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Immunostimulatory effect of a marine yeast *Candida sake* S165 in *Fenneropenaeus indicus*

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Abstract

The efficacy of a marine yeast *Candida sake* as source of immunostimulant to Indian white shrimp *Fenneropenaeus indicus* was estimated. Biomass of *C. sake* was prepared using malt extract agar and incorporated at graded levels into a standard diet to prepare yeast diets of varying biomass concentrations (1%, 10% and 20%). *F. indicus* were fed on these diets for a period of 28 days and challenged orally with white spot syndrome virus (WSSV) and immune parameters such as total haemocyte count, phenoloxidase and nitroblue tetrazolium reduction (NBT) were determined. Ten per cent *C. sake* in the diet was found to support an optimum immune response in the animals in general and their enhancement could be observed on the second and third day following challenge with the virus. The study has demonstrated that marine yeast *C. sake* at 10% in diet (w/w) may be used as an effective source of immunostimulants in *F. indicus*.

Keywords: *Candida sake*; *Fenneropenaeus indicus*; Immunostimulants; White spot syndrome virus; Total haemocyte count; Phenoloxidase; Superoxide anion; Survival

1. Introduction

The immune system of crustaceans is mainly non-specific and relies on phagocytosis, encapsulation and agglutination alongside the phenoloxidase-mediated production of melanin through the pro-phenoloxidase cascade (Smith and Soderhall, 1983). They have three distinct types of haemocytes, i.e. hyaline cells, semi-granular cells and granular cells, each with distinct morphological features and physiological functions (Johansson et al., 2000). The β-1,3-glucans of certain fungi and yeasts have been successfully used as immunostimulants to enhance the defence potential of fish and shellfish against bacterial and viral infection (Oliver et al., 1986; Robertsen et al., 1990; Sung et al., 1994; Song et al., 1997; Chang et al., 2000, 2003). Sung et al. (1994) reported an enhanced resistance to vibriosis in *P. monodon* postlarvae administered with β-glucan. Increased post-challenge survival was observed in glucan-fed *P. monodon* to WSSV (Song et al., 1997). Chang et al. (2003) showed that oral administration of β-glucan at an optimal level of 10 g kg^{-1} diet for 20 days effectively enhanced the immune system, resulting in improved survival against WSSV infection in *P. monodon*.

However, only a few studies have been reported on the use of yeast as a source of immunostimulants in penaeids and investigations have not been conducted with marine yeasts. Scholz et al. (1999) compared the efficacy of five

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different yeast-supplemented diets in shrimp and reported that *Phaffia rhodozyma* incorporated into the feed gave better performance in terms of bacterial clearance and increased phenoloxidase activity in haemolymph. Recently Burgents et al. (2004) reported enhanced disease resistance in the pacific white shrimp *Litopenaeus vannamei* against experimental *Vibrio* infection when fed with *Saccharomyces cerevisiae* supplemented feed.

In the present study, the immunostimulatory effect of a marine isolate of *Candida sake* S165 was examined in *Fenneropenaeus indicus.* Total haemocyte count, phenoloxidase activity and superoxide anions (O_2^-) were measured and a challenge test was carried out using white spot syndrome virus.

2. Materials and methods

2.1. Yeast strain

C. sake S165 was isolated from coastal waters off Cochin and maintained in the Microbiology laboratory of the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences. Selection of this isolate was based on the preliminary tests with *F. indicus* that showed growth increments and improved survival upon WSSV challenge for postlarvae fed on a diet supplemented with seven marine yeasts (Sajeevan et al., 2003). A lawn of *C. sake* was prepared using malt extract agar (malt extract, 20 g; mycological peptone, 5 g; agar, 20 g, 20 ppt seawater, 1 L; pH 6) and cell biomass was harvested at the exponential phase with sterile seawater (20 ppt). Cells were separated by centrifugation at 7500 ×*g* for 10 min at 4 °C for incorporating in to the feed.

2.2. Experimental diet

Three experimental diets were prepared by incorporating yeast biomass (wet weight) at concentrations of 1%, 10%, and 20% (w/w) to a standard shrimp diet. Diet without supplementation of yeast was used as control. Composition of the diets was as follows. Control diet: fish meal, 28 g; prawn shell powder, 20 g; rice bran, 10 g; soyabean meal, 10 g; groundnut oil cake, 8 g; vitamin mix, 2 g; refined wheat flour, 20 g. In the experimental diets wheat flour was substituted by yeast biomass at different quantities. i.e. 1, 10 and 20 g for the preparation of 1%, 10% and 20% yeast diets respectively. Ingredients except the yeast biomass were mixed well into a dough with 100 ml water and was steamed for 10 min in an autoclave. Into the test feeds yeast biomass was added at a graded levels 1, 10 and 20 g and pelletised using a laboratory model pelletiser having 1 mm die. The feeds were air dried for 2 h and stored at −20 °C until used.

2.3. Animals used

A batch of apparently healthy adult *F. indicus* (mean body weight 15.6±1.5 g) was brought to the laboratory from a shrimp farm located at Kannamali, Cochin. The shrimps (60 Nos), after seven days quarantine, were transferred to four aquarium tanks of 500 L capacity and acclimatized for a week.

2.4. Feeding experiment

Group 1 received the control diet, Group 2, the feed containing 1% yeast, Groups 3 and 4 the diets containing 10% and 20% yeast, respectively. Feed was given twice daily (8 A.M. and 7 P.M.) at a rate of 10–15% body weight per day. Physico-chemical parameters of the rearing water such as salinity, NH_3–N, NO_2–N, NO_3–N and dissolved oxygen were monitored regularly (APHA, 1995) and maintained at optimal levels by water exchange (Table 1).

2.5. Challenge test

After 28 days of feeding the animals were challenged with white spot syndrome virus by feeding white spot virus infected frozen tissue at the rate of 1 g/animal. Thereafter they were maintained on their respective diets and the immune parameters assayed at defined intervals. The percentage survival in each group was also recorded for a period of seven days.

2.6. Assay of immunological parameters

2.6.1. Collection of haemolymph

Haemolymph was withdrawn aseptically from the rostral sinus using specially designed sterile capillary tubes having a diameter of 0.5 mm and pre-rinsed with anticoagulant (Song and Hsieh, 1994). It was transferred to

Table 1
Rearing conditions and water quality

Stocking density	60 shrimp/tank
Tank capacity	500 L
Feeding level	10–15% body wt
Feeding frequency	Twice daily
Feeding period	28 days
Water temperature	24–27 °C
pH	7.5–8
Salinity	24–26 ppt
NH_3	0.01–0.02 mg L^{-1}
NO_3	Below detectable level
NO_2	0.00–0.01 mg L^{-1}
Dissolved oxygen	6–7 mg L^{-1}

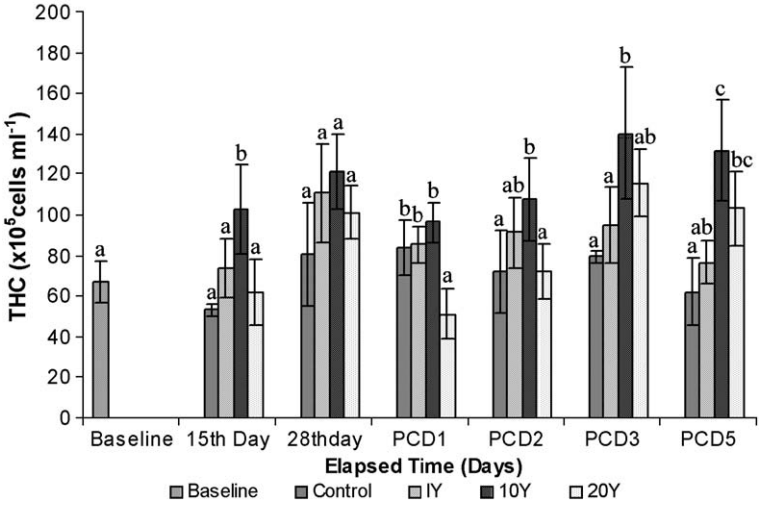

Fig. 1. Mean (±S.D.) THC of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV. Data at the same exposure time with different letters are significantly different ($p<0.05$). 1Y = 1% yeast, 10Y = 10% yeasts, 20Y = 20% yeasts. PCD = post-challenge day.

sterile microcentrifuge tubes containing cooled shrimp anticoagulant. The haemolymph collected from five shrimps ($n=5$) of each treatment group was assayed separately. Sampling was carried out at the beginning of the feeding experiment (0 day/base line), at day 15, day 28, and post-challenge at days 1 (PCD1), 2 (PCD2), 3 (PCD3) and 5 (PCD5).

2.6.2. Total haemocyte counts

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

2.6.3. Phenoloxidase activity assay

Phenoloxidase activity was measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate (Soderhall, 1981). The dopachrome formed was measured at 495 nm and phenoloxidase activity was then expressed as the increase in absorbance per minute per 100 μl haemolymph.

2.6.4. Superoxide anion (NBT reduction) assay

Respiratory burst activity of haemocytes was measured spectrophotmetrically as described by Song and Hsieh (1994) using nitro blue tetrazolium (NBT) as the substrate.

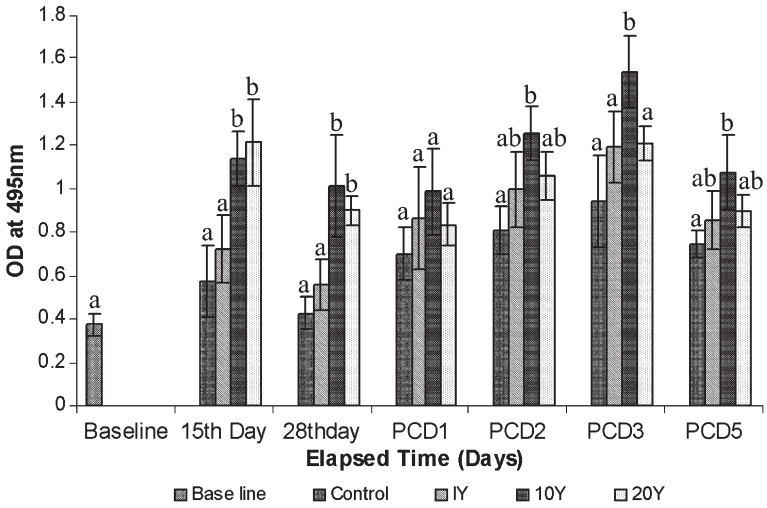

Fig. 2. Mean (±S.D.) phenoloxidase (PO) values of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV. Data at the same exposure time with different letters are significantly different ($p<0.05$).

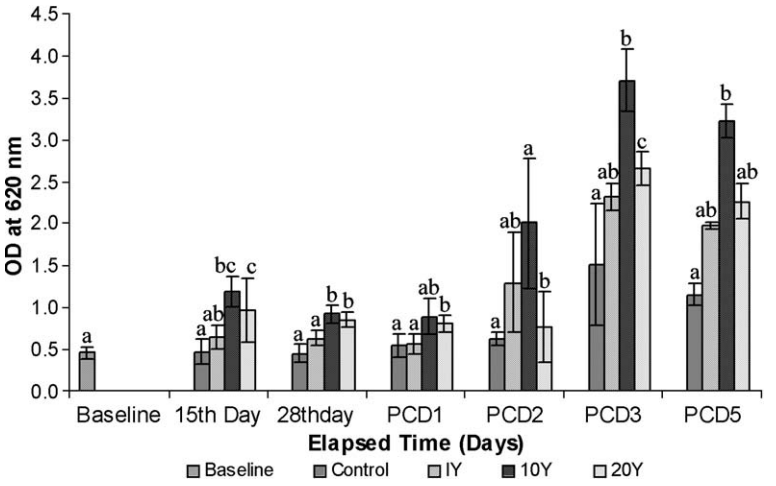

Fig. 3. Mean (±S.D.) Superoxide anion values of *F. indicus* fed on diets containing graded levels of yeast for 28 days and challenged with WSSV. Data at the same exposure time with different letters are significantly different ($p<0.05$).

Blue formazan compound is formed due to O_2^- reduction during phagocytosis by haemocytes. The absorbance at 620 nm was recorded and expressed as NBT activity per 100 µl haemolymph.

2.7. Statistical analysis

In order to determine significant differences, results were analyzed using one way analysis of variance (ANOVA) and Duncan's multiple comparison of the means using SPSS 10.0 for Windows. Differences were considered significant when $p<0.05$.

3. Results

The groups of shrimp fed a diet with 10% (w/w) yeast exhibited a significantly higher immune response compared to the control and other treatment groups ($p<0.05$) (Figs. 1, 2 and 3). The highest total haemocyte count, phenoloxidase activity and NBT reduction were observed on the 15th and 28th day of feeding prior to challenge with WSSV. Subsequent challenge with WSSV resulted in temporal increase in the above responses in groups of shrimp fed on different percentages of yeast. However, these responses were significantly higher ($p<0.05$) on the 3rd day post-challenge

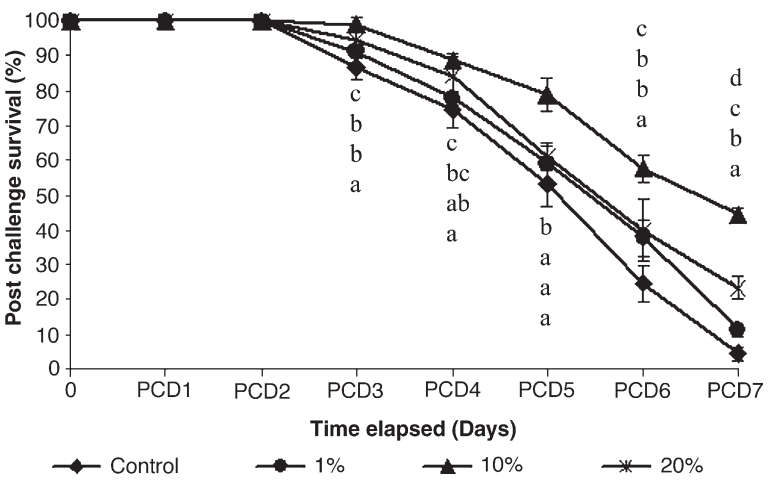

Fig. 4. Mean (±S.D.) post-challenge survival of *F. indicus* fed on diets containing graded levels of yeast for 28 days against experimental infection of WSSV. Data at the same exposure time with different letters are significantly different ($p<0.05$).

in the shrimp group fed the diet containing 10% (w/w) yeast. Meanwhile a uniform decrease in all immunological parameters was seen on the 5th day post-challenge in all challenge groups. Shrimps fed the diet containing 10% yeast showed 44±2% survival while groups fed on diets containing 1% and 20% yeast showed only 11±2% and 23±3% survival respectively (Fig. 4).

4. Discussion

Yeast is generally considered a good source of proteins, nucleic acids, vitamins and polysaccharides. Apart from cell wall glucans, the nucleotide content of the yeast might also contribute to immunostimulation. Although most cell types are capable of synthesizing nucleotides from purines and pyrimidines, de novo synthesis and salvage synthesis of nucleotides are thought to be energetically costly. An exogenous source of nucleotides may optimize the functions of rapidly dividing cells, such as those of the immune system, that lack the capacity to synthesize nucleotides and therefore must depend on a pre-formed source (Carver and Walker, 1995). However, information regarding the synthesis and metabolism of nucleotides in fish and crustaceans is extremely limited (Li and Gatlin, 2003).

Role of nucleotides in immunostimulation were demonstrated in fishes (Salmon, Common carp and striped bass) by various workers (Burrells et al., 2001a,b; Sakai et al., 2001; Li and Gatlin, 2003). Signal transduction in the prophenoloxidase-activating system of *Macrobarchium rosenbergii* and intracellular phenoloxidase activity in haemocyte lysate supernatant (HLS) were found to be increased after treating with CpG oligonucleotides (Chuo et al., 2005). Therefore it is presumed that the immunostimulatory effect of the yeast *C. sake* S165 is not only due to its glucan contents, but also due to its nucleotides.

Throughout our experiments the performance of the group fed 20% yeast was poor when compared to the group fed 10% yeast. This could be due to over-activation of the immune system and a condition similar to "immune-fatigue"(Chang et al., 2000). We observed an initial decrease on THC the first day after WSSV challenge. Chang et al. (2003) made a similar observation with WSSV infection in *P. monodon.* A probable explanation could be the infiltration of haemocytes, especially semigranular cells into connective tissues, stomach and gills upon WSSV infection (Munoz et al., 2002). Tsing (1987) and van de Braak et al. (2002) reported an increase in young and immature haemocytes just after an infection might indicate an intense proliferation of haematopoietic tissue. A very prominent elevation in NBT level on day 3 after challenge could be attributed to an increase in phagocytosis and the resulting production of more superoxide anions. It could be proposed that yeast might act both as a source of an immunostimulant and a nutritional supplement in penaeid shrimp. The danger of overdose with the possible immune-fatigue is also to be considered.

Most of the previous shrimp feeding experiments have been carried out with yeasts alien to marine or brackish water environment. However penaeid shrimp culture is usually carried out at salinities of about 15 to 35 ppt. The halotolerent property of the yeast *C. sake* would be an advantage in this context and it can be used in brackish water and seawater aquaculture where it would not result in cell lysis and associated water quality deterioration.

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