Chapter 4

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

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

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

Mock et al. (1980) reported that replacing algae with active dry baker’s yeast as feed for blue shrimp *P. stylirostris* larvae gave better result. Larvae of kuruma prawn *P. japonicus* and tiger prawns *P. monodon* fed with marine isolate of *Saccharomyces cerevisiae* to obtain high survival rates particularly at zoea stages have been reported (Aujero et al., 1985, Furukawa et al., 1973). Lactic acid yeast *Kluyveromyces fragilis* at a level of 13 % in diet for tiger prawns improved growth performance (cited in Hertrampf and Piedad-Pascual, 2003). Also under field conditions in Japan Squid meal was replaced successfully by 10 to 15 % lactic acid yeast in diet for kuruma prawns (cited in Hertrampf and Piedad-Pascual, 2003).
Scholz et al. (1999) compared the efficacy of 5 different yeast supplemented diets in prawns and reported that Phaffia rhodozyma incorporated feed gave better performance in terms of bacterial clearance and phenoloxidase value. Recently Burgents et al. (2004), reported enhanced disease resistance in pacific white shrimp Litopenaeus vannamei against experimental infection of Vibrio when fed with a Saccharomyces cerevisiae incorporated feed.

In the present study, the immunostimulatory effect of a marine isolate of Candida sake was tested in Fenneropenaeus indicus against white spot virus infection. Total haemocyte count, phenoloxidase activity and production of superoxide anions (O$_2^-$) were measured during the experimental period to assess the immune status of the shrimps and the possible role of yeast glucan and nucleotides as immunostimulants have been discussed.

4.2. Materials and methods

4.2.1. Yeast biomass

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

4.2.2. Experimental diet

Three experimental diets were prepared by incorporating yeast biomass at varying concentrations 1%, 10% and 20% to a referral shrimp diet (Table 2.3). The referral diet without supplementation of yeast was used as control. All the diets were stored at $-20^\circ$C until use.
4.2.3. Animals used
A batch of apparently healthy adult *Fenneropenaeus indicus* (mean body weight 15.6±1.5 g) were brought to the laboratory of School of Marine Sciences from a shrimp farm located at Kannamali, Cochin. The shrimps were randomly divided into four groups of 60 shrimps each into aquarium tanks (Fig 4.1) of 500 L capacity and acclimatised to the laboratory conditions for one week.

4.2.4. Feeding experiment
Among the four groups of experimental animals first group received control diet, second group the feed with 1% yeast, third group feed with 10% yeast and the fourth group received feed with 20% yeast. Feeding was done twice daily (8 A.M and 7 P.M) at a rate of 10-15% wet body weight of the shrimp. Physico-chemical parameters of the rearing water were monitored regularly. Salinity, NH3-N, NO2-N, NO3-N and dissolved oxygen were estimated (APHA, 1995) and maintained at optimal level by water exchange. Total culture period was extended up to 28 days and at the end of feeding experiment animals were challenged with white spot syndrome virus (WSSV) via orally. Maintaining the animals on same diet, the haematological parameters were assayed.

4.2.5. Assay of immunological parameters
4.2.5.1. Collection of haemolymph
Immunological assays were performed to study the immunostimulatory effect of the yeast diets. Haemolymph was withdrawn aseptically from the rostral sinus of the shrimps and transferred in to a sterile microcentrifuge tube containing measured quantity of cold sterile shrimp anticoagulant solution (0.02M sucrose, 0.01M tri-sodium citrate in 0.01M Tris-HCl, pH 7.6) (Song and Hsieh, 1994). A specially designed sterile capillary tube having a diameter of 0.5mm, pre-rinsed with anticoagulant solution was used for haemolymph collection. Haemolymph was collected from five shrimps of each treatment group and assayed separately. Sampling was done at the
Fig.4.1: Bioassay system used to study the efficacy of marine yeast and beta-1, 3-glucan as immunostimulant to *F. indicus* (adult).
beginning of the feeding experiment (0 day/base line), day 15, day 28 besides post challenge day 1 (PCD1), post challenge day 2 (PCD2) and post challenge day 3 (PCD3). Samples were diluted three-fold with shrimp salt solution (450mM NaCl, 10mM KCl, 10mM EDTA.Na₂, 10mM HEPES, pH 7.3) as described by Vargas-Albores and Ochoa (1992) and analysed individually.

4.2.5.2.Total haemocyte count
Total haemocyte count (THC) was taken by using a Neubauer improved haemocytometer and expressed as THC ml⁻¹ haemolymph.

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

4.2.5.4.Superoxide anion (NBT reduction) assay
Respiratory burst activity of haemocytes was measured spectrophotometrically as per the method described by Song and Hsieh (1994) with minor modifications. Nitro blue tetrazolium (NBT, SRL Chemicals, India) was used as substrate that gives a blue formazan colour due to its reduction by O₂⁻ produced during phagocytosis of haemocytes. 100 μl of haemolymph was taken into a microcentrifuge tube precoated with 0.2% poly- L-lysine (Sigma). Poly- L-Lysine coating increases the haemocyte adhesion to the microcentrifuge tube. 100 μl NBT solution (2 mg/ml) prepared in Tris-HCl buffer (pH 7.8) was added to the haemolymph and incubated at room
temperature for 30 min. Tubes were centrifuged at 300 x g for 10 min. in a cooling centrifuge. Discarded the supernatant and stopped the reaction by adding 1ml absolute methanol followed by incubation for 10 min. Spun down the tubes again, discarded the supernatant and left the tubes for air-drying for 30 min. The tubes were washed thrice with 50% methanol and a final washing was done using PBS of pH 7.6. 2M KOH (120 µl) followed by 140 µl dimethylsulphoxide (DMSO, SRL Chemicals) were added to the tubes. Finally 2ml distilled water was added. The optical density at 620nm was recorded using UV-Visible Spectrophotometer (Hitachi. U-2001) against a blank consisting of reagents (KOH and DMSO) and 2 ml distilled water and expressed as NBT activity per 100µl haemolymph.

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

4.3. Results
4.3.1. Total haemocyte count (THC)
Total haemocyte count was maximum in prawns fed with 10% yeast diet both during the feeding experiment and on post challenge. An increase in haemocyte count could be noticed during the feeding experiment in all the treatment groups followed by sudden decrease in the count on post challenge. However, a substantial increase could be noticed on day 3 post challenge showing the immune boost up to face the challenge (Fig 4.2 and Table 4.1 of appendix). Diet wise comparison showed that 10% yeast diet supported maximum cell count and this difference in performance was significant on day 3 post challenge. Generally the count proved to be
remarkably low for prawns fed 20% yeast diets except on day 3 post challenge.

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

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

4.4. Discussion
Administration of immunostimulants like glucan, peptidoglycan and lipopolysaccharides have been found to increase the disease resistance of penaeid shrimps against pathogenic microbes (Sung et al., 1994; Itami et al., 1994, 1998; Karunasagar et al., 1996; Song et al., 1997; Newman, 1999; Takahashi et al., 2000; Chang et al., 2000, 2003). Among these immunostimulants, glucans derived from yeast gained much importance as an immunostimulant in crustacean aquaculture. Glucans are the structural components of cell wall of yeast and certain fungi and principally contains
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Glucose molecules linked by β-1,3 linkages with occasional 1-6 branching. Extraction of glucan from yeast cell wall requires harsh chemical treatments and the method of extraction may affect the immunostimulatory property of glucan. This can be the reason why some authors report the inefficiency of glucan in stimulating the immune response (Scholz et al., 1999). Glucan extraction is a labour intensive process involving both alkali and acid treatments resulting in the removal of valuable nutrients from the yeast cells.

Yeast is generally considered as a good source of protein, nucleic acid, vitamins and polysaccharides. In the present experiment a marine isolate of Candida sake was utilised as a feed ingredient at graded concentrations for F. indicus to study its immunostimulatory effect. The yeast C. sake was found to be nonpathogenic to penaeid shrimps by prawn blood agar haemolysis assay (Section 2.2.2). Of the 3 different concentrations of yeast-incorporated feeds 10% yeast feed showed better immunostimulatory property as evidenced from values of THC, phenoloxidase and NBT. The total haemocyte count (THC) of the treatment group, which received 10% yeast, showed higher values than that of other groups. Tsing (1987) and van de Braak et al. (2002) reported that an increase in circulation of young and immature haemocytes might have be an indicator of an intense proliferation of haematopoietic tissue. A slight drop in the THC just after WSSV challenge was noticed in all the test groups including control, but during the subsequent days it regained. So this may be due to the mobilisation/proliferation of haemocytes on recognizing an infection. A probable explanation for the slight decrease in total haemocyte count at the very beginning of infection could be the infiltration of haemocytes, especially semigranular cells, to connective tissues, stomach and gills on WSSV infection as reported by Munoz et al. (2002). Chang et al. (2003) made a similar observation in the reduction in total circulating haemocytes at the first day of WSSV infection in P. monodon. However, it is interesting to note that in the group fed with 20% yeast, THC count was less when compared to group that received 10% yeast and sometimes even lesser than the control.
At the end of 28 days of feeding the PO level of this group was doubled as compared to that of control on same day. During the first two days of post challenge this difference was not significant though, PO showed an elevated level and on third day of post challenge the difference was very significant. Since PO is considered as the key enzyme in shrimp defence the response of this enzyme during infectious period is having paramount importance in host resistance. NBT values also showed a similar pattern where the group fed with 10% yeast displayed a significant difference from all other groups. There occurs a gradual increase in NBT level during post infection periods and very prominent hike in the NBT level on the third day of post challenge. This can be attributed to an increase in phagocytosis resulting in the production of more super oxide anions for checking infection. Throughout the experiment the performance of the group fed with 20% yeast diet was poor when compared to 10% yeast diet fed group. This could be due to a higher dose of yeast-derived immunostimulant administered to the prawns resulting in over activation of immune system leading to a condition called "immune-fatigue".

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

Nucleotides are low molecular weight biological components that play a major role in almost all biological processes like encoding genetic information, mediating energy metabolism, and signal transduction (Carver and Walker, 1995; Aggett et al., 2003) They are generally considered as non-essential nutrients because deficiency signs have not been observed (Carver and Walker, 1995). Although most cell types are capable of synthesising nucleotides from purines and pyrimidines, de novo synthesis and salvage synthesis of nucleotides are thought to be a costly process in
terms of energy requirements. An exogenous source of nucleotides may optimise the functions of rapidly dividing cells, such as those of immune system, which lack the capacity to synthesise nucleotides and therefore must depend on pre-formed nucleotides (Carver, 1994; Carver and Walker, 1995). Moreover information regarding the synthesis and metabolism of nucleotides in fishes and crustaceans are extremely limited to date (Li and Gatlin III, 2003).

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

Low et al. (2003) examined the relative expression of certain immune genes of Scophthalmus maximus after feeding with nucleotide supplemented diet and showed that there occurs an increased expression of genes of IgM, interleukin etc. Recently Chuo et al. (2005) studied the signal transduction of the proPhenoloxidase activating system of Macrobrachium rosenbergii haemocytes and reported that intracellular phenoloxidase activity in haemocyte lysate supernatant (HLS) was increased after treating with CpG oligonucleotides. Here the oligonucleotides function as an immunostimulant,
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similar to that of glucan, where it increases the phenoloxidase activity and
degranulation of granular haemocytes, but failed to induce
proPhenoloxidase synthesis at transcriptional level.

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

Most of the shrimp feeding experiments were performed with either baker’s
yeast or brewer’s yeast, which are, quite alien to a marine or brackish water
environment. The penaeid shrimp culture practice is being restricted to such
brackish or seawater conditions having a salinity of about 20 ppt to 35 ppt.
In this context it is reasonable to state that the fresh water forms like S.
cerevisiae may find sea water as hyperosmotic and cause the cell rupture
due to osmotic shock which further leads to pollution of culture waters
(Kawano and Ohsawa 1971). One practical solution to this problem is to
identify a salt resistant, halophilic form, which can be used to substitute the
common freshwater yeast as a feed ingredient in prawn culture systems.
The halotolerant property of yeast C. sake would be an advantage in this
context and can be used in brackish water or seawater aquaculture where it
will not cause problem of cell burst and related water quality deterioration.
Fig. 4.1. Mean (± S.D) THC of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV.
Phenoloxidase (increase in OD min$^{-1}$/100μl haemolymph)

<table>
<thead>
<tr>
<th>Feeds</th>
<th>Base line</th>
<th>15th Day</th>
<th>28th day</th>
<th>PCD1</th>
<th>PCD2</th>
<th>PCD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.376 ± 0.05*</td>
<td>0.575 ± 0.16*</td>
<td>0.427 ± 0.07*</td>
<td>0.703 ± 0.12*</td>
<td>0.810 ± 0.11*</td>
<td>0.942 ± 0.21*</td>
</tr>
<tr>
<td>IY</td>
<td>0.376 ± 0.05*</td>
<td>0.722 ± 0.15*</td>
<td>0.558 ± 0.11*</td>
<td>0.863 ± 0.33*</td>
<td>0.998 ± 0.17*</td>
<td>1.195 ± 0.16*</td>
</tr>
<tr>
<td>10Y</td>
<td>0.376 ± 0.05*</td>
<td>1.143 ± 0.12*</td>
<td>1.015 ± 0.23*</td>
<td>0.987 ± 0.19*</td>
<td>1.257 ± 0.12*</td>
<td>1.540 ± 0.16*</td>
</tr>
<tr>
<td>20Y</td>
<td>0.376 ± 0.05*</td>
<td>1.215 ± 0.2*</td>
<td>0.902 ± 0.67*</td>
<td>0.837 ± 0.1*</td>
<td>1.060 ± 0.11*</td>
<td>1.212 ± 0.08*</td>
</tr>
</tbody>
</table>

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

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

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

Fig. 4.2. Mean (±S.D) phenoloxidase (PO) value of F. indicus fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV.
Fig. 4.3. Mean (± S.D) NBT value of *F. indicus* fed on diets containing graded levels of yeast for 28 days and then challenged with WSSV.