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Immune gene expression profile of *Penaeus monodon* in response to marine yeast glucan application and white spot syndrome virus challenge

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ABSTRACT

Immunostimulant potential of eight marine yeast glucans (YG) from *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 tested against WSSV challenge in *Penaeus monodon* post larvae (PL). Structural characterization of these marine yeast glucans by proton nuclear magnetic resonance (NMR) indicated structures containing (1-6)-branched (1-3)-β-D-glucan. PL were fed 0.2% glucan incorporated diet once in seven days for a period of 45 days and the animals were challenged with white spot syndrome virus (WSSV). The immunostimulatory activity of yeast glucans were assessed pre- and post-challenge WSSV by analysing the expression profile of six antimicrobial peptide (AMP) genes *viz*., anti-lipopolysaccharide factor (ALF), crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5 and 13 immune genes *viz*., alpha-2-macroglobulin (α-2-M), astakine, caspase, catalase, glutathione peroxidase, glutathione-s-transferase, haemocyanin, peroxinectin, pmCathepsinC, prophenol oxidase (proPO), Rab-7, superoxide dismutase and transglutaminase. Expression of seven WSSV genes *viz*., DNA polymerase, endonuclease, protein kinase, immediate early gene, latency related gene, thymidine kinase and VP28 were also analysed to detect the presence and intensity of viral infection in the experimental animals post-challenge. The study revealed that yeast glucans (YG) do possess immunostimulatory activity against WSSV and also supported higher survival (40–70 %) post-challenge WSSV. Among the various glucans tested, YG23 showed maximum survival (70.27%), followed by YG20 (66.66%), YG28 (60.97%), YG89 (58.53%), YG100 (54.05%), YG63 (48.64%), YG305 (45.7%) and YG340 (43.24%).

1. Introduction

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 [1,2]. *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 [3]. 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 [4–8]; chitin [9]; mannoproteins [10]; peptidoglycans [11]

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and lipopolysaccharides [12] known as biological response modifiers (BRMs) [13,14] have been proved to enhance immune responses in organisms, rendering higher survival rate and resistance to infections. Among these, 1, 3 β D–glucan of yeast origin is the most active compound [15].

β-Glucans are homopolysaccharides of glucose, present in the yeast cell wall (~50–60 %) and are potent stimulators of non-specific defense mechanisms in animals. Marine yeasts have been proved as an effective immunostimulant than Baker's yeast in *Fenneropenaeus indicus* [16]. Earlier researchers have showed the potent immunostimulatory properties of yeasts and glucans viz. β-glucan-incorporated feed [17]; alkali insoluble (1 → 3)-β-D-glucans [18]; β-Glucan extracted from *Candida sake* [7]; β-mercapto -ethanol- treated yeast [19] and glucan from a marine yeast *Candida tropicalis* [8].

The non-self-agent glucan, are known to invoke various *in vivo* responses in animals, such as change in haemocyte counts [20], induction of encapsulation [21], pro-phenoloxidase activity and melanisation [22] besides generating a range of immunoactive agents including peroxinectin and reactive oxygen species [23]. Subramanian and Philip [24] noted higher post-challenge survival and lower oxidative damage along with an elevation in antioxidant and immune parameters in 0.2% marine yeast glucan fed *F. indicus* on challenge with WSSV. Soonthornchai et al. [25] noted the elevated expression of immune genes (AMPs such as ALF, crustin and penaeidins) in *P. monodon* juveniles after challenge with *Vibrio harveyi*. Babu et al. [26] reported the up-regulation of immune and antimicrobial peptide (AMP) genes on administration of marine yeast *Candida aquaetextoris* incorporated diet followed by WSSV challenge.

The present study is focused on the characterization of various marine yeast glucans and their immunostimulatory potential in *P. monodon* post larvae conferring protection against WSSV challenge.

2. Materials and methods

2.1. Microorganisms used for glucan extraction

Eight marine yeasts (*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) 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 present work. These cultures were isolated from the sediment samples collected from Arabian Sea and Bay of Bengal.

2.2. Glucan extraction

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. [27] 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, resuspended in 3% NaOH and the procedure was repeated (2x). The residue was extracted using 0.5 N acetic acid at 75 °C for 6 h and separated by centrifugation. 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 and dried in vacuum for 48 h at RT and the final product (glucan) was used for the study.

2.3. Structural characterization of the cell wall glucan

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 were the following: 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 [28].

2.4. Determination of biocompatibility and IC_{50}

To determine the biocompatibility, XTT (2, 3-bis [2-methyloxy-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. [29] 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 minimal essential medium (MEM) 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 (0.05–6.4 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

Table 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