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Development of biofilm of *Vibrio alginolyticus* for oral immunostimulation of shrimp

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Abstract Biofilm (BF) of *Vibrio alginolyticus* was developed on chitin flakes. BF formation was studied at various nutrient concentrations and incubation time. Highest colony-forming units of BF cells were obtained with 0.15% trypticase soya broth and at 3 days of incubation. The BF cells could be completely inactivated at 80°C in 10 min and with 10% formalin in 24 h. SDS–PAGE profile of BF cells revealed repression of four proteins and expression of three new proteins compared to free cells (FC). The preliminary immune response studies showed that BF cells were superior to FC in stimulating the non-specific immune response of *Penaeus monodon*.

Keywords *Vibrio alginolyticus* · *Penaeus monodon* · Biofilm · Free cells · Immunostimulation

Abbreviations

TSB	Trypticase soya broth
CFU	Colony-forming units
PBS	Phosphate-buffered saline
TSA	Trypticase soya agar
TPC	Total plate count
PMSF	Phenylmethylsulphonylfloride
RPM	Rotation per minute

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EDTA	Ethylene diamine tetraacetic acid
THC	Total haemocyte count
PO	Phenoloxidase activity
L-DOPA	L-dihydroxyphenylalanine
ANOVA	Analysis of variance
BF	Biofilm
FC	Free cell
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SD	Standard deviation

Introduction

Use of immunostimulants is an effective means of increasing the immunocompetency and disease resistance in fish (Sakai 1999). Furthermore, oral stimulation of immune system is most economical and practical strategy in commercial culture of fish and shrimp. However, one of the important factors for the inconsistency and poor response to oral vaccination in fish is the digestive degradation of antigens in the foregut before the vaccine reaches immune responsive areas in the hindgut and other lymphoid organs (Azad et al. 2000). Bacterial biofilms, which are growing colony of bacteria on a substrate enveloped by glycocalyx (Costerton et al. 1981), are reported to be highly resistant to antibiotics (Brown et al. 1988), surfactants (Govan 1975) and antibodies (Kreig et al. 1988). Higher protective immune response in the form of elevated antibody titres was reported in common carp administered orally BF cells of *Aeromonas hydrophila* (Azad et al. 1999), which was attributed to superior antigen delivery to the lymphoid tissues as evidenced by antigen localization using monoclonal antibodies (Azad et al. 2000).

Changes in the protein profile of BF cells in comparison with free cells (FC) of *A. hydrophila* used in oral vaccination of fish have been reported (Asha et al. 2004). Cell-surface structures, cell wall composition, exopolysaccharide and capsular polysaccharides present in the bacteria vary with growth conditions. Bacteria infecting tissues produce cell-surface components not found on the bacteria grown in vitro (Brown and Williams 1985), and cell-surface changes may be produced in the cells of same species in response to variations in nutrient status, surface growth and other environmental factors (Lorian et al. 1985).

As shrimp lack specific immune response involving memory, there is need for oral immunostimulation that is long lasting and resistant to gut destruction. By considering the advantage of BF as a vaccine in teleost fish, we developed BF of *Vibrio alginolyticus* with an objective to standardize the biofilm development in vitro and test its expediency as an immunostimulant in *P. monodon*. There are no published reports on the application of BF as an immunostimulant in shrimp culture.

Materials and methods

Source of *V. alginolyticus*

The bacteria were obtained from an infected *P.monodon* from a culture pond at Vypeen near Kochi, Kerala, India during June 2006 by isolating on 1.5% TSB (Himedia, Mumbai)

containing 2% NaCl and identified based on biochemical characteristics. The isolate was preserved in 1.5% TSB containing 0.15% glycerol at −20°C. For use, an aliquot was revived on nutrient agar slant and stored at 4°C.

Nutrient requirement for BF formation

Ideal concentration of nutrients for optimum BF formation was standardized according to Azad et al. 1997. Six concentrations of TSB (0.025, 0.05, 0.1, 0.15, 0.2 and 0.25%) in duplicate were prepared and supplemented with 2% NaCl. Chitin (0.3%, W/V), as substrate, was added to each flask and autoclaved. Culture of *V. alginolyticus* in log phase ($1X10^7$ CFU) in 1.5% TSB was inoculated to each flask and incubated at room temperature for 24 h with 6-h agitation at 120 strokes min^{-1} on a mechanical shaker. After incubation, the supernatant solution was decanted into six sterile flasks. Chitin was washed thrice in sterile PBS (pH 7.4) by gently swirling the flask thrice, and the supernatant with loosely bound cells was decanted. Chitin was then transferred to 50-ml centrifuge tubes containing 10 ml PBS and agitated for 4 min on a cyclomixer to dislodge the BF cells. Dislodged cells were serially diluted in sterile saline and inoculated to TSA plates in triplicate by surface spread technique. TPC was obtained after incubation at room temperature for 24 h, and the counts were expressed as mean CFU g^{-1} chitin. The decanted supernatant solutions in sterile flasks were serially diluted in sterile saline after harvesting and washing twice in PBS. The diluted samples were inoculated to TSA plates in triplicate. Viable counts of FC were determined after incubating the plates at room temperature for 24 h and expressed as mean CFU ml^{-1}.

Period of incubation for optimum BF formation

For studying the bacterial kinetics, culture of *V. alginolyticus* in log phase ($1X10^7$ CFU) was inoculated to five flasks in duplicate containing 0.15% TSB (supplemented with 2% NaCl) and 0.3% chitin and incubated for five days at room temperature with 6 h of shaking day^{-1} on a mechanical shaker. Duplicate plates were removed at the end of every 24 h, BF and FC were harvested, and TPC was determined.

Heat and chemical inactivation of BF cells

For standardizing heat and chemical inactivation of BF cells, three-day-old BF cells (as per the results of the bacterial kinetics study) grown in 0.15% TSB containing 0.3% chitin were harvested and placed in water bath at 60, 70 and 80°C for 10 and 20 min. The samples were then plated on TSA plates, and per cent inactivation was enumerated. One-day-old FC in 1.5% TSB were harvested and placed in a water bath at 60, 70 and 80°C for 10 and 20 min after which the samples were plated on TSA plates and per cent inactivation was enumerated.

Three-day-old BF grown in 0.15% TSB containing 0.3% chitin was harvested and incubated in PBS containing 1, 5 and 10% formalin for 12, 24 and 48 h. One-day-old FC in 1.5% TSB were harvested and incubated in PBS containing 0.5% formalin for 24 h. After the incubation period, samples were plated on TSA plates and incubated for 24 h at room temperature, and per cent inactivation was enumerated.

SDS–PAGE analysis of BF and FC

Protein profile of *V. alginolyticus* BF and FC was analysed using SDS–PAGE according to Laemmli 1970. Three-day-old BF of *V. alginolyticus* on chitin was harvested by vortex mixing in PBS with 1 mM PMSF (Sigma, USA). The BF mass was pelleted after centrifugation at 86,400 *g* for 30 min at 4°C. One-day-old FC of *V. alginolyticus* were centrifuged at 7,100 *g* for 10 min, and cells were harvested and washed thrice in sterile PBS (pH 7.2). The samples were solubilized in reducing buffer and vortexed well. The solutions were then boiled at 100°C for 1 min and spun at 5,000 rpm for 2 min to settle down the debris. Protein content of the harvested BF and FC was analysed (Bradford, 1976). Twenty microlitres of sample supernatants containing 10 μg protein was loaded onto 4.5% stacking gel along with the protein marker (Sigma, USA) and resolved in a 15% separating gel at 150 V in a mini gel electrophoretic system. After electrophoresis, the resolved protein bands were visualized in coomassie brilliant blue (0.1% (W/V) in 40% methanol and 10% glacial acetic acid), stained for 1 h followed by destaining in 40% methanol and 10% glacial acetic acid till the background was clear.

Source, maintenance and feeding of *P. monodon*

Juvenile *P. monodon* (1.6 ± 0.3 g), obtained from Matsya fed prawn hatchery, Kannur, India, were acclimatized in the laboratory for 15 days in sea water (salinity 30 ± 1‰; pH 7.6 ± 0.2; temperature 26 ± 0.2°C) with continuous aeration. For the experiment, shrimp were divided into three groups and maintained in plastic tubs with triplicate tubs for each group containing 80 l sea water (salinity 30 ± 1‰; pH 7.6 ± 0.2; temperature 26 ± 0.5°C) with continuous aeration. Each tub was stocked with 10 shrimp. Required concentration of heat-inactivated BF cells on chitin flakes and heat-inactivated FC with autoclaved chitin was uniformly mixed with commercial shrimp feed with the help of a binder (Protogel, Orgavet Pharma, Vijayawada, India). First group was fed with BF cells at 10^8 cfu g^{-1} shrimp day^{-1} through feed for 7 days. Second group was fed with FC at 10^8 cfu g^{-1} shrimp day^{-1} through feed for 7 days. Shrimp in the third group, which received standard shrimp feed containing autoclaved chitin flakes, constituted the control group. Shrimp were fed at 5% of body weight twice daily. On day 8, haemolymph samples were collected from individual shrimp in their inter-moult stage directly from the heart using a tuberculin syringe fitted to a 26-gauge needle. When haemolymph collected from a single individual was not sufficient to fulfil analysis requirement, samples collected from 2 to 3 shrimp were pooled. Haemolymph (300 μl) was collected in 700 μl of marine anticoagulant solution (0.01 M trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.4) and subjected to enumeration of THC and PO activity (Liu and Chen 2004).

Enumeration of THC

A drop of the haemolymph–anticoagulant mixture was dispensed on a Neubauer slide, and the cells were allowed to settle for few seconds. The cells were counted in all five large squares and expressed as cells ml^{-1} of haemolymph.

Enumeration of PO activity

PO activity was measured spectrophotometrically by recording the formation of dopachrome from L-DOPA. Diluted haemolymph was centrifuged at 700 *g* at 4°C for 10 min.

After discarding the supernatant, the pellet was rinsed, resuspended gently in cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, and pH 7.0) and centrifuged again. The pellet was resuspended in 600 µl cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, and pH 7.0), and 100 µl of the cell suspension was incubated with 50 µl of trypsin (1 mg ml^{-1}), which served as an elicitor, for 10 min at 20°C. Fifty µl of L-DOPA (3 mg ml^{-1}) as a substrate was then added, and 800 µl of cacodylate buffer was added 5 min later. The optical density at 490 nm was measured using a Varion Carry-50 spectrophotometer. The PO activity was expressed as δ (490) 50^{-1} µl of haemolymph. The control solution that consisted of 100 µl of cell suspension, 50 µl cacodylate buffer (to replace trypsin) and 50 µl of L-DOPA was used for background PO activity. The background PO activity, which ranged from 0.02 to 0.08, was subtracted from the PO activity of shrimp for all test conditions.

Statistical analysis

CFU, THC and PO activity values were expressed as mean ± SD. The mean values of nutrient requirement for optimum BF formation and growth kinetics were statistically analysed by one-way ANOVA with tukey post-tests using GraphpadPrism (Version: 4) for Windows, GraphPad Software, San Diego, California, USA. THC and PO values were analysed by performing two-Way ANOVA with Bonferroni post-tests using GraphpadPrism (Version: 4) for Windows, GraphPad Software, San Diego, California, USA.

Results

Among six nutrient concentrations studied, significantly ($P < 0.001$) higher CFU of BF cells were obtained with 0.15% TSB supplemented with 2% NaCl (Fig. 1). Highest CFU g^{-1} of chitin flakes was obtained on third day of incubation that was significantly ($P < 0.001$) higher than the CFU on 1, 2, 4 and 5 days (Fig. 2). Highest CFU ml^{-1} of FC was obtained on second day of incubation.

Complete inactivation of 3-day-old BF was achieved at 80°C for 10 min, whereas complete inactivation of FC was possible at 60°C for 10 min (Table 1). In case of formalin inactivation, complete inactivation of BF cells was achieved with 10% formalin in 24 h, while FC were completely inactivated with 0.5% formalin in 24 h (Table 2).

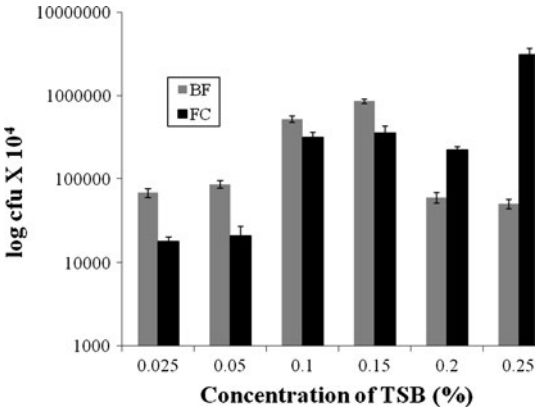

Fig. 1 Growth of BF and FC of *V. alginolyticus* under different nutrient concentrations (*vertical bars* indicate SD)

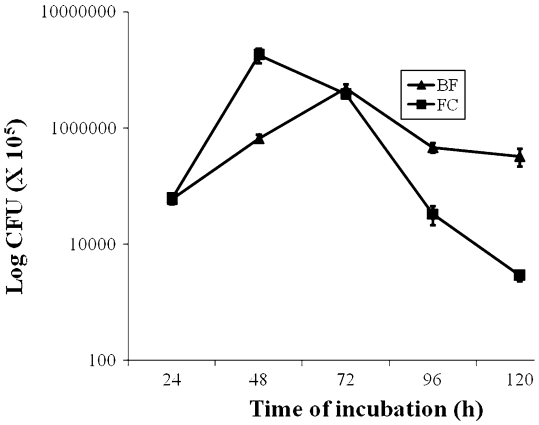

Fig. 2 Growth kinetics of *V. alginolyticus* BF and FC (*vertical bars* indicate SD)

Table 1 Heat inactivation of three-day-old BF and one-day-old FC of *V. alginolyticus*

BF cells			FC		
Temp (°C)	Time (min)	% Inactivation	Temp (°C)	Time (min)	% Inactivation
60	10	91.26	60	10	99.99
60	20	96.76	60	20	99.99
70	10	99.46	70	10	99.99
70	20	99.96	70	20	99.99
80	10	99.99	80	10	99.99

Table 2 Formalin inactivation of three-day-old BF and one-day-old FC of *V. alginolyticus*

% Formalin	% Inactivation of FC after	% Inactivation of BF cells after		
	24 h	12 h	24 h	48 h
0.5	99.99	N	N	N
1	N	N	N	92.22
5	N	96.66	99.22	99.99
10	N	99.92	99.99	N

N Not tested

SDS–PAGE of 3-day-old BF and FC revealed the separation of a total of 22 proteins from FC. With BF cells, an additional expression of three proteins of 45, 50 and 55 kDa was recorded. Furthermore, a repression of four proteins of 31, 44, 47 and 53 kDa was also found (Fig. 3).

Mean values of both THC and PO activity were significantly ($P < 0.001$) higher in BF group on day 7 when compared to FC fed or control shrimp (Fig. 4). Also, values of THC and PO activity were significantly ($P < 0.001$) higher on day 7 compared to that on day zero in both BF and FC groups.

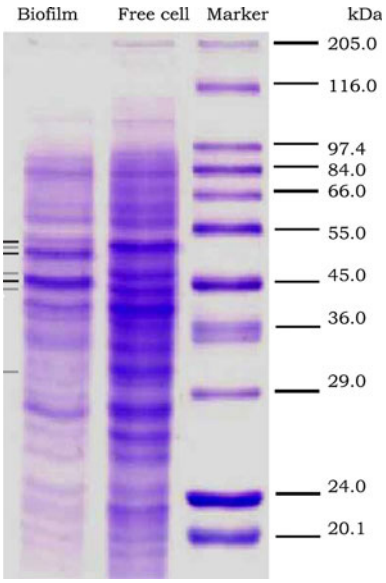

Fig. 3 SDS–PAGE profile of BF and FC of *V. alginolyticus*: *grey lines* on the left indicate repressed proteins, *black lines* indicate newly expressed proteins

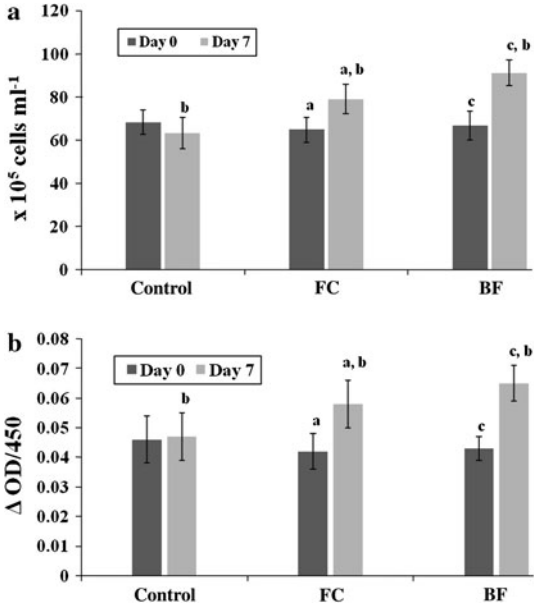

Fig. 4 Mean immune response values of *P. monodon* fed with BF and FC of *V. alginolyticus*: *a* THC; *b* PO activity (*vertical bars* indicate SD; means with common letters have significant difference between them, $P < 0.001$)

Discussion

Among various TSB concentrations studied, highest CFU of BF cells was obtained with 0.15% supplemented with 2% NaCl. Azad et al. 1997 used four different concentrations of TSB and obtained highest CFU of BF cells of *A. hydrophila* with 0.225% TSB concentration. Bacterial kinetics showed that increased colonization with time resulted in an

increase in BF population with a corresponding decrease in population of FC. Similar observations were reported in case of *Staphylococcus aureus* (Anwar et al. 1992) and *A. hydrophila* (Azad et al. 1999).

Three-day-old BF cells were completely inactivated at 80°C in 10 min and 10% formalin in 24 h. In contrast, FC of *V. alginolyticus* were completely inactivated at 60°C in 10 min and 0.5% formalin in 24 h. BF cells of *A. hydrophila* were completely inactivated at 90°C in 30 min (Azad et al. 1999). The present study revealed that BF cells of *V. alginolyticus* were more resistant to heat and chemical treatment compared to FC. Role of BF as barriers of heat exchange has been reported. Bacteria have been known to colonize water-cooled metal surfaces in heat exchangers, resulting in reduced thermal exchange abilities. Aged BF cells embedded in multilayered glycocalyx are shielded against heat and chemical action, and hence, the cells in the deeper layers survive (Costerton et al. 1981).

With BF cells, an additional expression of three proteins and repression of four proteins were observed. BF cells of *A. hydrophila* showed repression of nine proteins and additional expression of three proteins of 72, 36 and 22 kDa (Asha et al. 2004). Similar observations were also made in *Pseudomonas putida* where 15 proteins were up-regulated and 30 proteins were down-regulated following attachment to silicon surface (Sauer and Camper 2001). Another related study under iron-limited conditions has shown that *P. aeruginosa* expressed additional 77 and 85 kDa proteins (Anwar et al. 1984). BF of *P. aeruginosa* developed on glass wool also revealed changes in protein profile (Steym et al. 2001). Studies on starved cells of *A. hydrophila* have shown that about five outer membrane proteins were lost with the expression of three new proteins compared to FC (Rahman et al. 1998). Also, starved cells of *Vibrio* sp. lost many cellular proteins and synthesized new proteins called starvation proteins (Amy and Morita 1983; Nelson et al. 1997). Expression of additional proteins in BF cells could be a stress response, which might help the bacteria to survive in the new mode of life (Buchmeier and Hefron 1990).

BF of *V. alginolyticus* could stimulate the immune response of *P. monodon* as evidenced by elevated THC and PO activity. Also, BF cells were found superior to FC in stimulating the immune response. This might be due to the better delivery of antigen from BF, since the cells are embedded in the glycocalyx matrix thus facilitating a slow and sustained release of antigens when compared to FC antigens that are normally rapidly digested and released resulting in short-lived and insufficient immune activation (Azad et al. 1999). Improved antigen delivery and protective responses in carp administered BF cells of *A. hydrophila* by oral route have been demonstrated (Azad et al. 2000). Elevated THC and PO activity were also reported when shrimp were administered various immunostimulants like probiont bacterium (Rengpipat et al.2000), β-1, 3-glucan (Lopez et al. 2003), spent brewer's yeast β-glucan (Suphantharika et al. 2003), chitin/chitosan (Wang and Chen 2005), *Sargassum fusiforme* polysaccharide extracts (Huang et al. 2006) and marine yeast (Sajeevan et al. 2006).

In the present study, heat-inactivated BF cells were used as oral immunostimulant, since large number of immunogenic epitopes were expressed leading to higher protective response in heat-inactivated bacterin than formalin-inactivated cells (Lamers and van Muiswinkel 1986).

Present study established that *V. alginolyticus* BF can be developed on chitin flakes. BF cells were found to be more resistant to heat and chemical action when compared to FC. Also, BF cells showed changes in protein profile when compared to FC. Preliminary immune response studies showed that BF cells were superior to FC in stimulating the immune system of *P. monodon*.

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