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# *Vibrio harveyi* biofilm as immunostimulant candidate for high-health pacific white shrimp, *Penaeus vannamei* farming

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ABSTRACT

The study was to develop *Vibrio harveyi* biofilm-based novel microbial product and its oral delivery for high health *Penaeus vannamei* farming. Yield of bacterial biofilm was optimized on chitin substrate (size: < 360, 360–850 and 850–1250 µm; concentration: 0.3, 0.6 and 0.9%) in tryptone soy broth (0.15%). The biofilm was characterized by crystal violet assay, SEM and LSCM imaging; protein profiling by SDS-PAGE and LC-ESI-MS/MS. The immune stimulatory effect of the biofilm in yard experiments was evaluated by relative quantification of immune genes using real-time PCR effect on overall improvement on health status under field trials. The highest biofilm yield ($6.13 \pm 0.2 \times 10^7$ cfu/ml) was obtained at 0.6% of < 360 µm chitin substrate. The biofilm formation was stabilized by 96 h of incubation at 30 °C. Protein profiling confirmed expression of six additional proteins (SDS-PAGE) and 11 proteins were differentially expressed (LC-ESI-MS/MS) in biofilm cells over free cells of *V. harveyi*. Oral administration of the biofilm for 48 h confirmed to enhance expression of antimicrobial peptides, penaeidin, crustin and lysozyme in *P. vannamei*. Further Oral administration of biofilm for two weeks to *P. vannamei* (1.8 ± 0.13 g) improved the growth (2.66 ± 0.06 g) and survival (84.44 ± 1.82%) compared to control (2.15 ± 0.03 g; 70.94 ± 0.66%) Nursery trials showed a significant reduction in occurrence of anatomical deformities like antenna cut (12.67 ± 0.66%), rostrum cut (4.66 ± 0.87%), and tail rot (3.33 ± 0.88%), compared to animals fed with normal diet which was 24.33 ± 2.72; 14 ± 1.52 and 10.66 ± 1.45% respectively. *In vitro* and *in vivo* studies suggest inactivated biofilm cells of *V. harveyi* on chitin substrate express additional antigenic proteins and when administered orally through feed at regular intervals stimulates immune response and improve growth, survival and health status of shrimp.

## 1. Introduction

Fish and fishery products have been the most traded food items in the world reaching USD 152 billion in 2017, and at an annual growth of 8% and about 35% of global fish produce enters international trade. Shrimp along with salmon and some species of bivalves constitute one-fourth of the global aquaculture trade. India is the second-largest fish producing country in the world, and Indian aquaculture is expected to grow at 44.1% to 8.21 million tons by the year 2030 [1]. Frozen shrimp constitutes more than 85% of the country's USD 7.02 billion fish, and fishery product exports. Economic loss due to diseases has been one of the most important causes of concern in intensive shrimp culture operations worldwide, including India [2].

Globally, *Vibrio harveyi* is the most important bacterial pathogens causing mass mortalities in shrimp hatchery and retarded growth in grow-out farms leading to severe economic loss [3–7]. Further, *Vibrio* spp are reportedly involved in several of the shrimp diseases like oral and enteric vibriosis, appendage and cuticular vibriosis, localised vibriosis of wounds, shell disease, systemic vibriosis, septic hepatopancreatitis, tail rot disease, bacterial white tail disease and 'Bright-red' syndrome [7–10].

Raising consumer awareness of food safety has emphasised the need to develop novel microbial products for high health aquaculture. In the latter half of the century, antimicrobial agents gained prominence as effective therapeutic measure to control the bacterial diseases. However, the emergence of anti-microbial resistance has necessitated

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the development of alternative prophylactic/therapeutic measures to substitute the antimicrobials. With the implementation of regulatory restrictions on the use of undesired pharmacologically active substances in aquaculture, administration of immune-stimulating agents has been suggested [11]. The immune-stimulating ability of formalin-inactivated vibrio bacteria administered to marine invertebrates has been reported extensively [12–17].

Shrimp being an aquatic animal, is continuously challenged by microbial pathogens and in such a situation, disease incidences due to mixed aetiologies are observed. Commonly deformities and fouling over the appendages due to bacteria, fungal, parasitic or algal infection causes chronic poor health status in shrimp than acute mortalities [18,19]. Therefore, application of effective immunostimulant to elevate innate immunity of the shrimp cultured in farms will improve the health status and fight against the pathogens. Humoral immune agents like antimicrobial peptides (AMPs) play major role in eliminating such pathogens and elicit cascading secondary immune system. Effective antimicrobial activity of AMPs such as penaeidins, crustins and lysozymes ensures better health status of the shrimp [20].

Bacterial biofilms are well-structured, multicellular communities, capable of adhering and growing on various biological and inert surfaces [21]. They encase in self-produced extracellular (glycocalyx) matrix called extra polymeric substances (EPS) which are resistant to surfactants, antibiotics, antibodies and extracellular enzymes [22]. Artificially formed inactivated bacterial biofilms have been previously evaluated as vaccines against pathogens of human, veterinary and aquatic animals [23–28]. Bacterial biofilms express several additional antigenic proteins and protection by glycocalyx matrix from the digestive enzymes make them a highly suitable candidate for oral delivery [29]. Chitin, being natural, biodegradable and non-toxic material is a suitable substrate to grow bacterial biofilms which reportedly has the immune-stimulating activity [30,31]. *Vibrio alginolyticus* biofilm grown on chitin flakes has been reported enhancing the immune status of tiger shrimp, *P. monodon* [32]. The present work deals with optimisation and characterisation of *Vibrio harveyi* biofilm on chitin substrate, understanding additional antigenic proteins synthesised by the biofilm and its potential role as immunostimulant in production of high health *Penaeus vannamei* in commercial shrimp farms.

## 2. Materials and methods

### 2.1. Nutrient and substrate standardization for Vibrio harveyi biofilm (BF) production

The bacterium, *V. harveyi* isolated from a diseased shrimp (GenBank Accession No. JF 264473) was used in this study. Bacterial biofilm production was developed in nutrient-depleted (0.15%) Tryptone Soy Broth (TSB) supplemented with 2% NaCl with the addition of chitin as substrate [32] with modifications. Briefly, media was provided with chitin (India Seafoods, Cochin, India) as substrate at three different sizes 350, 350–850 and 850–1250 μm and at three different concentrations 0.3, 0.6 and 0.9%. *V. harveyi* culture was inoculated to each flask and incubated at 30 °C for 96 h with 6 h of agitation at 120 rpm every 24 h on a mechanical shaker while; free cell was grown in 1.5% TSB containing 2% NaCl for 24 h without addition of chitin substrate.

Biofilm was harvested by washing the chitin flakes in 50 mM phosphate-buffered saline (PBS) by gentle swirling to remove unbound cells and dislodged by vortex mixing in 10 mM PBS. The bacterial yield was quantified by plating the serially diluted biofilm or free cells on Tryptone Soy Agar Himedia, Mumbai, India supplemented with 2% NaCl and numbers were expressed as cfu/ml. Size and concentration of chitin flakes yielding highest biofilm were selected for further studies.

### 2.2. Biofilm biomass quantification using crystal violet assay

*V. harveyi* biofilm was grown on 350 μm chitin flakes (0.6% w/v) and sampled at 24, 48, 72 and 96 h for quantification of biomass by crystal violet assay [33]. The biofilm on chitin flakes was fixed in 99% methanol followed by staining with aqueous crystal violet (0.1%). The stain was eluted with acetic acid (33%) and the elute was measured at 590 nm (Tecan Spark10 M, Switzerland).

### 2.3. Characterisation of biofilm formation using scanning electron microscopy and laser scanning confocal microscopy

*V. harveyi* biofilm grown on 350 μm chitin flakes was sampled every 24 h until 96 h and processed for scanning electron microscopy (SEM) and laser scanning confocal microscopy (LSCM). For SEM, washed biofilms were fixed using Karnovsky's fixative on polylysine coated coverslips. Fixed biofilm was dehydrated using alcohol and layered with *t*-butyl alcohol for freeze-drying and sputter coated. All samples were imaged in a JSM-IT300 Scanning Electron Microscope (JEOL JSM-IT300, Japan) to observe the formation of the biofilm.

For LSCM, the biofilm was stained with acridine orange base (Sigma, USA) and visualized under a confocal laser scanning microscope (Carl-Zeiss, Germany). Images were obtained using LSM confocal software (LSM 700 ZEN).

### 2.4. Protein profiling

*V. harveyi* biofilm and free cells were grown and harvested, as explained earlier. Dislodged biofilm/free cells were pelleted by centrifugation at 8000*g* and subjected to SDS-PAGE analysis with 4% stacking and 12% resolving gel. Following the electrophoresis, the resolving gel was stained with Coomassie brilliant blue, and differential protein expression between the biofilm and free cell was recorded.

Proteomic analysis of *V. harveyi* biofilm and free cells was done using cytosolic and membrane protein fractions in PBS containing protease inhibitor cocktail (For bacteria, M307 Amresco). Cell lysates were quantified for protein concentration, followed by cysteine alkylation by iodoacetic acid and trypsin digestion. The digested protein samples were desalted and subjected to LC-ESI-MS/MS analysis (TripleTOF5600). The data obtained from LC-ESI-MS/MS was quantified by Sequential Window Acquisition of All Theoretical Fragment-Ion Spectra (SWATH) based on information-dependent acquisition (IDA) with false discovery rates (FDR) analysis at National Chemical Laboratory, Centre for Scientific and Industrial Research (CSIR), Pune, India.

### 2.5. Feeding trials

The *V. harveyi* biofilm was produced on 350 μm chitin flakes (0.6% w/v) as explained earlier. The biofilm biomass was inactivated using 5% formalin for 24 h at room temperature [32] and the complete inactivation was reconfirmed by inoculating into nutrient broth. The inactivated biofilm equivalent to $10^5$, $10^7$ and $10^9$ cfu/kg feed was prepared as feed top dressing with binder, 0.1% guar gum (Himedia, Mumbai, India). Feed coated with chitin flakes (0.6%) with and without inactivated free cell ($10^8$ cfu/kg feed) served as control.

*P. vannamei* (1.8 ± 0.13 g), were obtained from a commercial shrimp nursery, Nellore, Andhra Pradesh, India and acclimatised to laboratory conditions. Twenty shrimp each were randomly distributed to 50 L tanks in triplicate and fed experimental diets at 5% body weight thrice daily for 14 days. The percentage survival and growth were monitored at an interval of seven days. Another experiment was conducted by administering biofilm ($10^9$ cfu/kg feed), free cell and chitin for 48 h. The shrimp (n = 5) was sacrificed to collect gill tissue and stored in RNA protector (Takara Bio, India) for relative gene expression studies.

#### 2.5.1. Relative mRNA expression of immune related genes

Total RNA was extracted from gill using RNAiso Plus (Takara Bio,

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**Table 1**
Media and substrate composition was standardised to optimize the production of biofilm.

| Sl No. | Chitin (%) | Chitin size (μm) | Biofilm cfu/ml |
|---|---|---|---|
| | 0.3 | < 360 | $3.89 \pm 0.34 \times 10^{7c}$ |
| | 0.3 | 360–850 | $2.40 \pm 0.21 \times 10^{7d}$ |
| | 0.3 | 850–1250 | $4.53 \pm 0.11 \times 10^{4f}$ |
| | 0.6 | < 360 | $6.13 \pm 0.20 \times 10^{7a}$ |
| | 0.6 | 360–850 | $5.60 \pm 0.24 \times 10^{7b}$ |
| | 0.6 | 850–1250 | $4.00 \pm 0.15 \times 10^{4f}$ |
| | 0.9 | < 360 | $1.64 \pm 0.05 \times 10^{7e}$ |
| | 0.9 | 360–850 | $2.84 \pm 0.11 \times 10^{7d}$ |
| | 0.9 | 850–1250 | $1.48 \pm 0.01 \times 10^{7e}$ |

**Table 2**
Primer details used for relative quantification of immune genes in *Penaeus vannamei*.

| Sl No | Primer | Formaward | Reverse | Product size (bp) |
|---|---|---|---|---|
| | EF-1α | aaagatggttcccagcaagc | acggcaaaacgtccaaaagg | 72 |
| | Crustin | ttcggatcgcaggtttttgg | atcggtcgttcttcagatggtc | 92 |
| | Penaedin | acttttcaacgtcccagcag | tacaacgaaaggcagatggc | 86 |
| | Lysozyme | tcgcttggtttgtggcaatg | aaattcctgagccgaagtgc | 111 |

India) following manufacturer's instructions. Total RNA was subjected to DNase treatment (Recombinant DNase, Takara Bio, India) before reverse transcribed to cDNA using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio, India) and stored at −20 °C till further use.

Primers for gene expression study were designed using Primer3Plus (Table 2). Real-time PCR was carried out (7500 fast, Applied Biosystems®, USA) using SYBR® Premix Ex Taq™ II (Takara Bio, India) with initial denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 3 s and 30 s annealing at 60 °C. The relative expression level of penaeidin, crustin and lysozyme was calculated based on ΔΔ Ct method normalised with elongation factor 1-α as housekeeping gene.

### 2.6. Field trials

A preliminary field trial was conducted in a commercial shrimp (*P. vannamei*) nursery, Nellore, Andhra Pradesh, India. Three nursery ponds (1000 $m^2$, 1.5 m depth) each for treatment and control were fed with experimental diets. Medicated drug was prepared by topdressing the commercial feed to get the final concentration of $10^9$ cfu/kg feed and fed to the shrimp larvae for 7 days consecutively twice a month. Feeding behaviour was regularly monitored. The overall health status of the animals (n = 100) in three ponds were evaluated relative to the control group by comparing the anatomical deformities like antenna cut, rostrum cut and tail rot.

Statistical analysis was done using SAS 9.3. Significant difference between experimental groups was determined by one-way analysis of variance (ANOVA) with Tukey's Studentized Range (HSD) Test (α = 0.05). Student t-test was used for analysing the significant difference between two groups. The mean expression values and the standard error at each time point were determined.

## 3. Results

### 3.1. Yield optimisation of biofilm production on chitin flakes

Effect of size (< 360, 360–850 and 850–1250 μm) and concentration (0.3, 0.6 and 0.9%) of the chitin to enhance the yield of the biofilm production was studied. The study indicated highest yield of biofilm at 0.6% chitin of < 360 μm size ($6.13 \pm 0.20 \times 10^7$ cfu/ml) followed by 360–850 μm ($5.60 \pm 0.24 \times 10^7$ cfu/ml). Biofilm yield was inversely proportional to the size of the chitin at lower concentration (0.3 & 0.6%) however; this inverse relation was not observed at higher chitin concentration (0.9%) (Table 1).

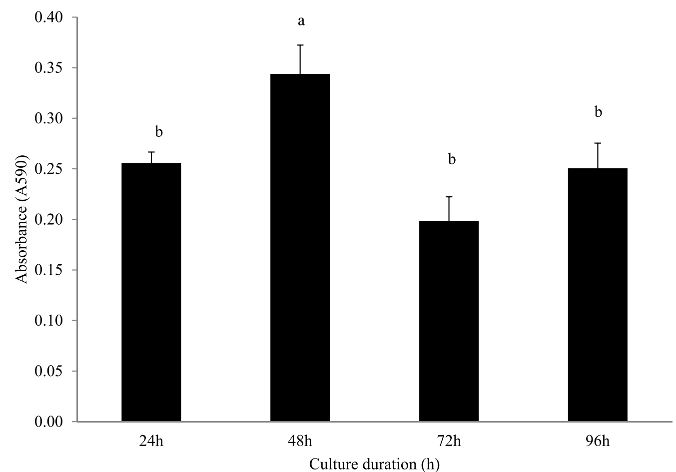


**Fig. 1.** Formation of *Vibrio harveyi* biofilm on chitin substrate, quantified using crystal violet assay. Vertical bars indicate standard error.

### 3.2. Biofilm biomass quantification

Biofilm biomass was quantified by crystal violet assay by measuring the absorbance at 24 h intervals. Intensity reached the peak at 48 h ($0.3439 \pm 0.0284$) culture, which was significantly higher ($p < 0.01$) than the absorbance at other time points (Fig. 1).

### 3.3. Characterisation of biofilm formation using scanning electron microscopy and laser scanning confocal microscopy

Development of the biofilm on chitin substrate was recorded at 24 h interval until 96 h in SEM. The images of *V. harveyi* biofilm at 24 h confirmed attachment of the bacterial cells on chitin surface. Maximum cell attachment was observed at 48 h. Attachment of bacterial cells on chitin substrate and formation of biofilm was compared with images of free cell and chitin substrate (Fig. 2).

Biofilm formation on chitin substrate was evaluated at 24 h interval until 96 h using LSCM. A progressive development of biofilm in the form of thick mat. The formation of biofilm was high at 96 h (Fig. 3) hence, 96 h incubation was considered for harvesting.

### 3.4. Antigen expression and proteomic analysis

Differential expression of proteins analysed using SDS–PAGE and LC-ESI-MS/MS shown an additional 6 proteins (21–45 kDa) in biofilm compared to free cell (Fig. 4). In total 24 proteins were identified to be common in biofilm and free cells, while 11 proteins were found to be differentially expressed in biofilm (Fig. 5). Six proteins (ATP-dependant Clp protease ATP-binding subunit, chaperone protein ClpB, nitronate monooxygenase-NMO, RecA bacterial DNA recombination family protein, beta-galactosidase-βGal and Urease) were significantly over-expressed while five proteins (D-erythrose-4-phosphate dehydrogenase, elongation factor Tu, glyceraldehyde-3-phosphate dehydrogenase, exoproteins, and one uncharacterized protein) were significantly repressed.

### 3.5. Effect of inactivated V. harveyi biofilm on immune response

The effect of oral delivery of inactivated *V. harveyi* biofilm on the relative expression of selected immune response genes (Lysozyme,

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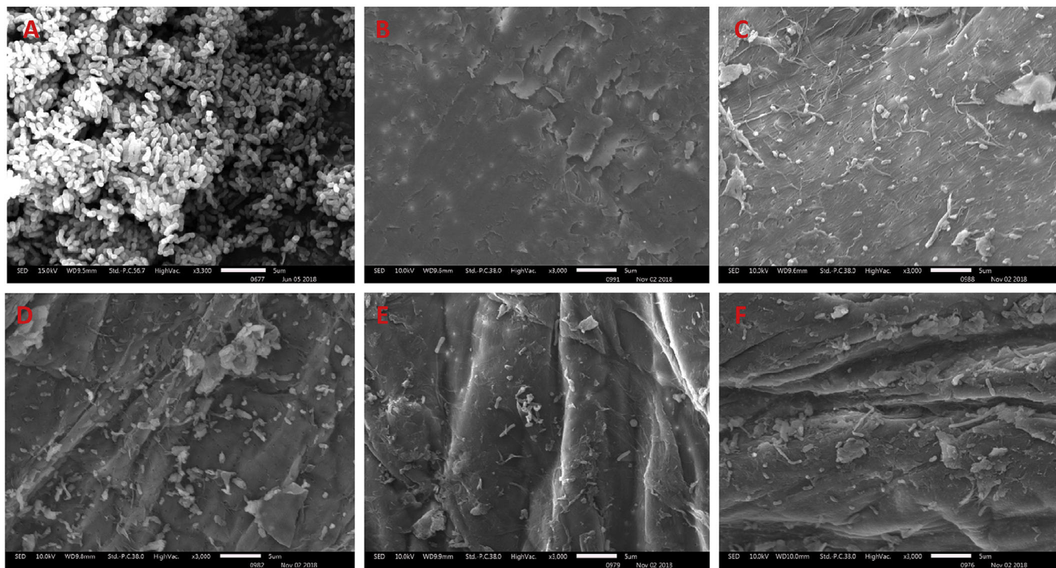


**Fig. 2.** Scanning Electron Microscope (SEM) images of free cell (A), chitin substrate (B) and progressive biofilm formation on chitin substrate at 24 (C), 48 (D), 72 (E) and 96 h (F).

Penaeidin and Crustin) in gill of *P. vannamei* was studied. Administration of the biofilm for a period of 48 h showed significantly elevated response of crustin and penaeidin compared to control, free cell and chitin groups in the shrimp. Both the AMPs over expressed in response to the biofilm treatment by 3.7 and 3.8-fold to control while the group administered with chitin substratum recorded no change from the control (Fig. 6). Though inactivated free cell administration marginally reduced response of the two AMPs, no significant difference was recorded. In the contrary, the treatment increased expression of lysozyme, where biofilm caused highest expression (7.1 fold) followed by chitin (6.7 fold) and free cell (4.5 fold) compared to control at 48 h treatment.

### 3.6. *Effect on growth and survival of pacific white shrimp P. vannamei*

Effect of inactivated vibrio biofilm on growth and survival was

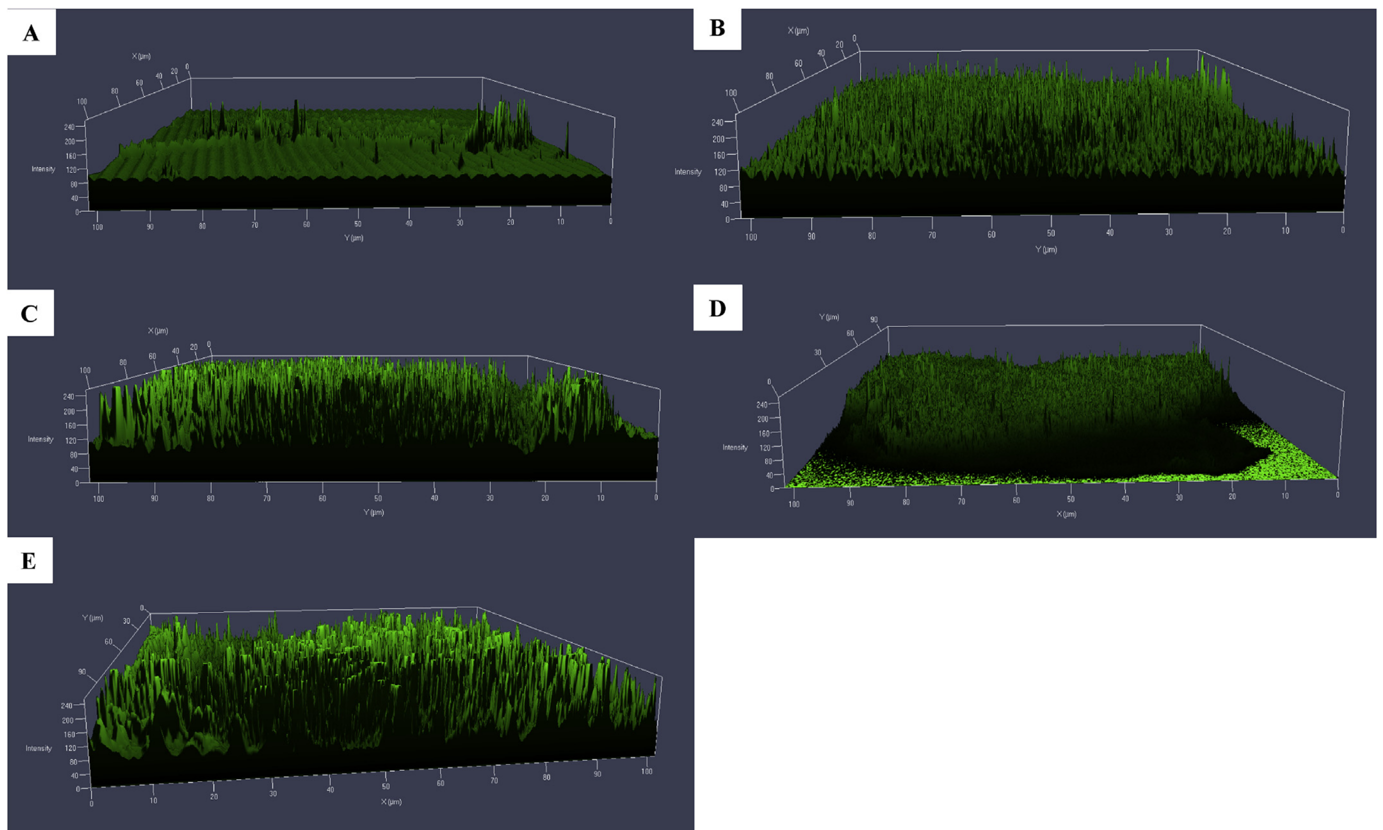


**Fig. 3.** Laser scanning confocal microscopy (LSCM) images of progressive biofilm formation of *Vibrio harveyi* on chitin substrate at 0 (A), 24 (B), 48 (C), 72 (D) and 96 h (E).

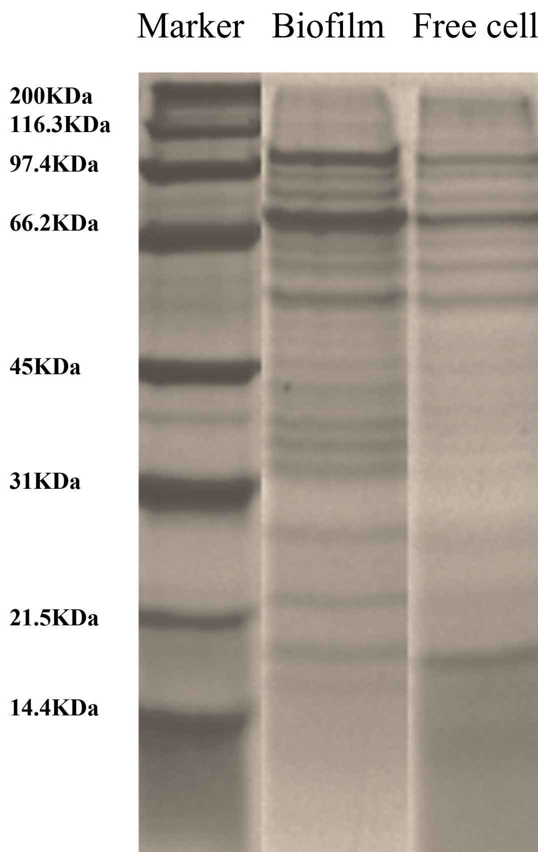


**Fig. 4.** SDS-PAGE profile of *Vibrio harveyi* biofilm showing the expression of six novel proteins (21-45 KDa) compared to free cell.

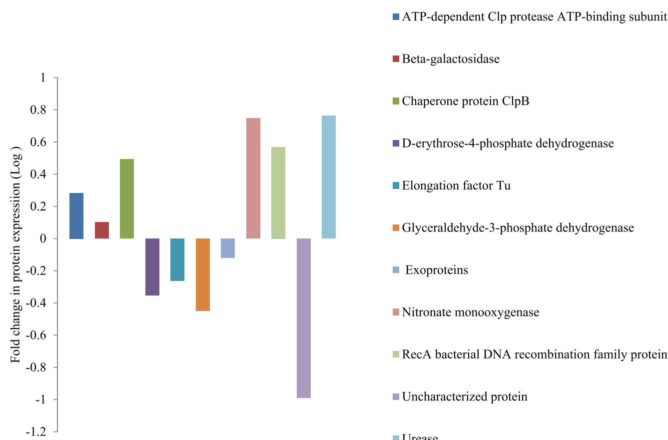


**Fig. 5.** Differential expression of proteins in *Vibrio harveyi* biofilm analysed using LC-ESI-MS/MS.

evaluated in yard experiments. Biofilm ($10^9$ cfu/kg feed) administered group showed significantly higher ($p < 0.05$) growth (2.66 ± 0.06 g) and survival (84.44 ± 1.82%) compared to control (2.23 ± 0.09 g; 76.33 ± 1.20%) and free cell (2.15 ± 0.03 g; 70.94 ± 0.66%) fed groups at the end of 2 weeks study period (Figs. 7 and 8).

In controlled nursery rearing trials, effect of biofilm coated feed on the general health condition of the cultured shrimp was observed as indicated by anatomical deformity assessment. The group administered with biofilm ($10^9$ cfu/kg feed) showed significantly ($p < 0.05$) lower levels of antenna cut (12.67 ± 0.66), rostrum cut (4.66 ± 0.87) and tail rot (3.33 ± 0.88) in comparison to control group (Fig. 9).

## 4. Discussion

There is a renewed interest in developing immune stimulating agents originating from plant, animal and microbes to control bacterial infections as an alternative to antibiotics in food producing animals including aquaculture. Bacterial biofilms are an excellent source of additional antigenic proteins and their potential as immunostimulating agents and vaccines has been reported in terrestrial and aquatic animals. Present study reports the application of vibrio biofilm as oral immunostimulant in *P. vannamei.*

Chitin is the widely used natural biodegradable substrate for formation of biofilms of *Aeromonas hydrophila*, *V. alginolyticus*, *V. parahaemolyticus, Salmonella typhimurium, V. cholerae* [23,32,34–37]. Concentration and size of the substrate determines the yield of biofilm. In the present study, effect of different size with concentrations on yield of biofilm showed < 360 μm chitin at 0.6% concentration was ideal. The observed importance of substrate size at lower concentrations could be attributed to enhanced surface area. This advantage was not observed in higher chitin concentrations, which might be due to saturation of culture conditions. This observation could be significant for cost effective industrial production of biofilm-based vaccine.

Biofilms are generally quantified using crystal violet staining assay [33,37]. The basic triarylmethane dye binds to negatively charged molecules such as bacterial cell surface proteins or components of the extracellular matrix and can be quantified after being re-dissolved using ethanol or acetic acid [37]. In the present study the highest biomass was observed at 48 h which stagnated thereafter. However, based on the confocal imaging, production of biofilm on the chitin surface was peaked at 96 has observed with production of *A. hydrophila* biofilm [23] and at 72 h in *V. alginolyticus* [29] and *V. parahaemolyticus* [34]. This difference in the observations could be attributed to the bacterial species, size and concentration of the substrate used and method of quantification used.

Proteinaceous components of the bacterial biofilms include molecules involved in adhesion, cellular appendages and other proteins which are known to have antigenic and immunogenic properties [38]. Several additional proteins have been reported to be expressed in bacterial biofilm cells compared to free cells [29] in response to stress for survival under hostile environmental conditions [39]. SDS-PAGE analysis of *V. harveyi* in this study revealed an additional expression of six proteins (21–45 kDa). In similar studies three extra proteins of 22–72, 45–55 and 32–55 kDa respectively in *A. hydrophila* [29], in *V. alginolyticus* [32] and in *V. parahaemolyticus* [34] were observed.

The proteomic analysis using LC-ESI-MS/MS, revealed 11 proteins specific to *V. harveyi* biofilm, among which 6 were overexpressed and 5 were repressed. Immunogenic urease and β-Gal expressed in *V. harveyi* biofilm might be involved in enhanced immune response and general health condition in biofilm fed *P. vannamei*. Ureases are found in numerous bacteria, fungi, algae, plants, and some invertebrates to protect against unfavourable conditions and also known to act as soil enzyme [40]. Since, it activates monocytes, neutrophils leading to the secretion of inflammatory cytokines, urease has been considered as a vaccine candidate for several bacterial and viral pathogens [41]. Similarly, structurally integrate βGal reported to induce T-Helper Type-1 immunity in the host [42]. The overexpressed proteins ATP-dependant Clp protease, ATP-binding subunit and chaperone protein ClpB are kind of heat shock proteins mainly involved in a prevention or correction of damage caused by misfolding of proteins [43–46]. Though function of NMO in bacteria is not clearly understood, it is suggested to be involved in detoxification, virulence and biofilm formation [47,48]. Further, the RecA protein has also been reported to be involved in stress induced biofilm formation [49,50]. Role of these proteins in candidate aquaculture species is scarce. Further studies on immunogenic function of these proteins will help in developing novel immune stimulating agents.

*P. vannamei* fed with biofilm cells of *V. harveyi* could provide better growth and survival compared to free cell and control diet fed animals.

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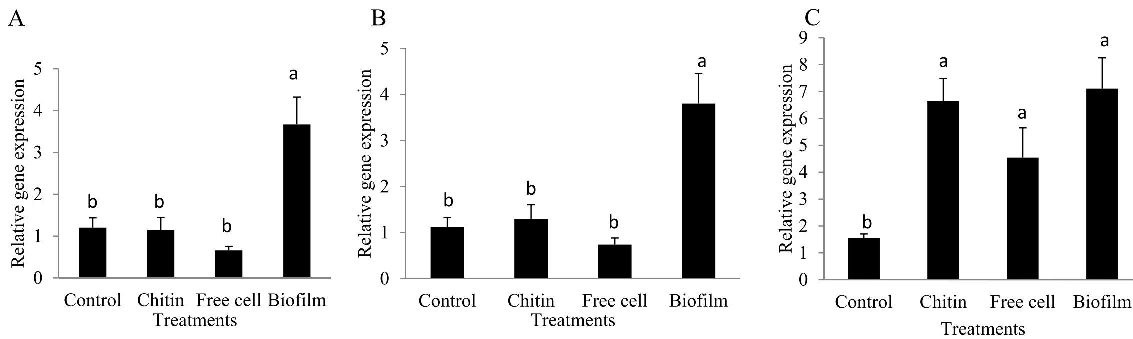


**Fig. 6.** Relative immune gene expression in gill of *Penaeus vannamei* (n = 5), orally administered with $10^9$ cfu/kg feed of *Vibrio harveyi* biofilm as compared with $10^8$ cfu/kg feed of *Vibrio harveyi* free cell and chitin. Expression level of Penaeidin (A), Crustin (B) and Lysozyme (C) was normalised against internal control elongation factor 1-α.

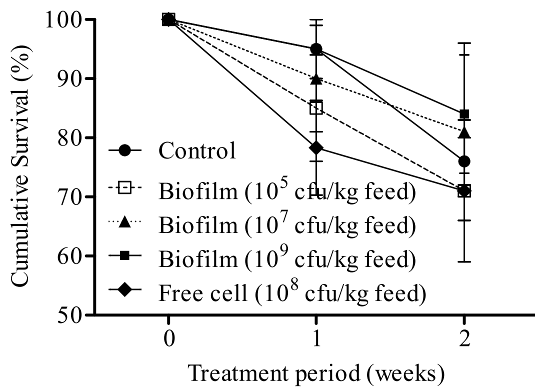


**Fig. 7.** The survival pattern of shrimp post oral administration of different concentration of *V. harveyi* biofilm and free cell. Biofilm ($10^9$ cfu/kg feed) administered group showed significantly higher ($p < 0.05$) survival, two weeks post administration.

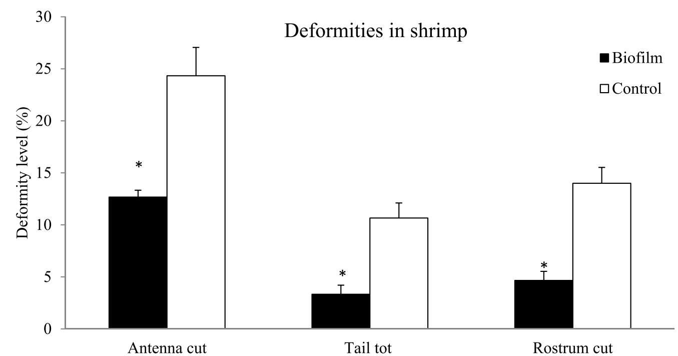


**Fig. 9.** Effect of oral administration of *V. harveyi* biofilm vaccine on percentage of animals with appendage deformities (mean ± SE) in *P. vannamei* nursery rearing (n = 3). Shrimp fed with biofilm diet had significantly less deformities ($p < 0.05$).

The biofilm treatment for 48 h significantly improved the expression of penaeidin, crustin and lysozyme in shrimp. Interestingly continuous feeding for 14 days showed supressed response of these genes (data not shown) suggesting, the possible tolerance effect as previously reported [51]. The crustaceans lacking adaptive immune system, entirely depend on innate immune response in its fight against the infectious pathogens. Furthermore, the AMPs play pivotal role in their first line of defence system, hence elevated level of expression of penaeidin, crustin and lysozyme could be crucial in anti-microbial immune response [20]. The present trial in shrimps confirmed oral administration of the biofilm for a shorter spell could effectively enhance the AMPs in shrimp and support the host in its fight against pathogens. Further detailed studies may elucidate the mechanism of immune response in biofilm fed animals. The increase in survival and growth, immune response in biofilm fed animals may be correlated to the sustained antigen delivery from biofilm, since the cells are embedded in the glycocalyx matrix facilitating a slow and sustained release of antigens [23]. Uptake and processing of *A. hydrophila* biofilm antigens by oral route have been demonstrated in carps [52]. Sharma et al. [27] showed the protective response of *V. alginolyticus* biofilm delivered orally to *Penaeus monodon*. Increased antibody titre and protective response against *A. hydrophila* has been demonstrated by oral delivery of *A. hydrophila* biofilm in carps and catfish [23,25,28].

Anatomical deformities like antennae cut, tail rot and rostrum cut

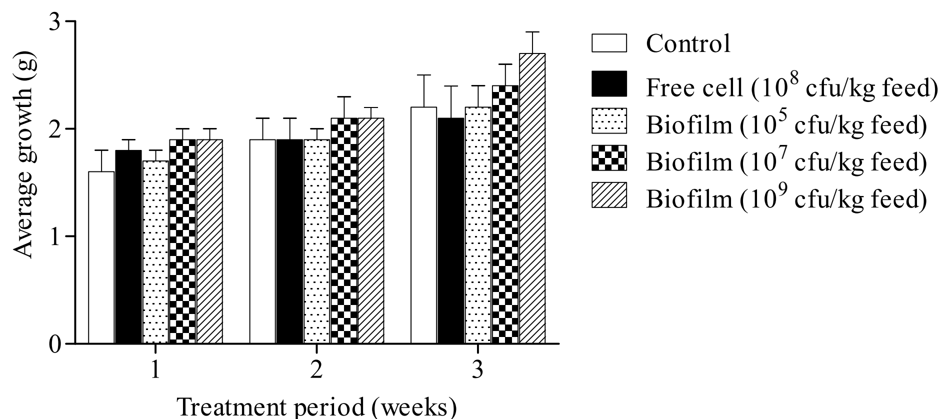


**Fig. 8.** Weight gain of shrimp post oral administration of different concentration of *V. harveyi* biofilm and free cell. Biofilm ($10^9$ cfu/kg feed) administered group showed significantly higher ($p < 0.05$) growth, two weeks post administration.

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could be an indirect indicator of suppressed immune status leading to possible subclinical bacterial infections in crustaceans [18]. Effect of immune stimulating agents on overall health status of the shrimp could be evaluated by monitoring occurrence of these deformities [8,19]. In the nursery trials, significant reduction in anatomical deformities observed could be attributed to enhanced immune status following application of the biofilm product, similar to previous observation after administration of inactivated vibrio bacterin [19].

Oral delivery of microbial biofilm product as 'immune stimulating agent' could be a novel approach for high health shrimp farming. Production of high yield *V. harveyi* biofilm on chitin substrate expressing immunogenic proteins when administered orally improve immune response, growth, survival and health status of *P. vannamei* both in laboratory and field trials. Observations of the present study in conjunction with previous studies using *A. hydrophila, V. alginolyticus* and *V. parahaemolyticus* biofilm suggest possible role of biofilm based microbial products as immunomodulation agents in farmed food animals.

## Declaration of competing interest

No conflicts of interests are declared.

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