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SHORT COMMUNICATION

Application of bacterins and yeast *Acremonium dyosporii* to protect the larvae of *Macrobrachium rosenbergii* from vibriosis

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The fresh water prawn, *Macrobrachium rosenbergii*, has proven potential for use as an aquaculture species (Hanson & Goodwin, 1997; Kurup, 1984). In India alone, culture of this species of prawn in low saline areas requires about 200 million seed per year (Kurup, 1984). In hatcheries poor survival rate has been associated with vibriosis at different stages of the larval cycle. Members of the family Vibrionaceae associated with the larvae of *M. rosenbergii* were shown to be pathogenic under laboratory conditions (Bhat *et al*., 2000, in press). Vibrios have been associated with mortality of penaeid prawns by several workers (Aquacop, 1977; Hameed, 1993; Karunasagar *et al*., 1994). Two methods have been suggested to protect both the larvae and juveniles from vibriosis; one is the administration of bacterins prepared from pathogenic strains (Itami *et al*., 1989, 1991; Adams, 1991; Song & Sung, 1990; Sung *et al*., 1991) and the other is the utilization of yeast β 1-3 and 1-6 glucans as immunostimulants for enhancing the non-specific defense system (Sung *et al*., 1994; Song *et al*., 1997). In the light of these observations it was hypothesised that bacterins and yeast glucans may also be effective in protecting the larvae of *M. rosenbergii* from vibriosis as has been achieved in the case of penaeids. To examine this hypothesis, the ability of bacterins and an extracellular glucan-producing yeast to increase the overall survival and metamorphosis of larvae in a hatchery, as well as to protect against an experimental challenge under laboratory conditions, was evaluated.

A strain each of *Vibrio* (ANM 708) and *Photobacterium* (AAC 727) isolated from diseased larvae of *M. rosenbergii* and found to be affiliated to *V. fisheri, V. logei* or *V. marinus* and *P. angustrum*, respectively, at G+C ratio level (determined from T*m* values), but phenotypically dissimilar to the type strains and proven to be pathogenic to the larvae under laboratory conditions (Bhat *et al*., 2000, in press) were used for the preparation of bacterins. The bacteria were cultured in nutrient agar (Peptone 0·5%, Beef extract 0·5%) (HI Media Laboratories, Bombay, India), aged sea water (15 ppt), pH 7·5 at 28° C for 48 h and harvested in phosphate buffered saline (PBS) composed of NaH_2PO_4 6·42 g; Na_2HPO_4 34·316 g; NaCl 10 g (SRL, Bombay, India), distilled water 1000 ml. The cultures were diluted in PBS to obtain 0·5 OD (Abs_{600}) and were exposed to a final concentration of 0·2% (v/v) formalin for 24 h at room temperature and subsequently stored at 4° C for 14 days. To confirm inactivation an aliquot of 1 ml

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bacterin was inoculated into 500 ml nutrient broth and incubated at room temperature for 7 days and checked for turbidity. The procedure followed was comparable to that suggested by Itami *et al.* (1989) who used 0·5% final concentration (v/v) formalin for inactivation.

The yeast, *Acremonium dyosporii* (MTCC 1316; IMTECH, Chandigarh) was grown in Saboraud dextrose broth for 30 days at room temperature (28° C) and harvested by centrifugation at 2800 × *g* for 30 min at 4° C. The yeast developed a mucilaginous mat with pseudomycelium and on acquiring weight sank to the bottom with a new mat forming on the surface. This facilitated a separation of the yeast from broth by filtration through muslin cloth. An incubation period for 30 days yielded 5 g (wet weight) biomass per 100 ml medium. The supernatant was kept at 4° C and the pellet was dried at 80° C, powdered and stored at 28° C in airtight containers.

The bacterin for immersion was prepared by mixing aliquots (100 ml each) of inactivated cell suspensions of the strains ANM 708 and AAC 727 having 2·14 and $2{\cdot}0 \times 10^{12}$ cells ml^{-1} determined by counting the cells smeared on microscope slides and having OD values of 0·5 and 0·18 at Abs_{600}, respectively. To this cell suspension 100 ml supernatant saved from the broth culture of *Acremonium dyosporii* was added to obtain $1{\cdot}42 \times 10^{12}$ cells ml^{-1}.

To incorporate bacterin and yeast cells into feed, 100 ml aliquots of each inactivated bacterial suspension prepared as described above from the strains ANM 708 and AAC 727 were centrifuged at 10 600 × *g* at 4° C, washed with sterile PBS and re-suspended in 5 ml aliquots of PBS to obtain $4{\cdot}28 \times 10^{14}$ cells ml^{-1}. To this, 1 g yeast cell powder as the whole cell immunostimulant was mixed and maintained at 4° C until use. The above preparation was incorporated in egg custard composed of three chicken eggs (150 g wet weight and 30 g dry weight; Clam meat 180 g wet weight (54 g dry weight), Agar agar 15 g dry weight and Guagum 6 g dry weight. Chicken eggs and clam meat were cooked separately, blended and fibres removed by passing through a nylon mesh of 500 μm porosity and boiled with Agar agar for 10 min. When the temperature dropped to 55° C the bacterins and yeast cells were added along with guagum, stirred well and kept undisturbed until it solidified (*c.* 10 min). The control feed was without bacterins and yeast cell components. Both the preparations were maintained in small aliquots at −20° C and thawed at the time of use, broken up into small pieces and fed to larvae.

Freshly hatched larvae were maintained in 11 ppt sea water in 250 l fibre glass tanks with a stocking density of 80 larvae l^{-1} under continuous aeration. Exposure of the larvae to bacterin was given every 7 days for 35 day by reducing the volume to 50 l and by adding 352 ml stock bacterin to attain a final cell number of 1×10^{10} cells ml^{-1}, for 90 min under vigorous aeration. The volume of water was subsequently brought back to the original volume by adding fresh 11 ppt sea water. Oral administration of the bacterin/yeast cell preparation commenced on the nineth day post-stocking with 10 g (wet weight) diet/tank/day for the entire rearing period. Water quality parameters such as temperature, NH_4^+-N (following Strickland & Parson, 1968) and total, heterotrophic bacterial population (following standard plate count using ZoBells agar) were monitored throughout the experimental period. From the ZoBell's agar plate 20 colonies were selected at random and identified to genera following Oliver (1982) and Buchanan & Gibbons (1974).

The rate of survival of larvae was determined once in 7 days and the extent of metamorphosis was assessed 35 days post-stocking. A stress test was performed on the larvae 20 days post-stocking by transporting both the experimental and control larvae to the laboratory and maintaining them at a high stocking density of 100 larvae l^{-1} without aeration, water exchange and feeding sparsely (three *Artemia* nauplii $larva^{-1}$ day^{-2}). The overall survival rate and the number of post-larvae settled were noted on the seventh day. Simultaneously, non-stressed larvae were challenged with the pathogens ANM 707 and AAC 727 20 days post-stocking. To revive virulence, the pathogens were passaged three times consecutively through muscle infusion prepared from *M. rosenbergii* by incubating for 24 h at 28° C. The infusion was prepared by macerating 20 g muscle tissue in 200 ml PBS and centrifuging at 1000 × *g* for 10 min at 4° C and the

Table 1. Physico-chemical and microbial quality of rearing water in the hatchery during the experiment

Rearing water quality	Treated tank	Control	Stock water
A. Physico-chemical			
1. pH	7–7·5	7–7·5	7–7·5
2. Salinity (PPT)	8–13	8–13	8–13
3. Temperature (° C)	26–28·5	26–28·5	26–28·5
4. NH_4^+-N ($\mu g \,.\, L^{-1}$)	0–2·5	0–1·67	0–0·44
5. TPC (CFU mL^{-1})	$1{\cdot}60 \times 10^7$ to $4{\cdot}80 \times 10^9$	$1{\cdot}80 \times 10^7$ to $2{\cdot}50 \times 10^9$	$0{\cdot}3 \times 10^7$ to $1{\cdot}1 \times 10^8$
6. Genera (%)			
Pseudomonas	54·55	52·5	58·7
Aeromonas	32·72	33·0	30·5
Alteromonas	7·27	6·9	8·0
Enterobacteriaceae	3·67	4·0	2·5
Vibrio	1·82	3·6	0·3

supernatant filter-sterilized first by passing through a Seitz filter and second through membranes (Sartorius India pvt. Ltd, Bangalore, India) of 0·22 μ porosity. The larvae were kept in groups of 20 in 500 ml 11 ppt sea water in plastic troughs, in triplicate, which were inoculated with the passaged pathogens to obtain a final concentration of 10^8–10^9 cells ml^{-1} as bath. The larvae were fed uniformly with *Artemia nauplii* and egg custard devoid of bacterins and yeast cell powder. Ammonia nitrogen, temperature and pH were monitored. Cumulative mortality within 48 h was recorded from which relative per cent survival was calculated using the following equation:

$$\text{R.P.S} = \left(1 - \frac{\text{\% mortality in the group administered with bacterin and yeast}}{\text{\% mortality in the control group without the above administration}} \right) \times 100$$

In the hatchery during the trial all measured water quality parameters such as pH, salinity, temperature and NH_4^+-N were within the accepted range (Table 1). The total bacterial population was moderately high and dominated by *Pseudomonas* and *Aeromonas* which did not vary much between the experimental and control tanks and the stock water.

On completion of the experiment the overall per cent survival of the group of larvae in the hatchery, administered with the bacterins and dry yeast cell preparations, was only marginally higher than that of the control group (Table 2). However, there was a considerable increase (18·8%) in the percent metamorphosis of larvae in this group. However, under stressed conditions there was an increase in the percent survival of the test group by 16·5% with 7·0% increase in metamorphosis. On challenging larvae with the bacterial strains ANM 708 and AAC 727, the group of larvae fed with bacterins and yeast cell powder (treated) exhibited significantly higher ($P<0{\cdot}01$) percent survival compared with the untreated group (Table 3). The untreated and unchallenged groups also registered mortality under the experimental conditions, but to a lesser extent compared with the challenged group.

Virulence of the two bacterial strains used for the challenge experiment is also demonstrated in Table 3. Strain AAC 727 was found to be virulent as it caused significantly higher mortality ($P<0{\cdot}05$) in untreated larvae compared with unchallenged larvae, while the strain ANM 708 was not so virulent. On the basis of this observation, the percent survival of treated larvae subsequent to the challenge with AAC 727,which is almost identical to the percent survival of the unchallenged group, assumes importance as the administration of bacterins and yeast cell powder was found

Table 2. Survival and metamorphosis of larvae under varying conditions subsequent to the treatment with bacterins and yeast cell powder

Varying conditions	Overall percentage survival	Percentage metamorphosis to post-larvae
A. Under field conditions		
1. Test	41·5	29·4
2. Control	38·0	10·6
B. Under stressed condition in laboratory		
1. Test	31·5	26·98
2. Control	15·0	20·0

Table 3. Survival of larvae on challenging with the pathogenic strains ANM 708 and AAC 727 after treatment with bacterins and yeast *Acremonium dyosporii*

Pathogen	Treatment	Survival (%)	*t*-value	df	*P*	Relative survival (%)
ANM 708	Treated[1]	90·83 ± 1·86	4·922	7	<0·01	72·5
	Untreated[2]	66·67 ± 10·27				
AAC 727	Treated[1]	90·66 ± 7·01	5·728	7	<0·01	76·80
	Untreated[2]	59·66 ± 6·20				
Unchallenged	Treated[1]	92·02 ± 5·99	4·847	5	<0·01	71·22
	Untreated[2]	72·20 ± 0				

[1]The group of larvae treated with bacterins and yeast cell powder.
[2]The group of larvae untreated and used as control.
t-value: Student's *t*-test.
df: Degrees of freedom.

to be effective in protecting the larvae from the virulent pathogen. Results comparable to this have been obtained by earlier works with bacterins and immunostimulants, especially in penaeid prawns. Itami *et al.* (1989, 1991) obtained survival rates of 50·3 and 19·5%, respectively, in treated and untreated groups. Song & Sung (1990) and Sung *et al.* (1991, 1994) observed increased growth in penaeid post-larvae on administration of *Vibrio* bacterins. Comparable to this, in this study, the bacterins and immunostimulant treated group quickly molted to post-larvae.

It is worth pointing out that the hatchery system where the experiment was carried out was very well maintained and had a consistently overall survival of 30–40%. From the information gathered on bacterial diversity in the larval rearing system it was found that the existing bacterial flora was composed of more *Pseudomonas* than *Aeromonas* and *Vibrio* and this is characteristic of healthy larval rearing systems for *M. rosenbergii*. This might have led to the situation of obtaining only a marginal increase in the rate of survival of treated larvae in the hatchery over that which already existed. This implies that had the bacterin been applied in a hatchery system where higher mortality of the larvae usually occurred, a better performance of the preparation, by increasing the survival rate, could have been obtained.

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