogen and sulphur compared to sepia melanin and synthetic melanin. However, the carbon content was almost similar to synthetic melanin but higher to sepia melanin.

Table 4.2 CHNS analysis of melanin samples

Sample No.	Sample Name	C%	H%	N%	S%
1	Yeast melanin	49.71	9.52	7.83	1.03
2	Synthetic Melanin	49.77	3.39	6.18	-
3	Sepia Melanin	32.61	3.11	5.87	0.33

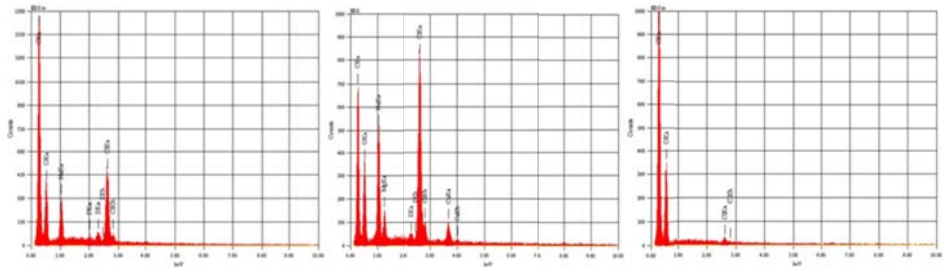
4.3.2.7 SEM-EDS analysis of melanin

The morphology and elemental composition pattern of the melanin samples were resolved by Scanning electron microscope-Energy dispersive spectrometer (SEM-EDS) analysis. The appearance of R23 melanin is in a crystalline form while synthetic melanin appears to be amorphous and sepia melanin in defined spherical forms (Fig. 4.6). The surface elemental composition pattern of R23 melanin revealed that it contains 6 elements, in which the major amount was carbon followed by moderate amounts of oxygen and chlorine; sodium, sulphur and phosphorous were also detected. Whereas, sepia melanin consisted of 7 elements, in which carbon is abundant followed by chlorine, oxygen and sodium in moderate amounts, very small amount of magnesium and calcium and trace of sulphur were observed. But, synthetic melanin contains only 3 elements including large quantity of carbon, moderate amounts of oxygen and a trace of chlorine (Fig. 4.7) (Table 4.3).



a) R23 Melanin b) Sepia Melanin c) Synthetic Melanin

Fig. 4.6 Scanning electron microscopy images of melanin samples



a) R23 Melanin b) Sepia Melanin c) Synthetic Melanin

Fig. 4.7 Energy dispersive spectra of melanin samples

Table 4.3 Elemental composition of melanin samples by Energy-dispersive X-ray spectroscopy (EDS)

Element	(KeV)	R23 Melanin		Sepia Melanin		Synthetic Melanin	
		Mass %	Atom %	Mass %	Atom %	Mass %	Atom %
C	0.277	75.19	85.85	46.92	66.06	89.35	92.1
O	0.525	7.71	6.61	9.56	10.11	9.85	7.62
Na	1.041	4.01	2.39	10.41	7.66	-	-
P	2.013	0.74	0.33	-	-	-	-
S	2.307	1.21	0.52	0.48	0.25	-	-
Cl	2.621	11.14	4.31	27.36	13.05	0.8	0.28
Mg	1.253	-	-	2.38	1.66	-	-
Ca	3.690	-	-	2.90	1.22	-	-

4.3.3 Antioxidant activity

4.3.3.1 ABTS radical scavenging assay

The scavenging property of R23 melanin on ABTS radicals at various concentrations was observed. The scavenging ability of R23 melanin was found to be increased in dose dependent manner (Fig. 4.8). At a concentration of 5 µg ml^{-1} the inhibition was 33.3 ± 3.62% and increased in a concentration dependent fashion and attained an inhibition of 98.59 ± 0.69% at 50 µg ml^{-1} (Table 4.4). The concentration of an antioxidant needed to decrease the initial ABTS concentration by 50% (IC_{50}) is generally used to calculate antioxidant activity. Thus the inhibitory concentration (IC_{50}) of R23 melanin on the ABTS radicals was determined as 9.76 ± 4.10 µg ml^{-1}. The IC_{50} of standard trolox on ABTS scavenging was found to be 1.042± 0.06 µg ml^{-1}.

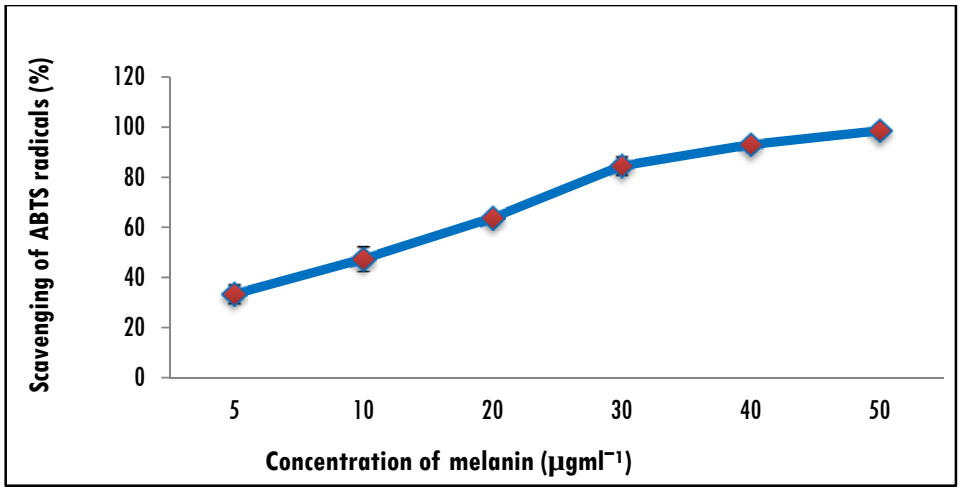


Fig. 4.8 ABTS radical scavenging activity of R23 melanin pigment

Table 4.4 ABTS Scavenging assay of R23 melanin and Trolox

Concentration of R23 (µg ml^{-1})	Inhibition % (I%)	Concentration of Trolox (µg ml^{-1})	Inhibition % (I%)
5	33.33± 3.62	0.6	18.9±1.47
10	47.37± 4.76	0.8	30.19±0.81
20	63.79± 1.19	1	44.27±4.13
30	84.53± 3.60	1.2	57.27± 0.69
40	93.02± 2.58	1.4	69.47±1.33
50	98.59± 0.69	1.6	81.9±4.29
IC_{50}	**9.76 ± 4.10 µg ml^{-1}**		**1.042± 0.06 µg ml^{-1}**

4.3.3.2 DPPH radical scavenging assay

The scavenging capability of R23 melanin on DPPH radical is shown in Fig. 4.9. There is an increase in the scavenging effect of melanin on DPPH radical which is evident when the concentration of melanin increased. At a concentration of 10 µg ml^{-1} the scavenging effect was 29.32±1.97%. When the concentration reached a maximum of 100 µg ml^{-1} the scavenging was 87.8±1.84%. The inhibitory concentration (IC_{50}) of R23 melanin on the DPPH radicals was calculated as 28.11±10.76 µg ml^{-1}. The IC_{50} of standard trolox on DPPH scavenging was determined as 1.334 ± 0.20 µg ml^{-1} (Table 4.5).

Table 4.5 DPPH Scavenging assay of R23 melanin and Trolox

Concentration of R23 (µg ml^{-1})	Inhibition % (I%)	Concentration of Trolox (µg ml^{-1})	Inhibition % (I%)
10	29.32±1.97	**0.5**	23.34±0.01
20	38.87±2.91	1	39.4±1.01
40	52.17±5.56	2	59.45±0.02
60	63.96±0.85	3	71.03±0.04
80	77.65±1.55	4	82.53±0.08
100	87.8±1.84	5	89.95±0.03
IC_{50}	**28.11±10.76 µg ml^{-1}**		**1.334 ± 0.20 µg ml^{-1}**

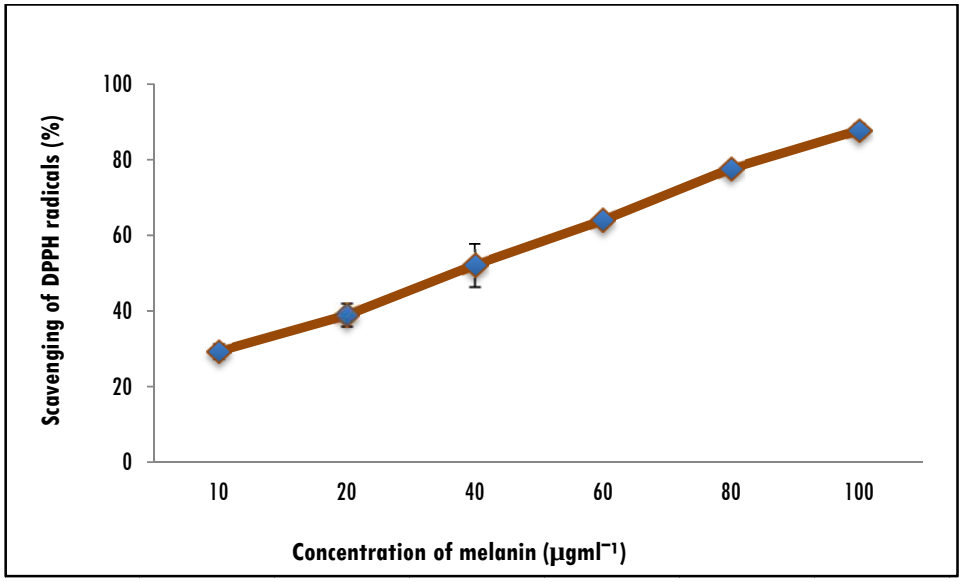


Fig. 4.9 DPPH radical scavenging activity of R23 melanin pigment

4.3.3.3 Lipid peroxidation inhibition assay

Non enzymatic peroxidation of egg yolk lipids takes place when egg yolk incubated with ferrous sulphate. The reaction will result in the subsequent formation of malondialdehyde (MDA) and other aldehydes and that form a pink colour with thiobarbituric acid. The inhibition effect of R23 melanin on non-enzymatic peroxidation was detected. On lipid peroxidation, the inhibition effect of R23 melanin increased with an increase in concentration (Fig. 4.10). At 75 µg ml^{-1}, the inhibition was 29.87 ± 4.68%. While the concentration of melanin increased from 150 - 750 µg ml^{-1} the inhibition effect also increased in a dose dependent manner from 45.56 ± 8.22 to 81.5 ± 2.53%. The IC_{50} of R23 melanin was calculated as 222.56 ± 43.49 µg ml^{-1} (Table 4.6). The positive control, butylated hydroxy toluene (BHT) exhibited an IC_{50} of 8.63± 4.38 µg ml^{-1} on lipid peroxidation.

Table 4.6 Inhibition percentage of R23 melanin and BHT on lipid peroxidation

Concentration of R23 (µg ml^{-1})	Inhibition % (I%)	Concentration of BHT (µg ml^{-1})	Inhibition % (I%)
75	29.87±4.68	5	36.1±1.01
150	45.56±8.22	10	51.4±2.36
300	54.15±5.00	20	67.28±2.99
450	62.85±12.98	30	76.77±1.24
600	70.43±0.73	40	81.94±1.35
750	81.5±2.53	50	88.63±2.03
IC_{50}	222.56 ± 43.49 µg ml^{-1}		8.63± 4.38 µg ml^{-1}

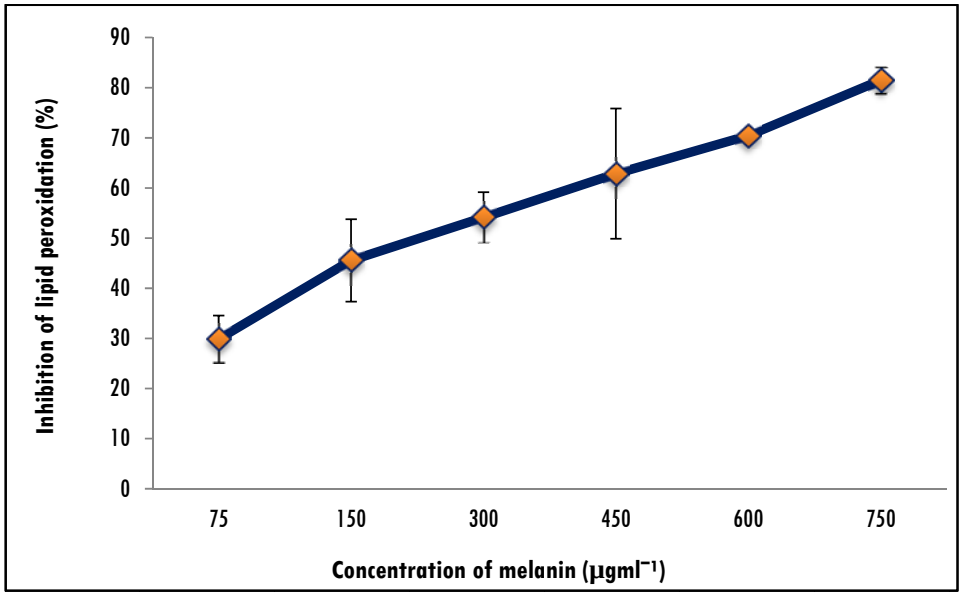


Fig. 4.10 Lipid peroxidation inhibition of R23 melanin pigment

4.3.4 Biocompatibility and cytotoxicity of R23 melanin

The viability of human HEp-2 cells on treatment with different concentration of marine yeast melanin was measured as a function of mitochondrial dehydrogenase activity and protein synthesis in healthy cells. In

sequential cytotoxicity assays by exposure of cell lines to different concentrations of R23 melanin is shown in Fig. 4.11. Melanin was nontoxic to HEp-2 cells in culture up to a concentration of 200 µg ml^{-1}, where only <8% inhibition in terms of mitochondrial dehydrogenase activity (XTT assay) and protein synthesis (SRB) were observed. The IC_{50} of melanin in terms of mitochondrial dehydrogenase activity was found to be 1.42±0.56 mg ml^{-1} and 1.25±1.32 mg ml^{-1} in terms of protein synthesis. Above 80% inhibition was observed at a concentration of 3.2 mg ml^{-1}. Both XTT and SRB showed <25% reduction in cell viability at 800 µg ml^{-1}, a concentration far higher than the dosage required for bioactivity.

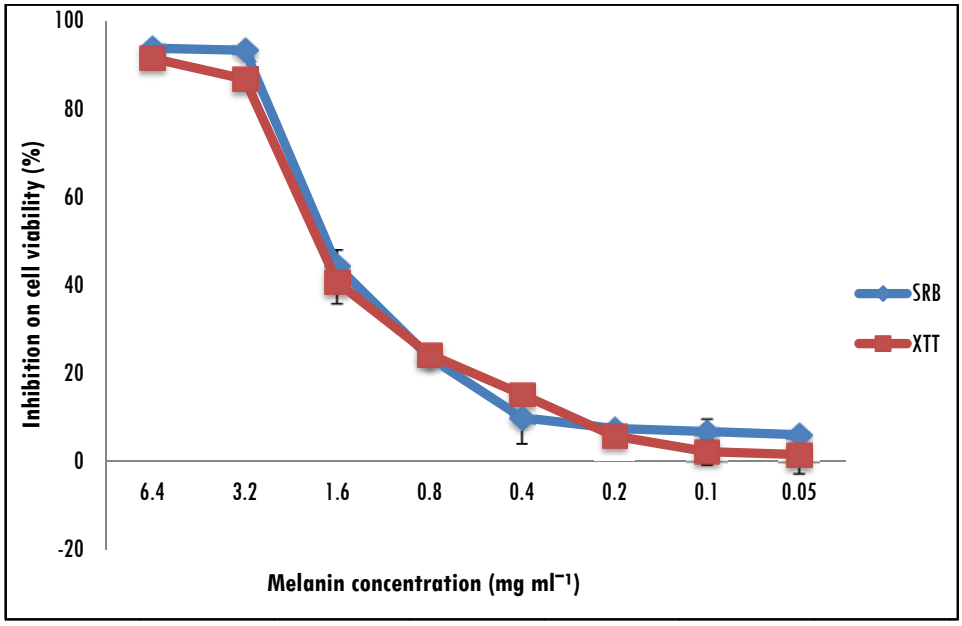


Fig. 4.11 Biocompatibility and cytotoxicity assay of melanin. 2,3-Bis-[2-Methoxy-4-Nitro-5-Sulfophenyl]-2H-Tetrazolium-5-Carboxanilide (XTT) and Sulforhodamine B (SRB) assay. The Y-axis shows the inhibition percentage of viable cells (n=3)

4.3.5 Phototoxicity assay of R23 melanin

Exposure of HEp-2 cell lines to ultraviolet radiation induced reactive oxygen species (ROS) and that was indicated by the increase in absorbance

(Fig. 4.12). The reactive oxygen species generated in UV exposed control cells was (35%) higher than the UV unexposed control cells. Generation of ROS was lower in melanin treated UV unexposed cells and the scavenging percentage was increased when the concentration of melanin increased. It is evident that melanin could act as a protection barrier and thus reduce the deleterious effect of ROS under normal condition of cells.

R23 melanin was used as the test and synthetic melanin as positive control. In melanin treated HEp-2 cells, melanin absorbed the UV rays and thus protected the cells from the UV-induced damage. As a result of UV exposure, the yeast melanin treated HEp-2 cells scavenged the reactive oxygen species in a dose dependent manner. R23 melanin scavenged 51.05% of the ROS generated at 50 μg ml^{-1} while the synthetic melanin (positive control) scavenged 37.93% of the ROS at the same concentration. At 100 μg ml^{-1} scavenging effect of R23 melanin increased to 60.12%. Photoprotection ability of R23 melanin is obvious from the reduction of ROS generation at a concentration of 200 μg ml^{-1} of melanin, where it scavenged 63.86% of ROS generated (Table 4.7). R23 melanin exhibited higher photoprotection than the positive control synthetic melanin.

Table 4.7 Phototoxicity assay of R23 melanin

Melanin (μg ml^{-1})	Scavenging % ROS (UV exposed)	Scavenging % ROS (UV unexposed)
Synthetic melanin-50	37.93	26.64
R23-50	51.05	36.33
R23-100	60.12	53.22
R23-200	63.86	56.72

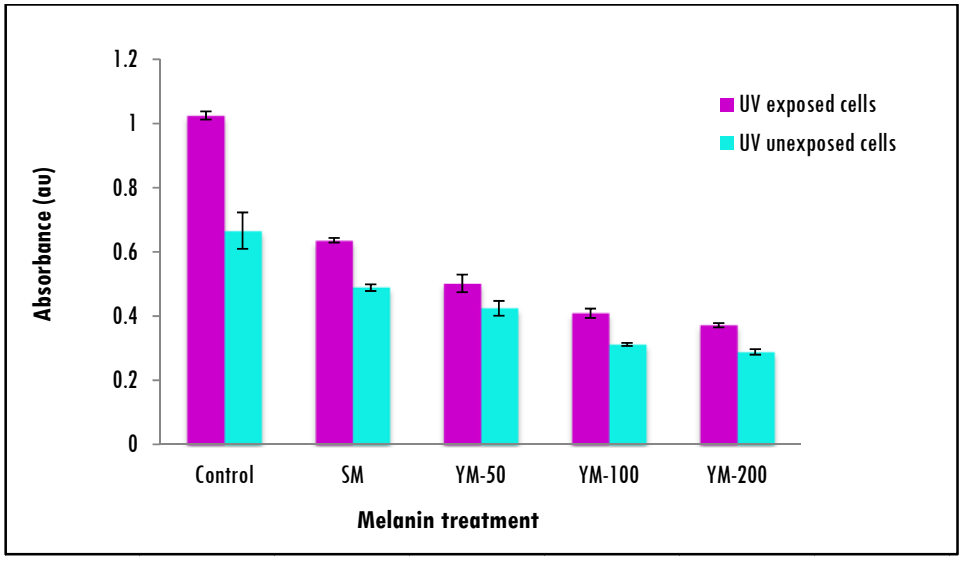


Fig. 4.12 Effect of melanin on scavenging ROS generated by UV. The absorbance (630 nm) indicated the mean concentration of ROS in HEp-2 cells in the presence of melanin

Control: HEp-2 cells (without melanin) UV exposed and unexposed. SM: Synthetic melanin (Positive control); YM-50: yeast melanin at 50 µg ml^{-1}; YM-100: yeast melanin at 100 µg ml^{-1}; YM-200: yeast melanin at 200 µg ml^{-1}

4.4 Discussion

Hues of pigments from marine sources have been documented in the scientific literature and many of the microorganisms which produce pigments have competitive advantage over various environmental conditions. The presence of dark brown pigment melanin provides the dark-coloured fungi a wide protective action and a competitive advantage under harsh environmental conditions (Butler et al., 2005; Yurlova et al., 2008). So far, there has been various reports regarding the isolation and identification of marine yeasts and not much information is available on characterization and biological activity of marine yeast melanin.

In the present study, marine black yeast *H. werneckii* R23 produced 0.0122 g dry weight melanin/g wet weight of yeast biomass on malt extract agar medium. Tu et al. (2009) reported the yield of melanin from Taihe Black-bone silky fowl i.e., 0.12 g/100 g on a wet weight basis. The extracted ratio of melanin from black tea (BT), black soybean (BS) and black-bone silky fowl (SF) were about 2%, 0.16% and 0.095%, respectively (Hsieh and Lien, 2012) which was comparable to the extracted ratio of R23 melanin (1.22%). The appearance of R23 melanin was found to be brownish black in colour and that was similar to synthetic melanin. Melanin pigments from a number of microbial sources exhibited a range of brown to black colour (Manivasagan et al., 2013; Yu et al., 2015; Kimura et al., 2015).

Physicochemical characterization of the extracted black yeast R23 melanin pigment exhibited characteristics which was comparable to the standard melanin. Melanin R23 was insoluble in most of the solvents tested except sodium hydroxide and aqueous ammonia and displayed solubilization property similar to that reported by Tu et al. (2009) and Kumar et al. (2013b).

The UV-Visible spectrum scanned from 230 to 600 nm for the extracted melanin pigment. The absorption spectrum showed characteristic absorption peaks in the UV region with an absorption maximum at 230 nm and absorbance was decreased as the wavelength increased towards the visible region. This phenomenon is typical to melanin and was due to the considerably complex conjugated structure of melanin (Sajjan et al., 2010; Tarangini and Mishra, 2014). Moreover, an additional plateau between 260-280 nm was also noticed in the absorption spectrum. The plateau in the UV range of 260-280 nm may be the result of aromatic amino acid presence in melanin as reported by Sun et al. (2016). Kumar et al. (2011) detected melanin pigment from a new strain of *Aspergillus bridgeri* which showed an absorption peak at 217 nm with a small

shoulder at 260-280 nm, in agreement with the present findings. These optical properties of melanin play a significant role in skin photoprotection, particularly as an optical screen that reduces the penetration of ultraviolet light and offers its multidimensional biological effectiveness in protection from light, oxidative stress and energy transduction.

The FTIR spectral analysis is an important tool for the structural elucidation of compounds. In the present study signal peaks were detected at 3283.34, 2919.08 and 2850.83 cm^{-1}. According to Magarelli et al. (2010) signals in the 3600-2800 cm^{-1} area are attributed to the stretching vibrations (O-H and N-H) of the carboxylic acid, phenolic acids and the aromatic amino functions present in the indolic and pyrrolic systems. Moreover, a C = C stretching or C=O stretching was detected at 1631.3 cm^{-1} in the R23 melanin. Similarly, a peak at ~1625 cm^{-1}, representative of aromatic C=C and/or carboxylate groups as well as nitrogen containing heterocycles from synthetic and natural melanin were noticed by Costa et al. (2012). Melanin extracted from *Auricularia auricula* exhibited characteristic absorption in C-H at 2925 cm^{-1} and the characteristic band at 1633.71 cm^{-1}(C=C, $–COO^{–}$, C=O), 1075.44 cm^{-1} (C–O) phenols or carboxylic groups, which were characteristic absorption peaks for fungal melanin and similar to that absorption spectrum of R23 melanin is also correlated (Sun et al., 2016). The two peaks at 1532.48 and 1411.12 cm^{-1} in the extracted R23 melanin are comparable to two similar peaks present in TBSF melanin (at 1539 and 1398 cm^{-1}), suggesting the presence of a pyrrole or indole NH group (Tu et al., 2009). Furthermore, the peak suggestive of the free carboxylic group found at 1707.9 cm^{-1} in R23 melanin and synthetic melanin, was not detected in sepia melanin. The most noticeable differences between R23 melanin with sepia and synthetic melanin were the peaks at 2919.08, 2850.83 and 1456.46 cm^{-1}, which implies the presence of a considerable amount of

aliphatic groups in R23 melanin. However, compared to the FTIR features of the standard melanin and melanin produced from various sources, the spectroscopic properties of the R23 melanin extracted from marine black yeast *H. werneckii* R23 correspond to the typical structure of melanin.

NMR spectra of R23 melanin and synthetic melanin exhibited similarity in particular regions. The NMR spectrum shows signals in both the aromatic and aliphatic regions. Peaks in the absorption region from 3.2 to 4.5 ppm can be assigned to protons on carbons attached to nitrogen and/or oxygen atoms and appeared comparable to the observed data of Katritzky et al. (2002) and Sun et al. (2016). Resonance between 0.5 ppm to 2.5 ppm are assigned to CH_3, CH_2 groups of alkyl fragments and those aliphatic carbons are due to proteinaceous material, based upon similar assignments as reported previously (Guo et al., 2014; Sun et al., 2016). In the aromatic regions of NMR spectra of R23 melanin, peaks between 6.5 and 8.5 ppm are assigned to the protons attached to indole and/or other differently substituted aromatic or heteroaromatic rings based upon similar peak assignments reported as previously (Nikodinovic-Runic et al., 2009; Jalmi et al., 2012). The well-defined peaks noticed in the aromatic region of R23 melanin were also noticed in the synthetic melanin as broad peaks. Moreover, aromatic region of synthetic melanin was very similar to human hair melanin spectrum reported by Katritzky et al. (2002).

The richness of metal ions such as Na, Ca, Mg, K and Fe in the R23 melanin sample shows the ability of melanin to bind various metal ions. Similarly, melanin pigment from *Pseudomonas stutzeri* was also rich in Na(I), Ca(II), Al(III), Mg(II), Fe(III), K(I) and Zn(II) ions (Kumar et al. 2013b). Meredith and Sarna (2006) reported the ability of both eumelanin and pheomelanin to bind metal ions, the most distinctive characteristic of this class of pigments. Synthetic melanin nanoparticles with an excellent binding capacity

of heavy metal ions such as lead, copper and cadmium have been reported (Kim et al., 2012).

Elemental analysis showed that the melanin from *H. werneckii* R23 mainly contained C, H, N and S elements. Higher C:N ratio indicated that the R23 melanin contains aliphatic groups which are almost closer to sepia melanin than synthetic melanin. The C:H ratio of R23 melanin is also nearer to sepia melanin. Sava et al. (2001) reported that higher carbon content and a higher C:H value indicates a higher level of aromaticity. The C:N ratio and C:H ratios are closely related to sepia melanin than synthetic melanin in case of R23 melanin. The S content of R23 melanin (1.03%) which is comparable to the S content of melanin from *A. auricular* (0.94%) along with C, H and N, suggested the presence of pheomelanin along with eumelanin as a preliminary conclusion based on elemental composition (Sun et al., 2016).

The morphological characterization and chemical composition of R23 melanin together with sepia and synthetic melanin have been analyzed by SEM-EDS and revealed that R23 melanin has a definite crystal shape with a defined structural order. Sepia melanin also shares the same property with a spherical shape and sepia melanin has been proposed as a standard for natural melanin (Centeno and Shami, 2008). Previous reports indicated that definite structural order is a property of natural melanin which is concordance with the present study (Simon et al., 2000; Costa et al., 2012; Mbonyiryivuze et al., 2015). Synthetic melanin exhibits an amorphous nature and lack structural integrity. Previous studies by Costa et al. (2012) and Tarangini and Mishra (2013) reported that synthetic samples appear to be amorphous solids while the natural samples appear to be small spheres with subunits have a diameter of 100-200 nm (Mbonyiryivuze et al., 2015).

Energy dispersive spectrometer analysis has been done to detect the chemical composition of melanin pigments and compositional variation has been recorded among samples. The elements detected in R23 melanin is more comparable with sepia melanin standard than synthetic melanin used in the present study and also in concordance with sepia melanin studied by Mbonyiryivuze et al. (2015). Compositional variation might be due to the change in production and growth medium composition as reported by Tarangini and Mishra (2013).

The dark coloured pigment melanin readily interacts with free radicals and other reactive oxygen species due to unpaired electrons present in the molecules. The antioxidant efficiency of melanin has been described by previous researchers and this property is mainly attributed to the chemical structure of melanin especially the phenolic group present in the molecule (Cai et al., 2004; Yao and Qi, 2016). Phenolic compounds are large group of natural antioxidants that can undergo reversible oxidation and reduction and exhibit the properties of donors or acceptors of electrons and protons (Shcherba et al., 2000). As a result of the formation of different reactive oxygen species (ROS) and their various mechanisms of action in living organisms, there is no universal method available to quantify the antioxidant activity accurately and quantitatively so that multiple assays are employed to evaluate the antioxidant activity. Evaluation of antioxidant activity of a compound is necessary and a useful tool in the initial selection of a compound that can be used in various pharmaceutical/cosmetic industries.

Earlier researchers have revealed the antioxidant potential of melanin from different sources such as *Lachnum* YM-346 (Ye et al., 2012) and *Pseudomonas* sp. (Tarangini and Mishra, 2013). The established methods such as ABTS and DPPH for radical scavenging activities are based on the ability of

antioxidants to donate a hydrogen atom or an electron to stabilize radicals by converting them to the non-radical species (Binsan et al., 2008). The scavenging effect of R23 melanin and standard trolox on the ABTS radical was found to be increasing in concentration dependent manner. ABTS radical scavenging property exhibited by the melanin pigment was significantly high; the percentage inhibition being 98.59 and 47.37% for melanin at 50 µg and 10 µg ml^{-1}, respectively. Similar observation was reported for melanin pigment from *Klebsiella* sp. GSK with an inhibition of 98 and 48.5 at 50 and 25 µg ml^{-1}, respectively (Sajjan et al., 2013). From this data it is inferred that even at a very low concentration (10 µg ml^{-1}), R23 melanin exhibited around 50% inhibition. In the present study, IC_{50} of R23 melanin (9.76 ± 4.104 µg ml^{-1}) on the ABTS radicals was found to be almost 9 times higher than the IC_{50} of standard trolox (1.042 ± 0.063 µg ml^{-1}). Zhong et al. (2011) reported that IC_{50} of ethyl acetate extract of *Streptomyces* Eri12 was 172.43 ± 22.19 µg ml^{-1} for ABTS which was about 220 times higher than that of the positive control (Trolox 0.76 µg ml^{-1}). When we compare this data, R23 melanin extracted from marine yeast *Hortaea werneckii* exhibited significant antioxidant potential in terms of radical scavenging activity and might be potentially used as a natural antioxidant. The activity of crude R23 melanin could be increased more by further purification of the compound.

The ability to scavenge DPPH radical is extensively used as an easy, rapid and sensitive way to assess free radical scavenging ability of natural antioxidants. This approach is based on the principle that DPPH radical is scavenged by an antioxidant through a donation of hydrogen to form a stable DPPH molecule. In the present study, DPPH radical scavenging effect of R23 melanin increased in a concentration dependent manner. At a concentration of 10 µg ml^{-1} the scavenging effect was 29.32 ± 1.97%. When the concentration

reached a maximum of 100 μg ml^{-1}, the scavenging was 87.8 ± 1.84%. IC_{50} of R23 melanin on the DPPH radicals was 28.11 ± 10.76 μg ml^{-1} and was 21 times higher than that of standard trolox (1.334 ± 0.196 μg ml^{-1}). Recently, melanin extracted from *Auricularia auricula* fruiting bodies (AAFB) exhibited antioxidant activity on DPPH radical with an IC_{50} value of 0.18 ± 0.03 mg ml^{-1} (Zou et al., 2015). The DPPH scavenging effect of melanin from *Klebsiella* sp. GSK revealed a higher scavenging activity at 50 μg ml^{-1} (74%) than at 25μg ml^{-1} (55%) (Sajjan et al., 2013). TBSF melanin from Taihe Black-bone silky fowl exhibited an IC_{50} value of 37.3 ± 2.62 μg ml^{-1} on the DPPH radical (Tu et al., 2009). In another report the scavenging rate of melanin from *Pseudomonas stutzeri* isolated from red seaweed *Hypnea musciformis* on DPPH radical reached 48.6% at 40 μg ml^{-1} and the EC_{50} value determined was 48.9 μg ml^{-1} (Kumar et al., 2013b). Different fractions of melanin extracted from chestnut shell exhibited antioxidant activity with an EC_{50} value of 66.5 ± 1.0 mg L^{-1} by fraction 3 with strongest scavenging efficiency (Yao and Qi, 2016).

All the reports mentioned here, shown that melanin had significant ability to scavenge free radicals and our experiment also supported the same. A dose dependent increase in antioxidant activity was shown by melanins extracted from different sources which are also concordance with R23 melanin. Melanin from marine yeast *Hortaea werneckii* revealed strong scavenging effect on both ABTS and DPPH radicals and which was more efficient on ABTS radical than DPPH radical. The capacity of melanin to react with and quench different radicals may vary relate to various factors such as the stereoselectivity of the radicals or the diffusiblity of melanin in different testing systems (Yu et al., 2002). Owing to the stronger antioxidant potential exhibited by R23 melanin, it can be suggests its use as a promising raw material for the cosmetic

industry for the formulation of creams that protect the skin against possible oxidative damage.

The antioxidant property of melanin in terms of lipid peroxidation inhibition was evaluated by earlier researchers. During lipid peroxidation process, peroxides are progressively decomposed to lower molecular weight compounds, among which MDA is an important derivative and considered to be a useful biomarker to investigate the final stage of lipid peroxidation. Kalka et al. (2000) reported that biomelanins are potent inhibitors of peroxidative damage and indicated that these compounds are useful antioxidants. Different fractions of melanin extracted from chestnut shells exhibited strong lipid peroxidation inhibition based on TBA method (Yao and Qi, 2016). As a measure of lipid peroxidation inhibition by TBARS assay, melanin from a microbial source EP28 exhibited a dose dependent increase in lipid peroxidation inhibition (Pithawala and Jain, 2014).

In the present study, the inhibitory effect of melanin on lipid peroxidation was also increased in a dose dependent manner. At a concentration of 75 μg ml^{-1}, inhibition was 29.87 ± 4.68% and it gradually increased to 81.5 ± 2.53% at a concentration of 750 μg ml^{-1}. This kind of dose dependent increase was also documented by Tu et al. (2009) for melanin extracted from Taihe Black-bone silky fowl (TBSF). The test compound R23 melanin extracted from marine black yeast *Hortaea werneckii* exhibited a lower IC_{50} than TBSF melanin (775 ± 40.3 μg ml^{-1}) (Tu et al., 2009). The IC_{50} is a parameter widely used to measure antiradical efficiency and lower the IC_{50} value, higher the antioxidant activity will be. In terms of lipid peroxidation inhibition also, the present study proves the antioxidant potential of R23 melanin.

Evaluation of cytotoxic effect of any newly isolated compounds is necessary for its acceptance in biological applications. Melanin as an antioxidant, assessment of cytotoxicity is prerequisite in healthy cells *in vitro*. Cytotoxicity of R23 melanin was analyzed by XTT and SRB assays. Cytotoxicity assays using HEp-2 cells revealed that up to a concentration of 800 μg ml^{-1}, only <25% reduction in cell viability was exhibited by both XTT and SRB assays. This is a concentration far higher than the dosage required for antioxidant activity by various antioxidant assays. Therefore R23 melanin from marine black yeast *Hortaea werneckii* is biocompatible for its use as antioxidant.

Melanins are dark brown biopolymers, play a crucial role in scavenging the reactive oxygen species (ROS) generated and that can effectively protect the host from UV radiation. Compounds that can act as antioxidants can protect the cells from ROS mediated DNA damage, which can result in mutation and succeeding carcinogenesis (Lopaczynski and Zeisel, 2001). Scientific records are available regarding the photoprotective role of various pigments from different sources including marine organisms (Young, 1991; Groniger et al., 2000; Moline et al., 2009; Pallela et al., 2010).

Photoprotective action of R23 melanin from *H. werneckii* was detected by the ability to scavenge reactive oxygen species generated upon UV irradiation on human epithelial cells, HEp-2. Photoprotective activity of melanin preparations from black yeast-like fungus *Aureobasidium pullulans* (var. *aubasidani*) during UV irradiation on human skin was studied and suggested that doses of melanin preparations should be empirically selected to achieve optimum photoprotective effect (Paramonov et al., 2002). Melanin acts as a neutral density filter reducing all wavelengths of light equally and thus act as photoprotectant (Kaidbey et al., 1979). Wan et al. (2007) reported DOPA melanin from a novel bacterial strain *Aeromonas media* as an effective photoprotectant to commercial bioinsecticide

BTI against UV and solar radiation. Recently, Swalwell et al. (2012) reported the role of melanin as a photoprotectant against cellular stressors such as UVA, UVB, and H_2O_2 in human melanoma cells.

In the present study, the reactive oxygen species generated by means of UV irradiation in melanin treated cells were considerably lower than control cells (melanin untreated). This may be due to the competency of melanin to act as an antioxidant and there by scavenging the reactive oxygen species generated through UV irradiation and thus aid photoprotection to the cells. Moreover, a dose dependent increase in scavenging of ROS could be observed in R23 melanin at different concentrations such as 50, 100 and 200 μg ml^{-1}. Interestingly, R23 melanin showed a greater scavenging effect than synthetic melanin on HEp-2 cells. R23 melanin from *H. werneckii* exhibited potential photoprotection ability as a means of reduction in UV induced ROS production and thus can act as an effective photoprotectant against the detrimental effects of UV radiation. In this perspective, R23 melanin will be a promising natural bioactive compound for the cosmetic industry for the formulation of creams that protect the skin against possible oxidative damage.

The use of pigments was primarily confined as colouring agents to various industries whereas during the past few decades, researchers have focused the divergent functions of pigments in pharmaceutical, cosmetic and food industries. From this study, it is evident that pigment R23 melanin extracted from marine black yeast *H. werneckii* possesses potential activity which would be promising in various industrial sectors. The physicochemical characteristic of R23 melanin is analogous to typical melanins. The antioxidant efficacy of R23 melanin was comparable to standard trolox in terms of ABTS and DPPH radical scavenging assays. Similarly, the photoprotection ability was also confirmed with regard to reduction in UV induced ROS production in HEp-2 cells and it was supreme to

the efficiency of positive control synthetic melanin. To conclude, the present investigation suggests that the isolated R23 melanin will be a potential natural product for use in cosmetic and/or pharmaceutical industries in an environment friendly perspective.

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Chapter 5

Evaluation of marine yeast glucan and melanin as anticancer agents by *in vitro* gene expression analysis

5.1 Introduction

Natural resources play a pertinent role in the expansion of novel therapeutic products. Several natural products from various sources such as plants, animals and microorganisms have been scrutinized till date. Many of them are currently in clinical trials or preclinical trials or undergoing further investigation. From the past few decades, there has been an upsurge in the search for new marine derived compounds in drug discovery. Compounds with unique structures and diverse pharmacological activities have been isolated from various marine derived flora and fauna (Faulkner, 2000; Haefner, 2003). Marine ecosystem harbor wide range of microorganisms with novel biosynthetic capabilities due to the existence of assorted environment. In recent years, efforts were taken to cultivate microorganisms and characterization of compounds, whereas this area of study is still in its infancy. In the continuous search for new bioactive compounds, marine derived products with unique mechanism of actions are being identified. Several pigmented compounds isolated from marine

microorganisms with promising biological activities have been reviewed (Soliev et al., 2011) which includes antibacterial, antifungal, antibiotic, immunosuppressive and anticancer activities. Polysaccharides from marine microorganisms especially those which survive in extreme environments may find possible applications in the industrial and pharmaceutical sector (Poli et al., 2010). It is anticipated that the marine environment will become an excellent source of novel compounds with potential medicinal application for various diseases including cancer.

Cancer is becoming a life threatening disease problem in the present scenario due to changing lifestyle. Worldwide cancer risk factors are almost similar which include smoking, insufficient physical activity, alcohol, diet, overweight and obesity. Infections account for a high proportion of cancers worldwide. Based on Worldwide cancer statistics (2012) reported an estimated 14.1 million new cases of cancer occurrence and 8.2 million deaths from cancer (Torre et al., 2015). 1,685,210 new cancer cases and 595,690 cancer deaths are projected to occur in the United States in 2016 (Siegel et al., 2016). Generally, the term cancer is referred as the abnormal cell division without any control and can invade various nearby tissues. There are several types of cancer which depend upon the site, from where they begin. It affects different part of the body and the chance of survival is determined by the cancer type and site of cancerous growth. According to worldwide cancer statistics (2012) lung, liver, stomach, and bowel are the most common causes of cancer death which accounts for nearly half of all cancer deaths. The advancement of modern science helps to treat cancer by unraveling the detailed mechanism of action of each newly isolated compound. A number of anticancer agents derived from various natural sources are in clinical trials and few of them are in use.

5.1.1 Anticancer agents from plant sources

From ancient times, extracts of plants, herbs and seeds are being used for the treatment of various diseases. Due to the intensification of cancer risk, there is also an increasing demand for anticancer medicines/therapy. Natural products play a relevant role in cancer therapy and are becoming an important research area for drug discovery (Orlikova et al., 2014; Rajesh et al., 2015). A number of classical plant derived compounds have been established for their anticancer effects. Vincristine, vinblastine, vindesine and paclitaxel are some of the effective plant derived anticancer drugs that have been approved for clinical treatment of cancer (Nobili et al., 2009). Thereafter a number of second generation compounds which include vinca alkaloids, taxanes and many new generation compounds derived with structural modifications have been developed (Fahy, 2001; Rowinsky and Calvo, 2006). So far, variety of anticancer compounds have been characterized from different plant sources with different mechanism of actions such as interaction with microtubules, inhibition of topoisomerases I or II, alkylation of DNA, and interference with tumour signal transduction (Hsiang et al., 1985; Hennequin et al., 1995; Jordan and Wilson, 2004; Carter and Keam, 2007).

Flavonoids are group of polyphenolic compounds that occur ubiquitously in plants. Studies have shown that dietary flavonol compounds have a variety of bioactivities such as antioxidant, antiinflammation, antiproliferative and antiangiogenic activities that can limit carcinogenesis and cancer progression (Russo et al., 2014; Sak, 2014; Liu et al., 2015; Xie et al., 2016). Recent studies have reported that one of the main flavonol compound quercetin, is able to reduce tumor cell viability, decrease the production of reactive oxygen species and induce apoptosis via modulation of a number of important signaling pathways, such as IRE1/JNK, PI3K/Akt, and FOXO3A (Tseng et al., 2012; Lu et al., 2015).

5.1.2 Anticancer agents from microbial sources

Search for anticancer agents from microbial sources was started from the beginning of the 20th century after the discovery of first antibacterial agent from microorganism. Intense research in this area has led to the discovery of the first antitumour antibiotic 'actinomycin D' from *Actinomyces antibioticus* by Waksman and Woodruff (1941). Thereafter, different *Streptomyces* species have been subjected for the study to detect the compounds for anticancer properties (Di Marco et al., 1964; Arcamone et al., 1969; Schulte and Neckers, 1998; Hartford and Ratain, 2007). Mitomycin C, bleomycin and doxorubicin, are the anticancer drugs derived from microbial sources. Recently, a bioactive metabolite 5-methyl phenazine-1-carboxylic acid betaine (MPCAB) was purified from a rhizosphere soil bacterium *Pseudomonas putida* PUW5. MPCAB exhibited selective cytotoxicity towards lung (A549) and breast (MDA MB-231) cancer cell lines. In these cancer cells MPCAB could induce G1 cell cycle arrest and apoptosis, and suggest that the MPCAB induces apoptosis through mitochondrial intrinsic pathway by the down regulation of Bcl-2 protein and activation of caspase-3 (Kennedy et al., 2015).

In the continuous search for anticancer agents, the anticancer property of the enzyme L-asparaginase was discovered. Although variety of plants and animals could produce this enzyme, microorganisms are the efficient and inexpensive source. A variety of microbes including bacteria, fungi, yeast, actinomycetes and algae are efficient producers of L-asparaginase, whereas L-asparaginase from *Escherichia coli* and *Erwinia caratovora* are used in the clinics (Narta et al., 2007; Nobili et al., 2009; Abakumova et al., 2012). L-asparaginases, have been used in the treatment of a range of lymphoproliferative disorders and lymphomas, especially in acute lymphoblastic leukaemia, in combination with other anticancer agents. Recently, Husain and coworkers

purified a glutaminase free asparaginase enzyme from *Pseudomonas otitidis* which exhibits anticancer activity by inducing apoptosis in human leukemia MOLT-4 cells and suggested that this enzyme induces cell death through activation of intrinsic apoptotic pathway (Husain et al., 2016).

Microbial polysaccharides are one of the attracting bioactive compounds with potential applications. Even though various applications of microbial polysaccharides have been reported, the potential of microbial polysaccharides in cancer research is scanty. Exopolysaccharides extracted from the endophyte *Bacillus amyloliquefaciens* sp. isolated from *Ophiopogon japonicus* exhibited antitumor activity against gastric carcinoma cell lines (MC-4 and SGC-7901) (Chen et al., 2013a). In another study, polysaccharides from the mycelia of *Rhizopus nigricans* (RPS) inhibited the proliferation of human gastric cancer BGC-823 cells and suggested the potential use of RPS as an anticancer agent against human gastric cancer. RPS induced mitochondria-mediated apoptosis which was associated with collapse of mitochondrial membrane potential, activation of caspase-9 and caspase-3, generation of intracellular reactive oxygen species and elevation of intracellular calcium in BGC-823 cells. RPS also exhibited cell cycle arrest in the G2/M phase of the cell cycle (Chen et al., 2013b).

Microorganisms produce various pigments which include carotenoids, melanins, flavins, quinines, prodigiosins and violacein. Microbial pigments also have been subjected for the anticancer studies. The red pigment isolated from *Streptomyces* sp. PM4 exhibited potential anticancer activity against HT1080, Hep2, HeLa and MCF7 cell lines by MTT assay (Karuppiah et al., 2013). Anticancer activity of the red pigment extract from *Serratia marcescens* has been evaluated against a panel of breast and prostate cancer cell lines by MTT assay and reported the anticancer potential of this microbial pigment (Athavale et al., 2013). In another study, the red pigment prodigiosin

from *S. marcescens* exhibited strong anticancer and apoptosis activity against the human cervix carcinoma cells (Kavitha et al., 2010). It is anticipated that all these studies and continuous research will facilitate the discovery of potential drugs from microorganisms against cancer.

5.1.3 Anticancer agents from marine sources

Marine environment is a highly competitive environment due to the combination of unique conditions, and hence force marine organisms to develop complex chemical adaptations. Scientific communities take this privilege for the expansion of new bioactive compounds from marine sources which include the marine flora and fauna. A number of marine derived products have been entered in to clinical trials that reveal the potential of marine derived products (Tasdemir et al., 2002). In the case of development of anticancer compounds also, the contribution is noteworthy. Anticancer agent, citarabine is the first drug originated from a marine source, Caribbean sponge *Cryptotheca crypta*. Followed by a number of compounds from various sources have been screened for their anticancer properties. Didemnin B, aplidine, and ET-743 are marine compounds derived from tunicates exhibiting antitumor properties (Rinehart, 2000; Delaloge et al., 2001). Furthermore, bryostatin-1 from marine bryozoan, Depsipeptide (NSC 630176) from *Chromobacterium violaceum*, Halichondrin B from the sponge *Halichondria okadai*, Discodermolide from a deep-sea sponge, *Discodermia dissolute*, Kahalalide F from the mollusc *Elysia rubefescens*, cryptophycins from cyanobacteria are some of the compounds isolated from various marine organisms from different parts of the world that have been noticed for their anticancer effects (Hornung et al., 1992; Ueda et al., 1994; Garcia-Rocha et al., 1996; Kowalsky et al., 1997). Recently, it has been reported that Isololiolide, a carotenoid metabolite isolated from the brown alga *Cystoseira tamariscifolia*, exhibited cytotoxic effects and induced apoptosis in

hepatocarcinoma (HepG2) cells through caspase-3 activation, increased p53 expression, PARP cleavage and decreased Bcl-2 levels (Vizetto-Duarte et al., 2016). Even though marine floras are potential source of anticancer compounds, they are least explored and among the anticancer compounds studied so far, the marine algae contribute 65.63%, the mangroves 28.12%, and the microbial flora 6.25% (Boopathy and Kathiresan, 2010) (Fig. 5.1).

The ability of microorganisms to produce various metabolites and their application in pharmaceutical sector has been there in history years back. Metabolites produced by marine microorganisms including fungi, actinomycetes, algae and bacteria also have been reported as antibacterial, antifungal, antiviral, antioxidant and anticancer agents (Shen et al., 2009; Arasu et al., 2013; Henriquez et al., 2014). Recent research has been started focusing on screening for anticancer property of metabolites from marine microorganisms over terrestrial counterparts.

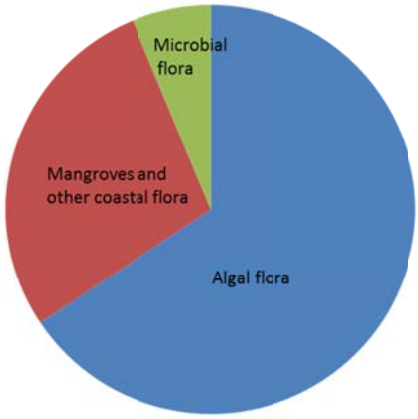

Fig. 5.1 Relative contributions of different marine floral components to anticancer compounds (Adapted from Boopathy and Kathiresan, 2010)

Marine microorganisms are considered to be an important source of bioactive molecules against various diseases and also have great potential as anticancer agents to increase the number of lead molecules in clinical trials.

Recently, Ramos and coworkers tested the anticancer activity of ethyl acetate extracts of four marine-derived fungi *Aspergillus similanensis* KUFA 0013 (E1), *Neosartorya paulistensis* KUFC 7897 (E2), *Talaromyces trachyspermus* KUFC 0021 (E3) and *Neosartorya siamensis* KUFA 0017 (E4) on a panel of seven human cancer cell lines by MTT assay, clonogenic assay, comet assay and nuclear condensation assay (Ramos et al., 2015). The extracts of *N. paulistensis* and *N. siamensis* exhibited selective antiproliferative and cell death activities in HepG2 (hepatocellular carcinoma), HCT16 (colorectal carcinoma), and A375 (malignant melanoma) cells, suggesting the potential anticancer effects from marine derived fungi. A new cytotoxic substance was isolated from marine-derived *Thermoactinomyces* sp. YM3-251, named mechercharmycin A which exhibited relatively strong antitumor activity against A549 cells (human lung cancer) and Jurkat cells (human leukemia) (Kanoh et al., 2005).

In another study, ethyl extracts from different strains of marine bacteria *Chromohalobacter salexigens*, *Halomonas meridian*, *Idiomarina loihiensis* and *Chromohalobacter israelensis* isolated from deep sea, exhibited significant anticancer potential against three human cancer cell lines such as MCF-7 (Breast adenocarcinoma), HeLa (Cervical carcinoma), and DU145 (Prostate carcinoma) through biological assays, such as MTT assay, mitochondrial membrane potential (MMP) assay, caspase assay, PARP-1 cleavage (representing DNA fragmentation) and expression of γH2Ax (indicating DNA damage). Significantly higher apoptosis have been noticed from these extracts which was followed by the sequence of apoptotic events involving MMP disruption, caspase-8 cleavage, activation of caspase-9, caspase-3/7 activity and PARP-1 cleavage suggesting the caspase mediated apoptotic pathways (Sagar et al., 2013). Recently, the potential anticancer effect of a marine microbial extract luminacin has been documented from the *Streptomyces* species against head and

neck squamous cell carcinoma (HNSCC) cell lines which was measured using cell viability, colony forming, and apoptosis assays (Shin et al., 2016). Currently, it appears that there have been no published reports focusing on bioactive compounds derived from marine yeasts to be used as anticancer agents.

5.1.4 Genes in cancer regulation

Cells procure several genetic changes during the period of cancer development. Consequence of these changes are the progressive acquisition of biological characteristics such as sustained proliferative signaling, insensitivity to growth suppressors, evading apoptosis, increasing genomic instability, activating mobility, invasion, metastasis and angiogenesis that might consequently progress into a malignant phenotype (Hanahan and Weinberg, 2011) (Fig. 5.2). Various novel strategies have been evolved which includes the use of microbial products such as proteins, enzymes, immunotoxins and secondary metabolites, which specifically target cancer cells and cause tumor regression through growth inhibition, cell cycle arrest or apoptosis induction (Bernardes et al., 2010).

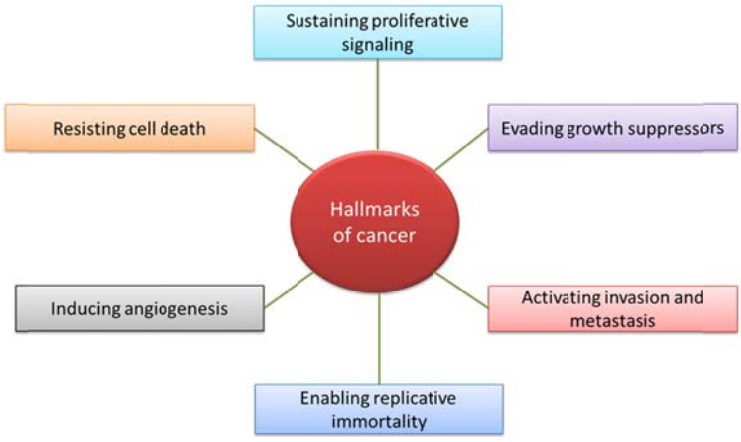

Fig. 5.2 Hallmarks of Cancer (Reproduced from Hanahan and Weinberg, 2011)

The most fundamental peculiarity of cancer cells involves their ability to sustain chronic proliferation. Tumor suppressor genes play a pertinent role in various ways to limit cell growth and proliferation. The two archetypal tumor suppressor genes which encode the RB (retinoblastoma-associated) and TP53 (tumor protein 53) proteins, that govern the decisions of cells to proliferate or, alternatively, activate apoptotic programs. The RB protein incorporates signals from various intracellular and extracellular sources and in response to that decides whether or not a cell should proceed through its growth-and-division cycle. Cancer cells with defects in RB pathway, missing the services of a critical gatekeeper of cell-cycle progression and whose absence permits persistent cell proliferation (Sherr and McCormick, 2002; Burkhart and Sage, 2008). On the other hand, if there is any alarm signals indicating devastating or irreparable damage to the cellular subsystems, TP53 can trigger apoptosis.

The concept of programmed cell death by apoptosis serves as a natural barrier to cancer development (Lowe et al., 2004). The apoptotic machinery is mainly composed of both upstream regulators and downstream effector components (Adams and Cory, 2007). The regulators receive and integrate various extracellular and intracellular signals and each culminates in activation of a normally latent protease such as caspases 8 and 9. That proceeds to initiate a cascade of proteolysis involving effector caspases such as caspases 3, 6 and 7 that are responsible for the execution phase of apoptosis. Wherein the cell is progressively disassembled and then consumed, both by its neighbors and phagocytic cells. The pro- and anti-apoptotic members of the Bcl-2 family of regulatory proteins actively participate in apoptosis to convey the signals between regulators and effectors. The prime example, Bcl-2, along with its

closest relatives such as Bcl-xL, Bcl-w, Mcl-1, A1 are inhibitors of apoptosis, while Bax and Bak are two pro-apoptotic triggering proteins (Willis and Adams, 2005). The ratio of Bax:Bcl-2 proteins increase during apoptosis (Leung and Wang, 1999) and that has been recognized as a key factor in regulation of the apoptotic process.

The immune system of an organism is very important to fight against foreign invaders and keeping the body healthy. Cytokines such as interleukins, interferons and growth factors are also considered as major components of immune system. These cytokines play an important role in cancer regulation through various immune mechanisms. Previous studies have shown that polysaccharide extracts from various sources enhance the immune functions, reduce the side effect of chemotherapy and radiotherapy and also act against cancer (Fujimiya et al., 1998; Lin and Zhang, 2004; Gong et al., 2005). It has been reported that IL-2 and IFN-γ are essential for the induction of cellular immunity, whereas TNF-α, IL-4, IL-5 and IL-10 play a key role in humoral immunity (Nishimura et al., 1999). Investigations on the anti-inflammatory and immunomodulation activity of fungal metabolites indicated that various active substances derived from mushrooms modulates immune system and as a result more enhanced innate and acquired disease resistance (Bao et al., 2001; Lull et al., 2005). The major immunomodulating effects of active substances derived from mushrooms include activation of immune effector cells, such as macrophages, lymphocytes and natural killer cells, resulting in the production of cytokines, comprising interleukins (ILs), tumor necrosis factor alpha (TNF)-α and interferon gamma (INF)-γ (Sorimachi et al., 2001; Jin et al., 2003). Sometimes compounds do not exhibit anticancer effect directly on cancer cells,

whereas produce their anticancer effects by activating different immune responses in the host. The role of β-glucans as immunomodulating agent with effect on both innate and adaptive immunity has been reported and different cytokines produced as a result of immune response may also play important role in cancer therapy (Chan et al., 2009).

IL-2 has been introduced as a cancer treatment since 1992 (McDermott and Atkins, 2006). Anti-tumoral activity of IL-12 has been reported which is mediated through its role in interferon gamma production as well as inhibition of angiogenesis (Duda et al., 2000; Ferretti et al., 2010). Interleukins are attractive target for cancer treatment due to the immune-modulatory role as well as their direct or indirect role in angiogenesis, apoptosis and other cancer development and progression pathways (Razavi and Allen, 2015). INF-β and TNF-α are also important cytokines that regulate cell growth and death, and INF-β has been well recognized for its anticancer activity (Repetto et al., 1993; Anderson et al., 2004). Several animal tumor model studies have revealed that cytokines have broad antitumor activity and this has been translated into a number of cytokine based approaches for cancer therapy (Lee and Margolin, 2011).

Effects of compounds on the expression of a set of genes implicated in cancer cells using PCR techniques helps to understand the gene based effects of each new isolated compound besides the traditional cytotoxic assays. In recent years number of studies have been reported based on potential effects of various extracts on regulating cancer related genes using gene expression analysis. Extract derived from *Zingiber officinale* exhibited potential antiproliferative effects on breast cancer cell lines MCF-7 and MDA-MB-231 by down-

regulating the expression of prosurvival genes, such as NF-κB, Bcl-X, Mcl-1 and Survivin, and cell cycle-regulating proteins, including cyclin D1 and cyclin-dependent kinase-4 (CDK-4). Then again, it increased expression of CDK inhibitor, p21 and also inhibited the expression of two prominent molecular targets of cancer such as c-Myc and the human telomerase reverse transcriptase (hTERT). This extract also mediated the apoptotic cell death by up-regulating the Bax and down-regulating the Bcl-2 (Elkady et al., 2012).

Recently, apoptosis inducing potential of *Origanum dayi* and *Ochradenus baccatus* extracts have been detected by semi-quantitative reverse transcription polymerase chain reaction. Both extracts displayed up-regulation of Bax, Bad, cytochrome c, caspase-3, caspase-7, caspase-9 and poly (ADP-ribose) polymerase in HepG2 cells through modulation of mitochondrial pathway which explains their antitumor activities (Bhatia et al., 2015). Although each gene has importance in regulating cancer development, various lines of evidence indicates that every single one of them operates as part of a larger network that is bounded for functional redundancy.

Despite knowledge about the potent anticancer activity of various extracts from marine environment, the anticancer effects of marine yeast compounds are not currently known. In this context, the present study was undertaken to investigate the impacts of three glucans extracted from marine yeasts *Candida parapsilosis* R20, *Hortaea werneckii* R23 and *Debaryomyces fabryi* R100, and also the melanin extracted from *Hortaea werneckii* R23 in NCI-H460 cancer cell lines. To understand the molecular mechanisms underlying this activity, a set of genes involved in cancer regulation have been considered for the gene expression study by quantitative real-time PCR analysis.

5.2 Materials and Methods

5.2.1 Microorganisms used for the study

Three marine yeasts *Candida parapsilosis* R20, *Hortaea werneckii* R23 and *Debaryomyces fabryi* R100 were used for glucan extraction. Black yeast *H. werneckii* R23 was used for melanin extraction. These cultures were isolated from the sediment samples collected from Arabian Sea and Bay of Bengal, and maintained in the Microbiology Laboratory.

5.2.2 Preparation of marine yeast glucan compound

Yeast biomass was prepared using swab inoculation on Malt Extract Agar plates (malt extract, 20 g; mycological peptone, 5 g; agar, 20 g; 20 ‰ seawater, 1000 ml; pH 6) and the biomass was harvested using sterile seawater. The harvested biomass was separated by centrifugation at 10,000 rpm for 20 min at 4°C in a refrigerated centrifuge (Kubota, Japan) and dried at 80°C for 24 h. Glucan was extracted from the dried yeast biomass following the method of Williams et al. (1991) with slight modifications. Briefly, 2 g dried yeast biomass was suspended in 40 ml of 3% NaOH and maintained at 100°C for 3 h in a water bath and was kept overnight at room temperature (RT). The suspension was centrifuged at 8000 rpm for 15 min, collected the insoluble residue, re-suspended in 3% NaOH and the procedure was repeated (2x). The insoluble residue was treated using 0.5 N acetic acid and heat to boil for 6 h and separated by centrifugation. The procedure was repeated (2x). This residue was treated with distilled water at 100°C and centrifuged at 8000 rpm for 10 min to collect the insoluble residue. The procedure was repeated (5x). After centrifugation, the insoluble fraction was re-suspended in ethanol, boiled and centrifuged at 8000

rpm to collect the residue and the procedure was repeated (3x). The residue was washed thoroughly with distilled water and dried under vacuum and the final product (glucan) designated as YG20, YG23 and YG100 which was used for conducting further experiments.

Soluble yeast glucan was prepared following Williams et al. (1992) with slight modifications. 0.5 g of glucan was dissolved in 12.5 ml of Me_2SO containing 9 g of 8 M urea. In another flask, Me_2SO (12.5 ml) and concentrated H_2SO_4 (1.25ml) were thoroughly mixed, and this mixture was added drop-wise to the previously mixed glucan-Me_2SO-urea solution with continuous stirring. This solution was heated to 100°C in a water bath with continuous stirring, and the reaction was carried out for 4 h. The solution was cooled to room temperature (28±2°C), diluted in 500 ml of MilliQ water, and passed through a Millipore prefilter (1.2 µl) to remove microparticulate glucan. This glucan solution was purified with an ultrafiltration system using a 10,000 MW cut-off filter, pH was adjusted to 7 and concentrated by lyophilisation. The solubilised glucans were filter sterilised and stored at -20°C until further use.

5.2.3 Preparation of marine yeast melanin compound

The black yeast, *H. werneckii* R23 was sub-cultured on to Malt Extract Agar plates, incubated at 28±2°C for five days and harvested with sterile saline (30‰). The cell suspensions were centrifuged at 10,000 rpm for 30 min in a refrigerated centrifuge to get the yeast biomass. From the harvested yeast biomass, melanin was extracted in accordance with the protocol of Gadd, 1982. Briefly, 1N NaOH was added to the harvested yeast biomass and autoclaved for 20 min at 121°C. After that it was centrifuged at 8000 rpm for 10 min and the supernatant containing melanin was separated. The supernatant

solution was then precipitated by adding concentrated HCl until the pH is reduced to 2. This solution was again centrifuged at 10,000 rpm for 10 min and the pellet was repeatedly washed with distilled water to get the purified melanin pigment. This was dried in a lyophilizer and stored at ⁻20°C till use and designated as R23 melanin.

Solubilisation of melanin was done by 1N aqueous ammonia (25%) and pH was adjusted to 7. The solution was filter sterilised with an ultrafiltration system and stored at -20°C until further use.

5.2.4 Cell lines and testing anticancer activity *in vitro*

The human non-small cell lung carcinoma cell line NCI-H460 was purchased from the American Type Culture Collection (ATCC). The cell line was grown in monolayer in (DMEM/F-12) medium supplemented with 10% fetal bovine serum (FBS) and antibiotic mixture containing 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The growth media was changed every other day. The prepared compounds glucan (YG20, YG23 and YG100) at a concentration of 5 mg ml^{-1} and melanin (R23 M) at a concentration of 0.5 mg ml^{-1} was added into the cultured cells and incubated for 24 h. The anticancer activity of marine yeast compounds glucan and melanin were evaluated *in vitro* by gene expression analysis.

5.2.5 Gene expression analysis

5.2.5.1 Total RNA isolation and reverse transcription

NCI-H460 cells grown in 25 cm^2 cell culture flask containing different glucan and melanin were subjected for total RNA extraction. For RNA isolation, the growth medium along with the test compound was removed; wells washed

with ice cold phosphate buffered saline (PBS) and 1ml TRI reagent (Sigma, USA) was added to each well. Complete lysis of cells was allowed to take place by repeated pipetting and the reagent was collected in 1.5 ml MCTs. The medium removed from the wells containing detached cells was centrifuged at 400 x g for 5 min, washed with ice cold PBS. Untreated cells were used as controls. The samples were stored for 5 min at RT to ensure complete dissociation of nucleoprotein complexes. An aliquot of 0.2 ml chloroform was added to 1 ml TRI reagent, shaken vigorously for 15 sec (CM101, Cyclomixer, REMI), and allowed to stand for 15 min. The resulting mixture was centrifuged (3K30, Sigma) at 12,000 x g for 15 min at 4°C. Colourless upper aqueous phase was separated carefully from the three layers formed and transferred to a fresh tube. An aliquot of 0.5 ml isopropanol was added and stored for 10 min at RT and centrifuged at 12,000 x g for 10 min. RNA was precipitated on the sides and bottom of the tube after centrifugation at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the pellet washed twice with 75% ethanol. The pelleted RNA was air dried and dissolved in 20 µl DEPC treated sterile water by repeated pipetting at 55°C. These RNA samples were subjected to DNase treatment with RNase-free DNase 1 (New England Biolabs). An aliquot of 0.2 units of the enzyme was added per µg of RNA and incubated at 37°C for 10 min. The enzyme was inactivated at 75°C for 10 min. Concentration and quality of RNA was measured by taking the absorbance at 260/280 nm in a UV-Visible spectrophotometer (U2800, Hitachi). Only RNAs with absorbance ratio (A260:A280) $\geq$ 1.8 were used for further experiments. One µg RNA was subjected to cDNA synthesis with 20 µl of reaction mix containing M-MuLV reverse transcriptase (80 U), RNase inhibitor (8 U), Oligo $(dT)_{12}$ primer (40

pmoles), dNTP mix (1 mM), RTase buffer (1x) and $MgCl_2$ (2 mM) at 42°C for 1 h. All reagents were purchased from New England Biolabs.

5.2.5.2 Real-time quantitative PCR analysis of target gene expression

Expressions pattern of the twenty three cancer pathway genes such as; Bcl-2, Bax, Caspase-3, Caspase-9, Cathepsin-G, Calpain-5, Rb1, P-53, Akt1, MAPK-1, JNK, IL-1β, IL-2, IL-6, IL-10, IL-12, IFN-β, IFN-γ, TNF-α, viperin, MX1, ISG15 and IFITM3 were measured by quantitative real-time PCR (qPCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize the expression of these genes. Amplification was performed using respective primers of the target genes (Table 5.1). The qPCR was carried out in the StepOnePlus real-time PCR system (Applied Biosystems) with each reaction run in triplicate. The amplifications were performed in a 96-well plate in a 20 µl reaction volume containing 10 µl of 2x SYBR Green PCR Master mix, 0.5 µl (each) gene specific forward and reverse primers, 1 µl of cDNA and 8µl PCR-grade water. The thermal profile for qPCR was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

Gene expression analysis and fold change were determined using the comparative threshold cycle (Ct) method. All target mRNA levels were normalized by the mRNA levels of internal control GAPDH. The results were expressed as the ratio of reference gene to target gene by using the following formula: ΔCt = Ct (Target genes) - Ct (GAPDH). To determine the relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct$ (Treated) - ΔCt (Control). Thus, the expression levels were reported as fold differences relative to the control.

Table 5.1 Primers used for the present study

Sl. No.	Primer Name	Sequence (5'-3')
1	GAPDH – F GAPDH – R	cggagtcaacggatttggtc agccttctccatggtcgtga
2	Bcl2 – F Bcl2 – R	acctgcacacctggatcca agagacagccaggagaaatcaaa
3	Bax – F Bax – R	aagctgagcgagtgtctccggcg cagatgccggttcaggtactcagtc
4	Caspase 3 – F Caspase 3 – R	ataccagtggaggccgacttc caaagcgactggatgaacca
5	Caspase 9 – F Caspase 9 – R	tgtcctactctactttcccaggtttt gtgagcccactgctcaaagat
6	Cathepsin G – F Cathepsin G – R	tcaagtttcctgccctggat cctgtgtccccgagaagaag
7	Calpain 5 – F Calpain 5 – R	caggtcctctcagaggcagatac acctctccagggaccttaacg
8	Rb1 – F Rb1 – R	gaacatcgaatcatggaatccct agaggacaagcagattcaaggtgat
9	P-53 – F P-53 – R	gggttagtttacaatcagccacatt gggccttgaagttagagaaaattca
10	Akt 1 – F Akt 1 – R	gcacaaacgaggggagtacat cctcacgttggtccacatc
11	MAPK – F MAPK – R	caatggcggtgtggtgttc agctcccttatgatctggttcc
12	JNK – F JNK – R	tggacttggaggagagaacca cgacgatgatgatggatgct
13	IL-1β – F IL-1β – R	gcagccatggcagaagtacctga ccagagggcagaggtccaggtc
14	IL-2 – F IL-2 – R	ctgctggatttacagatgatttga tggccttcttgggcatgt
15	IL-6 – F IL-6 – R	cctgacccaaccacaaatgc ccttaaagctgcgcagaatga
16	IL-10 – F IL-10 – R	ctgggttgccaagccttgt agttcacatgcgccttgatg
17	IL-12 – F IL-12 – R	cctggaccacctcagtttgg acggccctcagcaggtt
18	IFN-β – F IFN-β – R	ctcctgttgtgcttctccact ggcagtattcaagcctccca
19	IFN-γ – F IFN-γ – R	ctttaaagatgaccagagcatcca atctcgtttctttttgttgctattga
20	TNF-α – F TNF-α – R	cccagggacctctctctaatc atggctacaggcttgtcact
21	Viperin – F Viperin – R	cgtgagcatcgtgagcaatg gctgtcacaggagatagcga
22	Mx1 – F Mx1 – R	ccagctgctgcatcccaccc aggggcgcaccttctcctca
23	ISG15 – F ISG15 – R	tggcgggcaacgaatt gggtgatctgcgccttca
24	IFITM3 – F IFITM3 – R	tcccacgtactccaacttcca agcaccagaaacacgtgcact

5.3 Results

5.3.1 Effect of marine yeast β-D-glucan on the expression of cancer-related genes in lung cancer cells

In this study, glucan from marine yeasts *Candida parapsilosis* R20, *Hortaea werneckii* R23 and *Debaryomyces fabryi* R100 (YG20, YG23 and YG100) were prepared and used for *in vitro* experiments. To profile the expression of cancer-related genes in response to marine yeast glucan in the lung cancer cells (NCI-H460), we utilized the quantitative real-time PCR analysis. Quantitative RT-PCR analysis showed that cancer-related genes were differentially expressed in response to various marine yeast glucans (Fig. 5.3-5.7). The expression of p53 and Rb1 were found to be up-regulated in all the three glucan treated lung cancer cells and the highest up-regulation was exhibited by YG100 treated cells. Caspase-3, caspase-9, cathepsin G and calpain-5 were also found to be up-regulated in the glucan treated cells than control. Bax were found to be up-regulated in YG20, YG23 and YG100 treated cells, whereas Bcl-2 expression was down-regulated when compared to Bax. Enhanced up-regulation of JNK and MAPK-1 also could be noticed after glucan treatment. While considering three glucans, highest up-regulation could be attributed by YG100 in caspase-3, p53, Rb1 and MAPK-1. Other two glucans YG20 and YG23 were also noticed for highest up-regulation of apoptosis related genes in NCI-H460.

Glucan treatment also enhanced the expression of various interleukins such as IL-1β, IL-2, IL-6, IL-10 and IL-12. An up-regulated expression of other immune related genes such as IFN-β, IFN-γ and TNF-α also could be noticed after various marine yeast glucan treatments in lung cancer cells. Virus associated immune genes MX1 and IFITM3 were up-regulated whereas

viperin and ISG15 were down-regulated. Among the three glucans, YG23 could enhance the expression of most of the cytokines analysed.

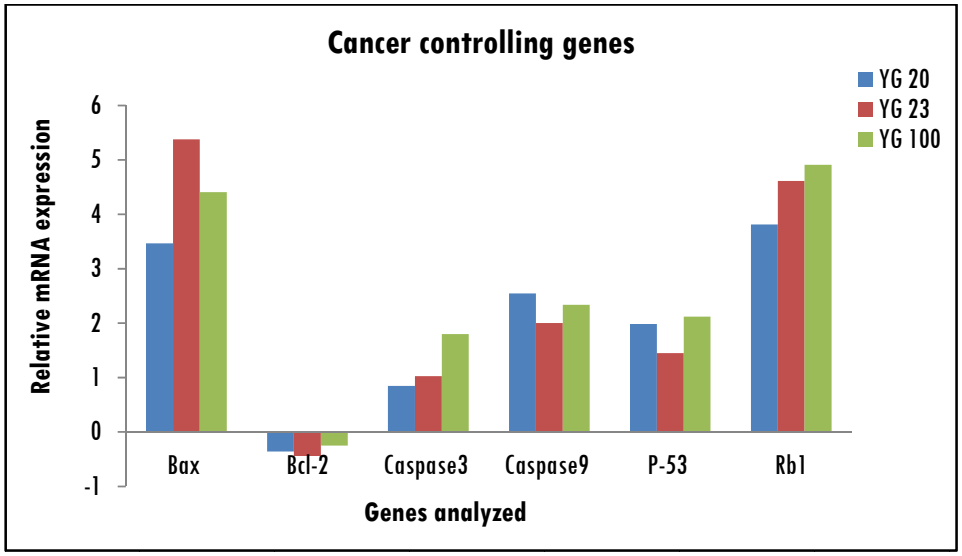

Fig. 5.3 Expression profile of cancer controlling genes in NCI-H460 cell lines in response to the exposure of different marine yeast glucans (YG20, YG23 and YG100)

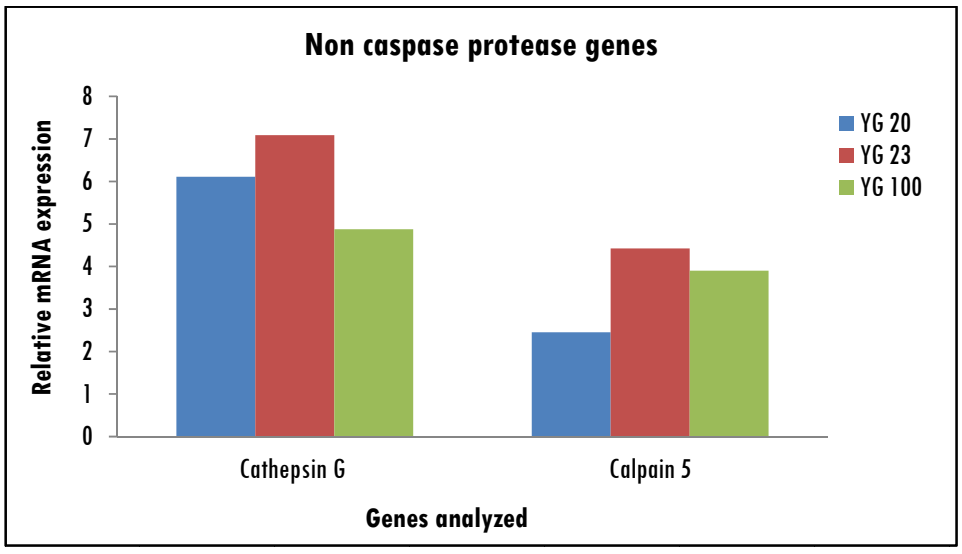

Fig. 5.4 Expression profile of non caspase protease genes in NCI-H460 cell lines in response to the exposure of different marine yeast glucans (YG20, YG23 and YG100)

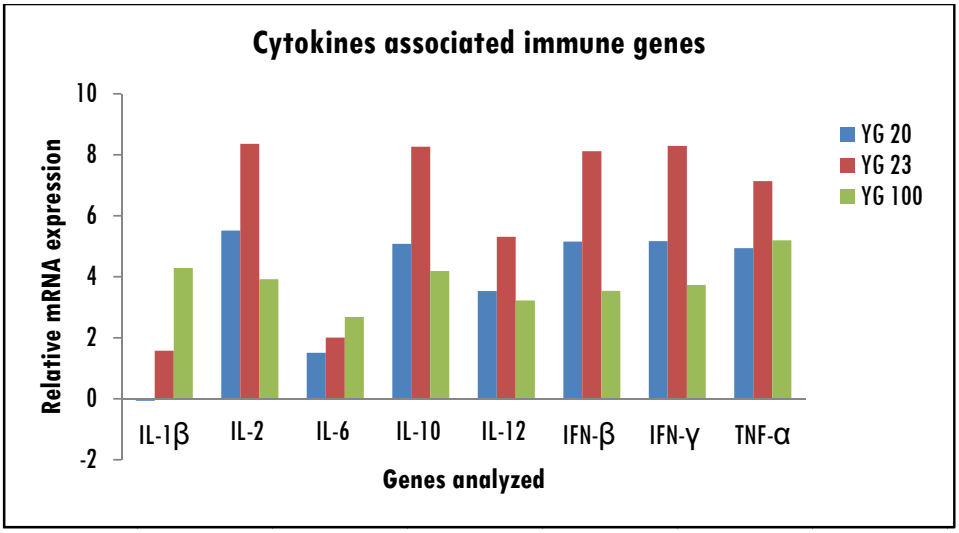

Fig. 5.5 Expression profile of cytokines associated immune genes in NCI-H460 cell lines in response to the exposure of different marine yeast glucans (YG20, YG23 and YG100)

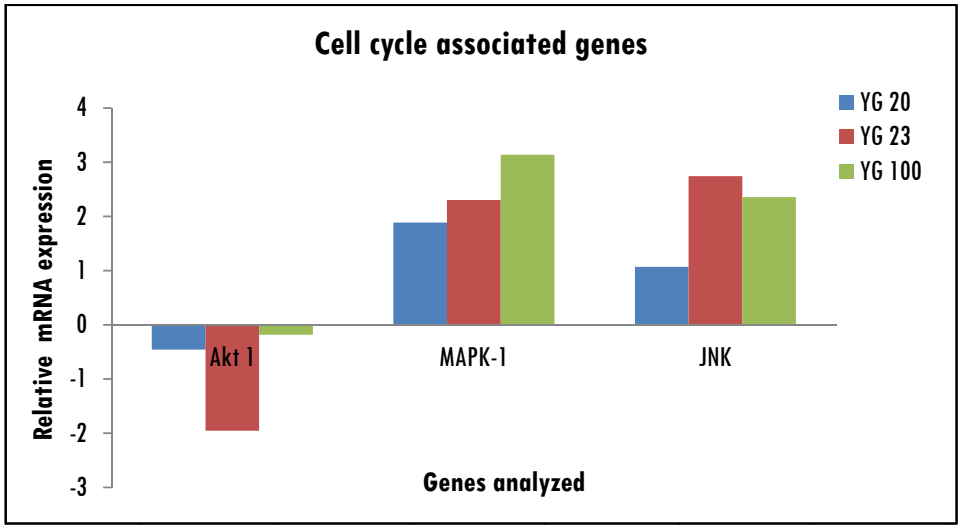

Fig. 5.6 Expression profile of cell cycle associated genes in NCI-H460 cell lines in response to the exposure of different marine yeast glucans (YG20, YG23 and YG100).

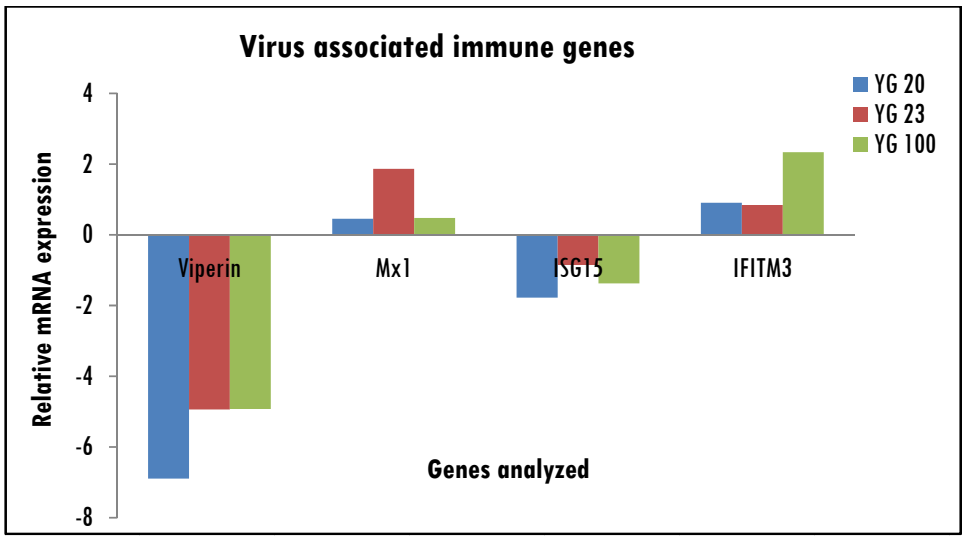

Fig. 5.7 Expression profile of virus associated immune genes in NCI-H460 cell lines in response to the exposure of different marine yeast glucans (YG20, YG23 and YG100)

5.3.2 Effect of marine yeast melanin on the expression of cancer-related genes in lung cancer cells

Melanin from marine black yeast *Hortaea werneckii* R23 (R23 melanin) was prepared and used for *in vitro* experiments. To profile the expression of cancer-related genes in response to marine yeast melanin in the lung cancer cells (NCI-H460), we utilized the quantitative real-time PCR analysis. Quantitative RT-PCR analysis showed that cancer related genes were differentially expressed in response to marine yeast melanin (Fig. 5.8-5.12). In the case of melanin, most of the cancer controlling genes were up-regulated in response to melanin treatment. Expression of Bax, Caspase-3, Caspase-9, Rb1, P-53, Cathepsin-G and Calpain-5 were up-regulated than Bcl-2. Enhanced up-regulation of JNK and MAPK-1 could be noticed after melanin treatment.

Melanin treatment also enhanced the expression of various interleukins such as IL-1β, IL-6, IL-10 and IL-12. An up-regulated expression of other immune related genes such as IFN-β, IFN-γ and TNF-α also could be noticed

after melanin treatment in lung cancer cells. Virus associated immune genes MX1 and IFITM3 were up-regulated whereas viperin and ISG15 were down-regulated. Here also, few genes such as IL-2, ISG15, viperin, and Akt1 were found to be down-regulated in response to melanin treatment.

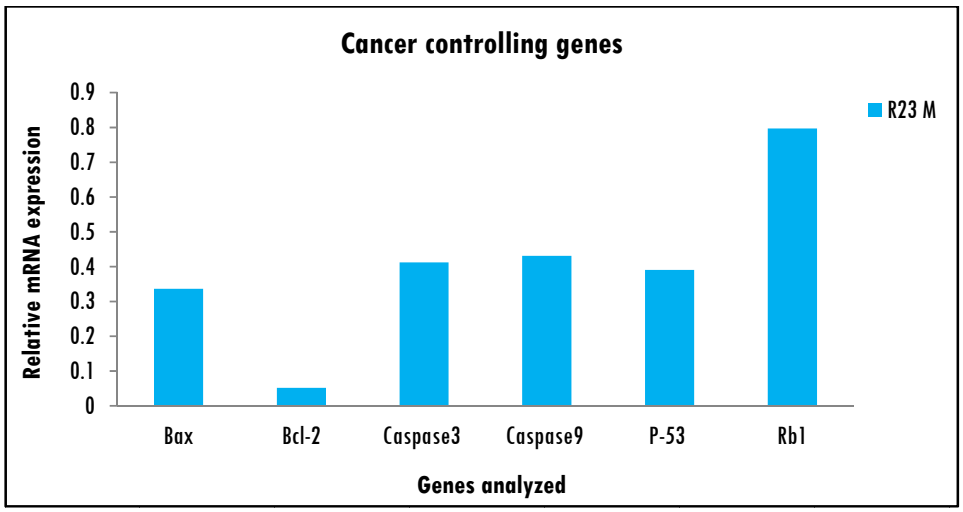

Fig. 5.8 Expression profile of cancer controlling genes in NCI-H460 cell lines in response to the exposure of marine yeast melanin (R23 M - R23 Melanin)

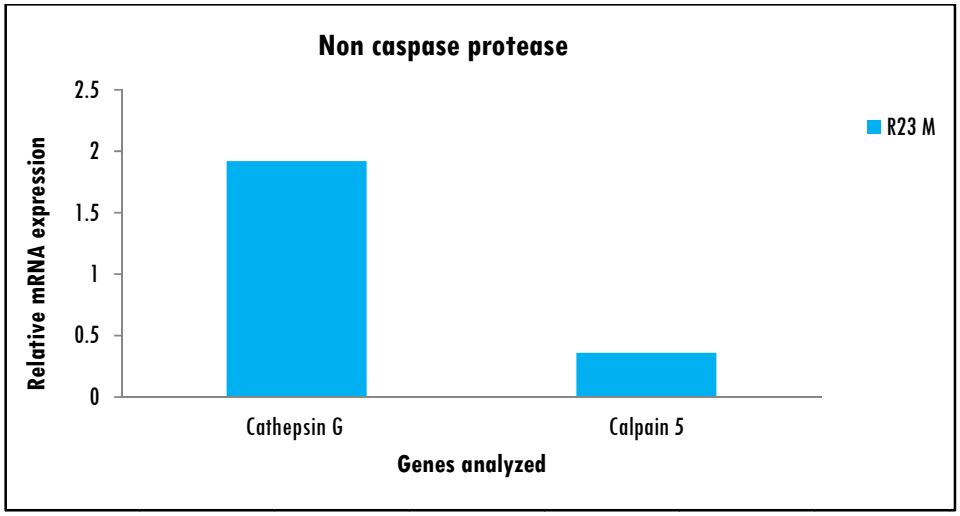

Fig. 5.9 Expression profile of non caspase protease genes in NCI-H460 cell lines in response to the exposure of marine yeast melanin R23 M (R23 M - R23 Melanin)

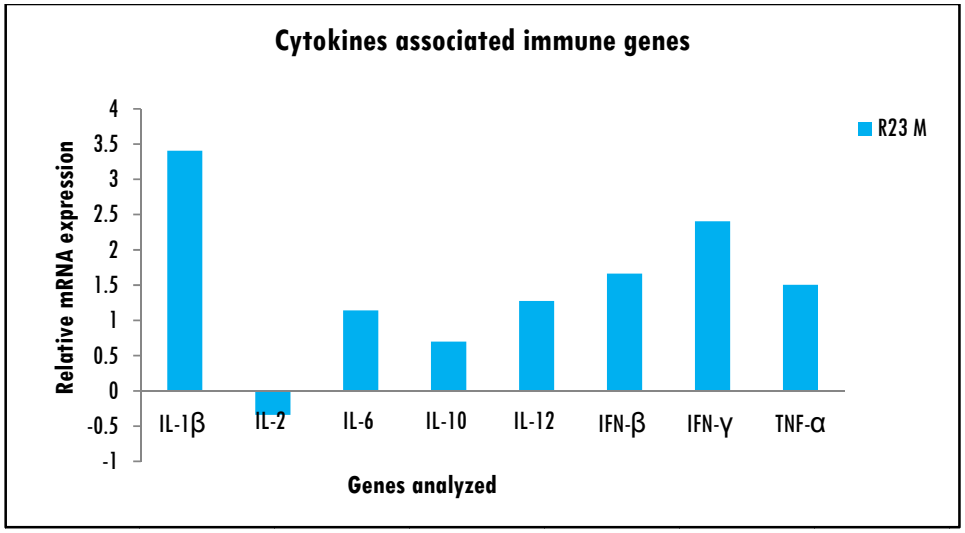

Fig. 5.10 Expression profile of cytokines associated immune genes in NCI-H460 cell lines in response to the exposure of marine yeast melanin R23 M (R23 M - R23 Melanin)

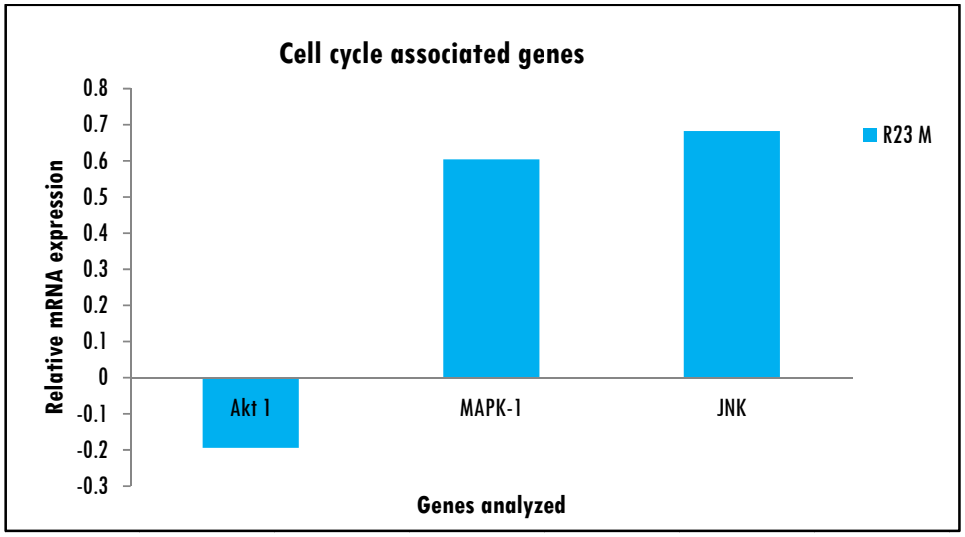

Fig. 5.11 Expression profile of cell cycle associated immune genes in NCI-H460 cell lines in response to the exposure of marine yeast melanin R23 M (R23 M - R23 Melanin)

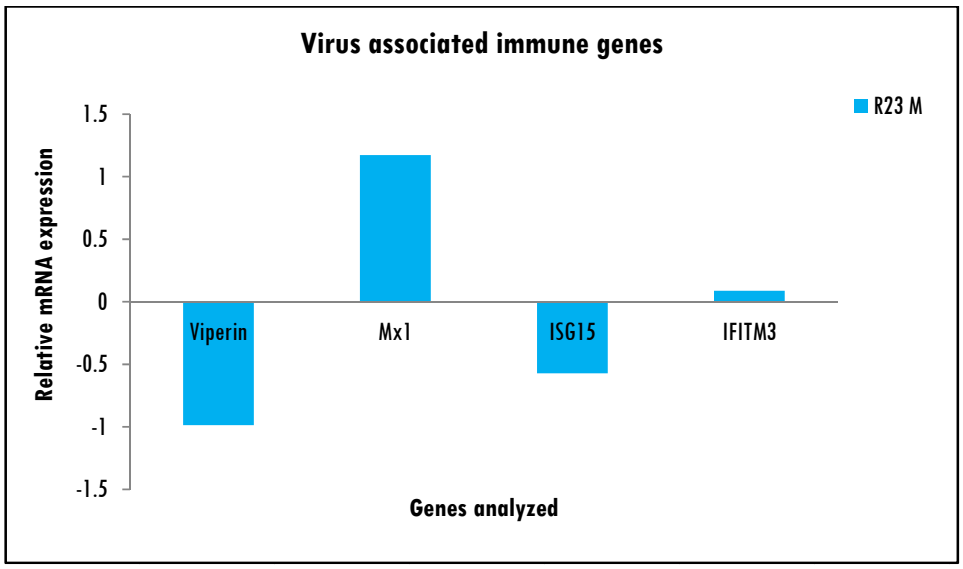

Fig. 5.12 Expression profile of virus associated immune genes in NCI-H460 cell lines in response to the exposure of marine yeast melanin R23 M (R23 M - R23 Melanin)

5.4 Discussion

Ocean is one of the greatest sources of bioactive natural products with fascinating chemical structures and potent biological properties. Up to now, an array of marine compounds have been identified and reported in the literature (Gerwick and Moore, 2012). Some of these compounds displayed significant tumor suppressing activity and are currently being considered in clinical trials or used as foremost compounds for development of new anticancer drugs (Shin et al., 2016). In recent years microorganisms have received great attention due to its various bioactive potential and it is amenable for better knowledge of the biosynthetic pathways that code for secondary metabolites in prokaryotes than those from eukaryotes. Marine microbial community which includes actinomycetes, cyanobacteria, microalgae and others have been recorded in literature for their bioactive potential. There are reports on compounds derived from marine microbial sources that have entered in clinical trials for cancer treatment.

Yeast is also an important marine microbial community and that have been isolated from different marine environment. The potential application of yeast in industrial sector is well known. Apart from the traditional applications, researchers have relocated on to new applications or evaluation of compounds from yeast in recent years. Marine yeasts also have proved its bioactive potential over their terrestrial counterparts. The immunostimulatory potential of marine yeasts and marine yeast glucans have been reported by earlier researchers (Sajeevan et al., 2009; Sukumaran et al., 2010; Antony et al., 2011c; Babu et al., 2013). Similarly antibacterial property of marine yeast melanin against a number of human and fish pathogens also have been reported (Kutty et al., 2013b; Rani et al., 2013). These activities of marine yeast compounds lead to search for the many more hidden potential of these unexplored marine yeast microbial community.

Effects of compounds, mostly from different plant sources have been reported on modulating cancer cell growth through regulation of multiple cell signaling pathways (Wang et al., 2012). β-glucans are considered to be 'biological response modifiers' since they exhibit immunomodulatory, wound-healing, antimicrobial, anticoagulation, anti-inflammatory, antitumoral and cholesterol lowering activities (Bohn and BeMiller, 1995; Kogan, 2000). Melanin from various sources also proved their antioxidant potential, free radical scavenging and photoprotection properties (Rozanowska et al., 1999; Paramonov et al., 2002; Tu et al., 2009). In this context the anticancer property of glucan and melanin from marine yeasts is considered for the present study on the basis of gene expression analysis of cancer related genes.

In cancer, there is an imbalance between cell division and cell death. Evasion of cell death is one of the indispensible changes in a cell that cause this malignant transformation (Hanahan and Weinberg, 2000). Apoptosis is an

important event in cancer regulation. Disrupted balance of pro-apoptotic and anti-apoptotic proteins, reduced caspase function and impaired death receptor signalling are the important mechanisms by which evasion of apoptosis occurs (Wong, 2011).

Proteins encoded by Bcl-2 gene family play a crucial role in the regulation of apoptosis which include both pro- and anti-apoptotic genes (Gross et al., 1999). Bcl-2 is an anti-apoptotic and Bax is a pro-apoptotic protein under the Bcl-2 family (Dewson and Kluc, 2010). In the present study, among the three glucans tested, the mRNA expression levels of Bax were significantly up-regulated after exposure of NCI-H460 cells to marine yeast glucan YG23 (5.37 fold) followed by 4.40 fold by YG100 and 3.46 fold by YG20, whereas the expression of Bcl-2 was down-regulated. When compared to Bax, down regulation of Bcl-2 could be noticed 0.44 fold in YG23 followed by YG20 (0.36 fold) and YG100 (0.25 fold). Study using the extracts from *Achillea teretifolia* Willd on human prostate cancer cell lines, exhibited powerful cytotoxic and apoptotic effects by the up-regulation of Bax and down-regulation of Bcl-2 mRNA gene expression (Bali et al., 2015). The present findings are in validation with significant apoptosis observed on the MDA-MB-231 human breast cancer cells treated with root bark of *Juglans regia* (RBJR) organic extracts with an increased expression of Bax and a down-regulated expression of Bcl-2 (Hasan et al., 2011). Melanin treated cells also exhibited an up-regulation of Bax (0.33 fold) than Bcl-2 which was only 0.05 fold. When compared to glucans, the apoptotic effect of melanin is less. Previous reports have shown that the ratio of Bax to Bcl-2 determines relatively, the susceptibility of cells to death signals. It is also known as these Bcl-2 family members as molecular targets in cancer therapy (Marzo and Naval, 2008). From the present data, it could be inferred that marine yeast

glucan has the potential to regulate the expression of apoptotic related genes such as Bax and Bcl-2. Furthermore, it is indicating that glucan treatment induced the apoptosis by shifting the ratio of Bax:Bcl-2 in favour of apoptosis and there by potential anticancer effect of glucan.

Caspases are one of the important players in the initiation and execution of apoptosis. Caspase-9 is an initiator caspases which are primarily responsible for the initiation of the apoptotic pathway and caspase-3 is one of the effector caspases which are responsible in the actual cleavage of cellular components during apoptosis (Fink and Cookson, 2005). In the present study, glucan treated cells exhibited an up-regulation of caspase-9 and caspase-3 in NCI-H460 cell lines. Highest level of mRNA expression for caspase-9 was exhibited by YG20 (2.54 fold) followed by YG100 (2.33 fold) and YG23 (2 fold) treatment. Glucan treatment also induced the expression of caspase-3 with a 1.79 fold increase by YG100 followed by YG23 (1.02 fold) and YG20 (0.85 fold). Exposure of melanin also could enhance the expression of caspase-9 and caspase-3 by 0.43 and 0. 41 fold. When compared to glucans, the effect of melanin on caspase activity is low which means lesser effect of apoptosis in NCI-H460 cells. It has been reported that polysaccharide extract from *Ganoderma lucidum* induced apoptosis by activating caspase-3 (Zhao et al., 2011). Hasan et al. (2011) reported the molecular mechanism of the extract from root and bark of *Juglans regia* (RBJR) on MDA-MB-231 human breast cancer cells by real time PCR. In their study, tested caspases-3 and caspase-8 gene transcripts were significantly up-regulated and demonstrated the induced apoptosis. Gene expression studies of *Origanum dayi* and *Ochradenus baccatus* on HepG2 cells also indicated an up-regulation of caspase-9, caspase-3 and caspase-7 and suggested the mechanisms involving mitochondria dependent apoptotic pathway (Bhatia et al., 2015). The mitochondria dependent apoptotic pathway has been involved in the

function of a majority of anticancer drugs. In the present study also presence of mRNA transcript of apoptotic related caspases-3 and caspase-9 indicates that mitochondrial release of cytochrome-c has taken place and thereby intrinsic apoptotic pathway has been activated. The tested marine yeast glucans have proved the potential apoptotic effect in NCI-H460 cells which implicates its anticancer potential.

Apart from caspase proteases, several other types of non caspase proteases may also play a role in the execution of apoptosis which include calpains and cathepsins (Johnson, 2000). Activated calpains degrade membrane, cytoplasmic and nuclear substrates, leading to the breakdown of cellular architecture and finally apoptosis. Calpain-5 is a non-classical member of the calpain family (Dear et al., 1997). In the present study, the three tested glucans significantly up-regulated the calpain-5 expression in NCI-H460 cells by a 4.42 fold increase in YG23 treated cells followed by YG100 and YG20. Melanin R23 up-regulated the expression of calpain-5 by 0.36 fold which was lesser compared to the effect of glucan. Investigation by Sareen et al. (2007) observed that calpain activity is significantly higher in resveratrol (RES) treated breast cancer cells and indicated that calpain is one of the key mediators of resveratrol-induced apoptosis in breast cancer. The importance of calpains in apoptosis has been demonstrated using number of calpain inhibitors (Squier et al., 1994; Wolf et al., 1999). The activation of calpain during apoptosis suggests an involvement of this non caspase protease in apoptosis execution process. In the present study, both the tested compounds glucan and melanin could up-regulate the expression of calpain-5 which was more in glucan treated cells indicating the apoptotic effect of glucans.

Earlier studies have proved the role of cathepsins with apoptosis. Cathepsin G is a serine protease of cathepsin family which includes the other

cathepsins, cathepsin A (serine protease), cathepsins D and E (aspartate proteases) and cathepsins B, C, H, K, L, S and T (cysteine proteases) (Johnson, 2000; Tan et al., 2013). It has been reported that in various models cathepsin D mediates apoptosis and the injection of cathepsin D induces caspase dependent apoptosis of fibroblasts (Roberg et al., 2002; Bidere et al., 2003). It was noticed that cathepsin G found in NB4 cells, cleaves human brm (homologue of the *Drosophila melanogaster* brahma and *Saccharomyces cerevisiae* SNF proteins) in a pattern similar to that observed *in vivo* during apoptosis (Biggs et al., 2001). Droga-Mazovec et al. (2008) proposed that degradation of anti-apoptotic Bcl-2 family members by lysosomal cathepsins synergizes with cathepsin mediated activation of pro-apoptotic Bcl-2 family member Bid to trigger a mitochondrial pathway to apoptosis and suggesting the role of lysosomal cathepsins in the stimulation of apoptosis. Three marine yeast glucans in the present study exhibited an up-regulation of cathepsin G with highest expression in YG23 treated cells (9.08 fold), followed by YG20 (6.11 fold), YG100 (4.87). Similarly, melanin R23 exhibited an enhanced up-regulation of 1.92 fold in NCI-H460 cells. Earlier it has been reported that cathepsin G, which is copiously expressed in neutrophils, can cleave and activate procaspase-7 *in vitro* (Zhou and Salvesen, 1997). These results illustrate the role of cathepsins in apoptosis, which is also noticeable with the present *in vitro* gene expression analysis using marine yeast glucan and melanin with an elevated expression of cathepsin-G and hence the potential role in cancer regulation by apoptosis.

p53 is an important tumour suppressor gene and its mutations are the most frequent genetic changes noticed in different types of human cancers. This p53 tumor suppressor turns to incorporate multiple stress signals into a series of diverse antiproliferative responses. One of the most important functions of p53 is its ability to activate apoptosis, and the disruption of this process can promote

tumor progression and chemoresistance (Fridman and Lowe, 2003). p53 protein prevents the proliferation of abnormal cells and thus inhibiting neoplastic development. The results of the present study shows an induction of p53 expression by marine yeast glucan extracts in treated cancer cells than untreated cells. This may be due to the possible anticancer effects of marine yeast glucan extract in the lung cancer cell line.YG100 treated cells exhibited 2.11 fold increases in the gene transcripts of p53, followed by YG20 (1.98 fold) and YG23 (1.45 fold). Melanin treated cells exhibited an induction of p53 with 0.39 fold increase in gene transcript. However, it could be noticed that marine yeast glucan is better apoptosis inducing agent than marine yeast melanin. In one of the recent study, the effect of gum methanol extract of *Boswellia thurifera* on the viability and p53 gene expression on cultured breast cancer cells was evaluated. The extract exhibited an induced p53 gene transcription and thereby suggests possible anticancer effect and toxicity in the cultured breast cancer cell line (Yazdanpanahia et al., 2014). Zhao et al. (2011) reported an up-regulation of p53 on ovarian cancer cells after the polysaccharide treatment from *Ganoderma lucidum* and suggest possible antitumor effect; this is also in validation with the present findings with an up-regulation of p53 upon marine yeast glucan treatment.

The protein encoded by RB1 gene is a negative regulator of the cell cycle and was the first tumor suppressor gene found. The protein pRb acts as a tumor suppressor protein, which means that it regulates cell growth and keeps cells from dividing too fast or in an uncontrolled way. pRb is a key component of the cyclin dependent kinase cell cycle pathway, responsible for cell division arrest at the G1/S checkpoint (Weinberg, 1995). Lower survival of patients with various types of lung cancer was correlated with the deficiency of Rb activity (Husgafvel-Pursiainen et al., 2000; Osada and Takahashi, 2002). In the present study

considerable up-regulation of Rb1 could be noticed in all the three marine yeast glucan treated cells with a highest expression in YG100 (4.91 fold), followed by YG23 (4.61 fold) and YG20 (3.81 fold). Melanin treated cells exhibited 0.79 fold increase in Rb gene transcript which is lower than the expression of glucan treated cells. One of the previous study using *Sargassum confusum* polysaccharide (SP) against S180 sarcoma *in vivo* exhibited tumor growth inhibitory effect with an increase in the expression levels of Rb and p53 genes at both the mRNA and protein levels (Liu and Meng, 2008). Similarly, various cytotoxic fractions of plant extracts on MCF-7 human breast cancer cell lines exhibited an up-regulation of Rb and p53 suggesting its modulatory effect (Aderonke et al., 2013). The present results also suggest the potential effect of marine yeast glucans and melanin in cancer cells and their positive impact on lung cancer cells.

The mitogen-activated protein kinases (MAPKs) are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide array of stimuli, including stress (Johnson and Lapadat, 2002; Werlen et al., 2003). MAPK family consist of three family members which includes the extracellular signal-regulated kinase (ERK), the c-Jun NH2-terminal kinase (JNK) and the p38-MAPK, in addition each family member has its own subfamilies. Upon stimulation, MAPKs phosphorylate their specific substrates and this can lead to a positive or negative regulation of substrates. Therefore, the MAPK signaling pathways modulate various cellular activities which includes gene expression, mitosis, proliferation, motility, metabolism, differentiation and apoptosis (Davis, 2000; Johnson and Lapadat, 2002; Werlen et al., 2003).

In the present study MAPK-1 expression was up-regulated with a 3.14 fold increase in YG100 treated cells followed by YG23 (2.30 fold) and YG20 (1.88 fold). R23 melanin treatment enhanced the expression of MAPK-1 with 0.60 fold increase in NCI-H460 cell line. Anticancer effects of flavagline

derivative (FL3) on human Oct4-expressing cancer stem-like cells (CSCs) have been reported via a p38 MAPK-dependent caspase-3-dependent pathway. FL3 treatment specifically triggered apoptosis in association with an induction of the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and caspase-3 activation in Oct4-expressing cancer stem cells (Emhemmed et al., 2014). One of the previous studies indicated that the combination of berberine (plant extract) and gamma-radiation enhanced the anticancer effects through the p38 MAPK pathway and ROS generation in the human hepatoma HepG2 cells (Hur et al., 2009). In a recent study, it has been reported that *G. lucidum* whisky extract can inhibit tumor proliferation and promote antitumor response through JAK/STAT and MAPK signaling pathways (Kao et al., 2016). The anticancer effect of Epigallocatechin-3-gallate (EGCG) on migration, invasion, and apoptosis has been reported by the activation of MAPKs activity (Min and Kwon, 2014). However in the present study, marine yeast glucan and melanin up-regulated the expression of MAPK-1 in the lung cancer cells and this may led to the phosphorylation of the substrates involved in various signaling pathways and may also result in the enhancement of anticancer effect of test compounds, glucan and melanin.

JNK (c-Jun N-terminal protein kinase) is a subfamily of the mitogen activated protein kinase (MAPK) superfamily (Hibi et al., 1993). They regulate many key biological processes, such as cellular proliferation and death, which function abnormally in cancer (Gozdecka et al., 2014). Evidence shows that JNK can function as a pro-apoptotic kinase (Lin, 2003). In mouse models of prostate and breast cancer, JNK exhibits potent tumor-suppressive properties (Cellurale et al., 2010; Hubner et al., 2012). From the present study it could be noticed that marine yeast glucan could enhance the expression of JNK in treated cells by an up-regulation of 2.74 fold in YG23 treated cells, followed by YG100

(2.35 fold) and YG20 (1.07 fold). Whereas, R23 melanin exhibited an up-regulation of 0.68 fold in treated cells. This is a positive indication that marine yeast glucan and melanin have potential role in cancer regulation by up-regulating JNK gene expression. Moreover, it has been reported that active JNK causes the release of apoptogenic factors, such as cytochrome c and SMAC (second mitochondria-derived activator of caspases)/Diablo from isolated mitochondria in a cell-free system (Aoki et al., 2002; Chauhan et al., 2003). These results point out that the activation of JNK directly regulates mitochondria-dependent apoptosis in a pro-apoptotic direction. Apoptosis induction associated with the stress response of endoplasmic reticulum through up-regulation of JNK in HeLa cells by gambogic acid extracted from *Garcinia hanburyi* was also reported (Krajarng et al., 2015).

AKT1 is a key intermediate of the AKT kinase signaling pathways. Activated AKT is a well-established survival factor, exerting antiapoptotic activity to a certain extent by preventing the release of cytochrome c from the mitochondria (Whang et al., 2004). AKT also phosphorylates and inactivates the pro-apoptotic factors BAD and procaspase-9 (Downward, 2004). Furthermore, AKT phosphorylates and inactivates the FOXO transcription factors, which mediate the expression of genes critical for apoptosis, such as the Fas ligand gene. Earlier reviews documented that frequent hyperactivation of AKT kinases or abnormal AKT signaling contributes to a wide variety of human solid tumors and hematological malignancies (Luo et al., 2003; Bellacosa et al., 2005). In the present study, down-regulation of Akt1 could be noticed in the marine yeast glucan treated cells. The highest down-regulation was exhibited in the YG23 treated cells, followed by YG20 and YG100. Similarly, R23 melanin also could down-regulate the expression of Akt1. It has been reported that several chemotherapeutic agents were found to prevent cancer cell growth and induce

apoptosis through the inhibition of the Akt pathway (Aggarwal and Shishodia, 2006). Genistein which is a flavonoid compound potentiates the anticancer effects of gemcitabine in human osteosarcoma via the down-regulation of Akt and nuclear factor-κB pathway (Liang et al., 2012). A down-regulation of Akt expression has been noticed on ovarian cancer cells treated with polysaccharide, which was reported for its antitumor effect (Zhao et al., 2011). Similarly, the present finding also exhibited a down-regulation of Akt1 by means of glucan and melanin exposure on NCI-H460 cell lines indicating a positive effect in cancer regulation.

Cytokines are molecular messengers that allow the cells of the immune system to communicate with one another to produce a coordinated, robust, but self-limited response to a target antigen. The cytokines are critical for tumor immunosurveillance and have validated therapeutic antitumor activity in murine models and in the clinical treatment of a number of human cancers (Lee and Margolin, 2011). Number of studies have shown that polysaccharides, especially β-D glucan, can modulate the functions of many components of the immune system, such as antigen-presenting cells, T and B lymphocytes, NK cells, neutrophil granulocytes, dendritic cells and cytokine production (Berovic et al., 2003; Chen et al., 2004). In the present study, cytokines tested i.e., the interleukins (IL) such as IL-1β, IL-2, IL-6, IL-10 and IL-12 have been up-regulated considerably in the glucan treated cells. Similarly, an up-regulation of interferons (IFN) such as IFN-β and IFN-γ and also tumor necrosis factor (TNF) TNF-α have been noticed. TNF-α is an activator of extrinsic pathway of apoptosis (Wang et al., 2009). Among the tested glucans, highest level of expression was exhibited by YG23 treated cells followed by YG100 and YG20. The highest fold increase of immune genes on NCI-H460 by YG23 were IL-2 (8.36), IL-10 (8.26), IL-12 (5.30), IFN-β (8.11), IFN-γ (8.29) and TNF-α (7.13) and by YG100 on IL-1β

(4.28) and IL-6 (2.68). These are consistent with the previous reports, that fungal cell wall polysaccharides have potential immunomodulatory function. Habijanic et al. (2015) noticed the immunomodulating role of fungal cell polysaccharide extracts from *Ganoderma lucidum*. The polysaccharide extracts induced the production of TNF-α, IFN-γ, IL-2, IL-4, IL-10, IL-12 and IL-17 in the human peripheral blood mononuclear cells (PBMC). The crude *G. lucidum* water-extract also induced the expression of cytokines, including IL-10 and TNF-α, IL-1β, IL-6 and IL-2 in human PBMC (Mao et al., 1999). These reports are in validation with the present findings that the marine yeast glucan also has the potential to induce the expression of various cytokines which are involved in modulation of the immune system.

In another study, β-glucans demonstrated the highest induction of cytokines TNF-α, IL-6 and IFN- γ that is higher than the capacity of a synthetic immunostimulant romurtide used in a supporting therapy in cancer patients treated with radiotherapy (Berovic et al., 2003). These cytokines are known as the proinflammatory cytokines and here in the present study also the up-regulation of proinflammatory cytokines have been noticed after treatment with β-glucans. The release of proinflammatory cytokines is essential for host survival from infection, and is also necessary for the repair of tissue injury (Kuo et al., 2006). From these studies it is evident that the tested polysaccharide glucan from marine yeasts also have the potential on modulating the induction of various cytokines and thereby an impact on the host cells.

Interferon (IFN) response is the first line of defense against viral infection, which triggers the induction of a broad array of antiviral proteins. IFN-inducible proteins, such as GTPase Mx1 (myxovirus resistance 1), ISG15 (IFN-stimulated protein of 15 kDa), Viperin (*v*irus *i*nhibitory *p*rotein, *e*ndoplasmic *r*eticulum-associated, *in*terferon-inducible) and IFIT (IFN-induced proteins with

tetratricopeptide repeats), have been functionally well characterized as antiviral effectors (Daffis et al., 2010; Pichlmair et al., 2011; Sadler and Williams, 2008). Recent studies have shown that these genes are mainly responsible for antiviral response to a large variety of viral infections (Lin et al., 2013; Zhang et al., 2013; Lin et al., 2015; Zhong et al., 2015). In the present study, a down-regulation of viperin and ISG-15 could be noticed in all the three glucan and R23 melanin treated cells, whereas an up-regulation of MX-1 and IFITM3 also has been noticed. However, much more work is required to clarify the multiple functions associated with these enigmatic molecules in relation with cancer.

Various *in vitro* studies have shown that β-glucan from different sources exhibit direct cytotoxic effects on cancer cells besides their modulation of immune system for the antitumour mechanism. The effect of β-glucan and β-glucan containing extracts from various mushrooms have proved their anticancer potential on various cell lines which includes the suppressed cellular proliferation (Martin and Brophy, 2010); reduced cell viability and induced G2/M cell cycle phase arrest (Aleem, 2011); reduction in proliferation and induction of apoptosis (Zhang et al., 2006) in MCF-7 breast cancer cells; inhibited cell proliferation and induced apoptosis in human leukemia HL-60 and U-937 cells (Hsieh et al., 2006); induced apoptosis with an up-regulation of caspase-3 in HT-29 colon cancer cells (Lee et al., 2009); induced G0/G1 arrest and apoptosis in human hepatoma HepG2 cells (Youn et al., 2008). These reports further supports the present findings related to the effect of β-glucan extracted from three marine yeasts and their potential effects in cancer regulation.

Pigments produced by microbes exhibit potential health benefits and play important role in various industrial sectors (Soliev et al., 2011; Malik et al., 2012; Gulani et al., 2012). Anticancer activity of various pigments have been

discovered by previous experimental studies (Kapadia and Rao, 2012; Karuppiah et al., 2013; Prashanthi et al., 2015). Marine microbes are also important candidates for pigment production and also have proved their potential growth inhibitory effects (Karuppiah et al., 2013). Recently, two marine carotenoids siphonaxanthin and fucoxanthin have ascertained their potent growth inhibitory and apoptosis inducing effect in HL-60 leukemia cells (Ganesan et al., 2011). The potency of each compounds may vary depending upon the cancer cell types/lines and the growth rate of the cells. In the present study, melanin pigment from marine yeast *H. werneckii* exhibited an up-regulation of apoptosis and tumour suppresor related genes; a noticeable effect could not be detected except the expression of cathepsin G in lung cancer cells. Most of the analyzed genes involved in apoptosis and tumor suppression was found to be meagerly expressed upon melanin treatment when compared to glucan exposure on NCI-H460 cell lines. Whereas a noticeable increase in gene transcript of interleukins such as IL-1β (3.40 fold), IL-6 (1.14 fold), IL-12 (1.27 fold) and also an up-regulation of IFN-γ (2.40 fold), IFN-β (1.66 fold) and TNF-α (1.50 fold) could be noticed when the cells were exposed to melanin. This finding indicates the possibility of melanin pigment in the immunomodulation effects in the lung cancer cells.

Riby et al. (2006) reported that 3, 3'-Diindolylmethane (DIM) up-regulates the expression of IFN-γ in human MCF-7 breast cancer cells. It has been suggested that this novel effect may contribute to the anticancer effects of DIM since IFN-γ plays an important role in preventing the development of primary and transplanted tumors and also the effectiveness of dietary indole against various tumor types. The indole content of melanin extracted from strains of *H. werneckii* was found to be high (81-90%) (Kutty et al., 2013b) and this may further confirms the up-regulation of IFN-γ upon melanin treatment and thereby a positive effect on lung cancer cells.

From the present study, it could be noticed that genes related to apoptosis and tumor suppression were positively regulated by marine yeast glucans in the lung cancer cells. It has been reported that apoptosis and p53 pathways are the targets of herbal-derived anti-lung cancer drugs (Ho et al., 2013). The cytokine pathways were modulated and thereby an immunological influence could be observed in the glucan treated cancer cells. Cytokines that are released in response to infection, inflammation and immunity can function to inhibit tumor development and progression (Dranoff, 2004). To a certain extent, melanin also could enhance the expression of immune related genes in cancer cells and these confirm the possibility of marine yeast pigment melanin in anticancer.

It is challenging to infer the pharmacological effect of a component using *in vitro* experiments alone. Although it is a pilot study, this report may help to investigate the bioactive compounds from marine yeast for their anticancer effects. Though some reports are available on anticancer effects of marine microbes, the marine environment is still a potential source for exploring new yeasts that can produce novel bioactive compounds with possible application in the industrial and pharmaceutical sector. Marine yeast glucans can certainly open new perspectives as anticancer agents against lung cancer. To conclude, these findings proved that marine yeast β-glucan exhibit potential anticancer effects on lung cancer cells by the modulation in expression of various genes involved in cancer regulation. The study further concludes that the yeast melanin also exerts immunomodulation effect on lung cancer cells. Although marine yeast glucan and to some extent the melanin promotes anticancer effects and immunomodulation effects by multiple mechanisms, further studies are needed to define the function of these compounds. Taken together, it has been proved that marine yeast is a promising source for various bioactive compounds with potential applications.

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Chapter 6

Summary and Conclusion

The biotechnological prospective of yeasts, including their use in food, fermentation and pharmaceutical industries is noteworthy. In comparison with terrestrial yeasts, the investigation on marine yeasts for the bioactive compounds is still at its early stage. However, some of the enzymatically and pharmaceutically interesting products such as amylases, inulinase, astaxanthin, siderophore and riboflavin have been investigated from marine yeasts. It is anticipated that exploitation of this fascinating microbial community will certainly provide space for unique bioactive compounds with potential applications in various industrial sectors with an environment friendly perspective.

The present work was focused on the extraction, characterization and evaluation of the bioactive potential of marine yeast isolates from Arabian Sea and Bay of Bengal. Marine yeasts already isolated from continental shelf and slope sediments of Arabian Sea and Bay of Bengal and maintained in the Microbiology Laboratory of School of Marine Sciences, CUSAT were used for the present study. Glucans were extracted from the marine yeasts and characterized by physicochemical analysis. Biocompatibility of the marine yeast glucans were assessed by XTT assay. Primary screening of marine yeast glucans as an immmunostimulant in *Penaeus monodon* postlarvae against WSSV challenge was carried out by analyzing the expression of various AMPs and immune related genes by semi-quantitative RT-PCR gene expression analysis. Post-challenge survival was also assessed. Evaluation of

selected glucans as an immmunostimulant in adult black tiger shrimp *P. monodon* against WSSV infection were carried out by analyzing the expression of various biodefense genes by quantitative real-time PCR gene expression analysis. The antioxidant and photoprotectant role of melanin was evaluated. Biocompatibility of yeast melanin was tested in HEp-2 cell lines by XTT and SRB assays. Anticancer effect of marine yeast glucans and melanin were tested in NCI-H460 cell lines by analyzing the gene expression pattern of various cancer related genes using quantitative real-time PCR.

The salient findings of the study are as follows:

- ✓ Eight marine yeasts viz., *Candida parapsilosis* R20, *Hortaea werneckii* R23, *Candida spencermartinsiae* R28, *Candida haemulonii* R63, *Candida oceani* R89, *Debaryomyces fabryi* R100, *Debaryomyces nepalensis* R305 and *Meyerozyma guilliermondii* R340 were used for the study. Glucans and melanin extracted from these marine yeasts were subjected to various assays for testing the bioactive potential.
- ✓ The yield of glucans extracted from various marine yeasts ranged from 12.5 to 27.55 % on dry weight basis (w/w).
- ✓ Glucans extracted from the marine yeasts were characterized and reported as (1-3, 1-6)-β-D-glucan by 1D NMR.
- ✓ Six of the marine yeast glucans were found to be linear and one glucan was found to be cyclic.
- ✓ Side chain length and branching frequency varied for each glucan depending upon the yeast species.
- ✓ Cytotoxicity assay (XTT) of the eight glucans revealed its biocompatibility with eukaryotic cells.

- ✓ Present study identified the immunostimulant potential of eight marine yeast glucans in *P. monodon* through a semi-quantitative PCR. *P. monodon* post larvae exhibited above 40% survival when fed various glucan incorporated diets and challenged with WSSV.
- ✓ Expression profile of WSSV related genes indicated the protective effect of marine yeast glucans in the experimental animals post-challenge.
- ✓ Three marine yeast glucans viz., YG20 from *Candida parapsilosis* R20, YG23 from *Hortaea werneckii* R23 and YG100 from *Debaryomyces fabryi* R100 were segregated based on their immunostimulant potential and percentage of survival in *P. monodon* post larvae.
- ✓ Immunostimulant potential of the selected yeast glucans (YG20, YG23 and YG100) were confirmed through immune gene expression profiling of glucan fed adult *P. monodon* by quantitative real-time PCR pre- and post-challenge WSSV.
- ✓ Characterization of the selected glucans by 2D COSY NMR study confirmed that the R20 marine yeast glucan is linear (1-3, 1-6)-β-D-glucan and R100 marine yeast glucan is cyclic (1-3, 1-6)-β-D-glucan, based on cross peaks.
- ✓ Assignments from the COSY spectrum helped to accomplish the linear versus cyclic structural analysis.
- ✓ The (1-6)-linked branching frequency was more and side chain length was shorter in R100 than for R20.
- ✓ The (1,3/1,6)-β-D-glucans exhibited above 40% survival in adult *P. monodon* upon WSSV challenge with the highest survival for cyclic glucan YG100 (60.41%) followed by YG20 (54.83%) and YG23 (46.87%).

- ✓ WSSV gene (VP28) expression profile in *P. monodon* post-challenge indicated the protective effect of marine yeast glucans.
- ✓ Melanin was extracted from marine black yeast, *Hortaea werneckii* R23 and the yield was 0.012 g dry weight/g wet weight.
- ✓ NMR spectra of melanin exhibited peaks in the aromatic and aliphatic region which resemble the spectra of synthetic melanin.
- ✓ FTIR spectra of melanin showed the presence of peaks at 3283.34 (OH and NH2 Groups), 2919.08 (aliphatic C-H group), 1631.3 (aromatic C=C or C=O groups), 1532.48 (NH bending) and 1456.46 cm^{-1} (aliphatic C-H group) characteristic of melanin pigment.
- ✓ Melanin from the black yeast, *Hortaea werneckii* R23 exhibited a defined crystal structure on SEM imaging similar to natural sepia melanin (defined structural order), while the synthetic melanin was amorphous.
- ✓ The surface elemental composition of R23 melanin showed 6 elements with an abundance of carbon followed by chlorine, oxygen and sodium. Trace amount of sulphur and phosphorous were also observed.
- ✓ ICP-AES revealed that the most abundant element in R23 melanin is sodium followed by calcium, magnesium, potassium and iron.
- ✓ CHNS analysis revealed that C:H and C:N ratio of R23 melanin is more similar to sepia melanin than synthetic melanin.
- ✓ Melanin exhibited significant antioxidant activity as shown by ABTS (IC_{50} = 9.76±4.104 µg ml^{-1}), DPPH (IC_{50} =28.11±10.75 µg ml^{-1}) radical scavenging and lipid peroxidation inhibition (IC_{50} =222.56±43.49 µg ml^{-1}) assays.

- ✓ In the present study, IC_{50} of R23 melanin (9.76 ± 4.104 µg ml^{-1}) on the ABTS radicals was 9 times higher than the standard Trolox (1.042± 0.063 µg ml^{-1}). The activity can be increased further on purification.
- ✓ The biocompatibility/non-toxicity of R23 melanin tested by standard assays proved that melanin was non-toxic to HEp2 cells in cultures up to a concentration of 200 µg ml^{-1}.
- ✓ R23 melanin at 50 µg ml^{-1} could scavenge 51.05% reactive oxygen species of HEp-2 cells generated on UV exposure, which showed a greater scavenging effect than the positive control synthetic melanin at 50 µg ml^{-1}.
- ✓ Melanin extracted from marine black yeast *H. werneckii* R23 revealed to be a promising antioxidant and photoprotectant which on further purification may possibly find wide pharmaceutical and industrial applications.
- ✓ Anticancer effect of marine yeast glucan and melanin were assessed through *in vitro* gene expression analysis on NCI-H460 lung cancer cell lines.
- ✓ Real-time quantitative RT-PCR analysis showed that cancer related genes were differentially expressed in response to various marine yeast glucans and melanin.
- ✓ Genes related to apoptosis and tumor suppression was positively regulated by marine yeast glucans in the lung cancer cells.
- ✓ The present study also proved the immunomodulation property of glucan in the treated cells.
- ✓ The apoptotic and tumour suppression genes were meagerly expressed upon marine yeast melanin treatment in the lung cancer cells.

- ✓ Marine yeast melanin could enhance the expression of the immune related genes in cancer cells.
- ✓ The expression of cell cycle associated and viral associated immune genes were also influenced by glucan and melanin treatment.
- ✓ Compared to marine yeast melanin, marine yeast glucans exhibited better anticancer and immunomodulation gene expression on lung cancer cells.
- ✓ These findings proved that marine yeast (1-3, 1-6)-β-D-glucan exhibit potential anticancer effects on lung cancer cells by the modulation in expression of various genes involved in cancer regulation.
- ✓ The present study proved that marine yeast is a promising source for bioactive glucan and melanin with potential applications such as immunostimulant, antioxidant, photoprotectant and anticancer agent.

The study proved that (1-3, 1-6)-β-D-glucan act as an immunostimulant both in *P. monodon* post larvae and adult, and confer protection against WSSV infection. Application of glucans would be highly helpful to the shrimp farmers to increase the immunocompetence of shrimps and prevent or minimise the devastating effects of diseases. Melanin extracted from marine black yeast *H. werneckii* R23 was proved to be a promising antioxidant and photoprotectant. Anticancer analysis of marine yeast compounds glucan and melanin revealed their potential anticancer and immunomodulation effect on lung cancer cells. Biocompatibility assay of both the compounds glucan and melanin proved them to be safe to eukaryotic cells. To conclude, the present study explored the bioactive potential of marine yeasts as a promising source for bioactive molecules, especially glucan and melanin, for application in aquaculture, pharmaceutical and cosmetic industries in an environment friendly perspective.

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Appendix I - GenBank Submissions

1. GenBank accession number **KJ937964.** 2014. **Wilson, W.,** Antony, S. P., Philip, R and Bright Singh, I. S. White spot syndrome virus clone w-tk1 thymidine kinase gene, partial cds.
2. GenBank accession number **KJ937965.** 2014. **Wilson, W.,** Antony, S. P., Philip, R and Bright Singh, I. S. White spot syndrome virus clone w-pk1 protein kinase gene, partial cds.
3. GenBank accession number **KJ937966.** 2014. **Wilson, W.,** Antony, S. P., Philip, R and Bright Singh, I. S. White spot syndrome virus clone w-lr1 putative latency-related protein gene, partial cds.
4. GenBank accession number **KJ937967.** 2014. **Wilson, W.,** Antony, S. P., Philip, R and Bright Singh, I. S. White spot syndrome virus clone w-en1 endonuclease gene, partial cds.

Appendix II · List of Publications

International:

1. **Wilsy Wilson**, Douglas Lowman, Swapna P. Antony, Jayesh Puthumana, I.S. Bright Singh, Rosamma Philip. 2015. Immune gene expression profile of *Penaeus monodon* in response to marine yeast glucan application and white spot syndrome virus challenge. **Fish & Shellfish Immunology**. 43: 346-356.
2. Rosamma Philip, Afsal V.V, Swapna P. Antony, Sruthy K.S, **Wilsy Wilson**, Divya T. Babu, Jayesh P, I.S Bright Singh. 2016. Antimicrobial peptides in crustaceans: Molecular and functional characterization. **Fish & Shellfish Immunology**. 53: 59-60.