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Studies on Development and Antigen Expression of Biofilm Cells of *Vibrio anguillarum* for Oral Vaccination in Aquaculture

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ABSTRACT

Background: *Vibrio anguillarum* is one of the major pathogen causing economic loss in the aquaculture industry. And, most of the microorganisms will use biofilm strategy for their survival in the host. Hence, less effectiveness of antibiotic and free cell (whole cell) vaccine may be observed in the aquaculture practice. Therefore the developed vaccine should be the mirror image of pathogen molecule, in regard; the present study was carried out to standardize the nutrient requirement for optimum biofilm production and antigen expression of biofilm and free cells of *Vibrio anguillarum.* Also, to identify the deferentially expressed protein in the biofilm mode.

Methods: Culture conditions were optimized for the maximum biofilm production of *V. anguillarum* on chitin flakes as a substrate in nutrient restricted conditions. Through proteomic approach such as SDS PAGE was conducted to know the protein profile of biofilm and free cells of *V. anguillarum* and further, nanoLC-MS/MS was used to identify the differentially expressed protein in the biofilm of *V. anguillarum*

Result: Maximum biofilm production was observed on the 3rd day on 0.1% TSB concentration supplemented with 0.3% chitin flakes and 2% NaCl. Significant changes in the antigen expression of biofilm of *V. anguillarum* were observed in SDS PAGE and it revealed that in the biofilm mode, three new novel proteins were expressed and about ten proteins repressed as compared to that of free cell counterpart.

Key words: Antigen expression, Biofilm, Oral vaccine, *Vibrio anguillarum*.

INTRODUCTION

Oral administration is highly advantageous compared to other methods being most convenient, economical, less time and labor-consuming and can be mass administered over a large area with minimum stress to the fish. Although oral vaccination is the preferred method from a fish farmer's perspective, the problems like poor immune response due to poor antigen delivery to immune responsive sites. This is because of low pH and high enzymatic as activity, the vaccine is destroyed in the foregut and causes poor antigen delivery to the hindgut and other lymphoid organs.

Biofilm of bacterial pathogen, having glycocalyx coat is considered appropriate to avoid destruction in foregut and also, biofilm antigen could be an appropriate vaccine candidate, as many pathogens exist in the host in biofilm mode (Lewis, 2001). Against this background, biofilm of *A. hydrophila* developed on chitin flakes has been successfully utilized as an oral vaccine in catla, rohu and common carp and obtained significantly higher antibody titer and protection than free cell vaccinated fishes (Azad *et al.,* 1997; 1999). Later biofilm of *A. hydrophila* was also evaluated for oral vaccination of walking catfish *Clarius batrachus* (Nayak *et al.,* 2004) and murrels *Channa striatus* (Siriyappagouder *et al.,* 2014). Biofilm of *V. alginolyticus* as immunostimulants to study the preliminary immune response in tiger shrimp (*Penaeus monodon*) showed that biofilm cells were superior to free cells in stimulating the non-specific immune response of *Penaeus monodon* (Sharma *et al.,* 2010).

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The biofilm acts as a biocapsule due to the presence of glycocalyx membrane resulting in safer transport of vaccine to the absorptive hindgut. Consistence high serum antibody concentration also indirectly supports the better performance of biofilm cells than free cells as fish do not possess physiological distinct memory cells; rather it appears to rely upon the increasing size of the pools of antigen sensitive cells. Furthermore, biofilm antigens are believed to be a mirror image of a pathogen *in vivo* and hence most ideal for a vaccine candidate. It has also been shown that the biofilm dependent changes in gene expression of bacteria lead to the production of candidate protein that can be used as antigens (Marco *et al.,* 2006).

In India and worldwide, the expansion of brackishwater aquaculture and mariculture, with intensified farming has led increase outbreak of vibriosis both in fish and shellfish caused by *Vibrio anguillarum*. In order to produce biofilm oral vaccine against *Vibrio anguillarum,* this study was carried out to standardization of nutrient requirement for optimum biofilm production and antigen expression of biofilm and free cells of *Vibrio anguillarum.*

MATERIALS AND METHODS

Vibrio anguillarum isolate

Vibrio anguillarum isolate was obtained from the Cochin University of Science and Technology (Dr. I.S. Bright Singh) and grown in 1.5% Tryptone soya broth with 2% NaCl. The isolate was confirmed by polymerase chain reaction (PCR) (Gyeong-Eun *et al.,* 2007). Further, pathogenicity of *V. anguillarum* was confirmed by infecting into *Lates calcarifer* and detected the pathogen in the infected tissues by PCR.

Standardization of nutrient requirement for optimum biofilm production of *V. anguillarum*

It was carried out according to Azad *et al.* (1997) and Sharma *et al.* (2010) with slight modifications. Briefly, six different concentration of TSB (0.025, 0.05, 0.1, 0.15, 0.2 and 0.25%) supplemented each with 2% NaCl and chitin flakes (0.3%, W/V) as substrate was prepared in conical flasks in triplicates and the media was autoclaved. One ml of *V. anguillarum* culture in log phase was inoculated to each flask and incubated at room temperature for 24 h with 6 h of agitation at 120 strokes per minute on a mechanical shaker. After incubation, the supernatant was carefully decanted into six sterile flasks. The chitin flakes were washed 3 times with sterile phosphate buffer saline (PBS) pH 7.2 by gently swirling the flasks three times in order to remove unbound cells. Chitin flakes with biofilm cells were collected in 10 ml PBS in a 50 ml sterile centrifuge tube and agitated for 4 min in a vortex shaker to dislodge the cells. The supernatant (1ml) containing the biofilm cells were serially diluted 10 fold in sterile physiological saline. From each dilution, 0.1 ml was transferred to trypticase soya agar (TSA) (supplemented with 2% NaCl) plates and spread uniformly. The plates were incubated for 24 h at room temperature. A number of colonies counted was expressed as cfu/g chitin flakes. The TSB concentration which gave the highest cfu/g chitin flakes of biofilm cells were considered for further studies. The supernatant containing planktonic cells was centrifuged at 1500 X g for 15 min and washed thrice with sterile PBS. The pellet was resuspended in 10 ml PBS and serially diluted. From each dilution, 0.1ml was placed on TSA plates and spread uniformly. After incubation for 24 h at room temperature, the colonies were counted and expressed as cfu/ml.

Growth kinetics of biofilm and planktonic cells of *V. anguillarum*

It was carried out according to Azad *et al.* (1997) and Sharma *et al.* (2010) with slight modifications. After determining appropriate TSB concentration for growth, *V. anguillarum*, six flasks in triplicate each with 0.3% (W/V) chitin flakes in 0.1% TSB supplemented with 2% NaCl were autoclaved. The flasks were incubated with 1ml of the inoculums of *V. anguillarum* in log phase for 6 days at room temperature with 6 h of agitation per day at 120 strokes/ min on a mechanical shaker. At the end of 1, 2, 3, 4, 5 and 6 days, the flasks were removed. Biofilm and planktonic cells were harvested as mentioned above and mean TPC was determined.

Analysis of protein profile of biofilm and free cells of *V. anguillarum* by SDS-PAGE: *Preparation of biofilm cells*

Three day old biofilm cells of *V. anguillarum* on chitin flakes were washed three times with PBS and harvested by vortex mixing in PBS with 1mM phenylmethylsulphonyfloride (PMSF). The biofilm mass was pelleted after centrifugation at 86,400 g for 30 min at 4°C.

Preparation of free cells

One-day-old free cell of *V. anguillarum* which was grown on 1.5% TSB with 2% NaCl was centrifuged at 7, 100g for 10 min and the cells were harvested and washed thrice in sterile PBS (pH 7.2).

Free cell and biofilm cells were solubilized separately in 4x sample buffer (500mM Tris-HCL pH 6.8, glycerol 0.8ml, 10% (w/v) SDS 1.6ml, 2- β mercaptoethanol 0.4ml, 0.05% (w/v) bromophenol blue 0.02%) and vortexed well. The solution was then boiled at 100°C for 1 min and spun at 5000 rpm for 2 min to settle down the debris. The supernatant (60 µl) was loaded on to 4.5% stacking gel and electrophoresed according to Laemmli in a 15% separating gel along with protein molecular weight markers (Broad range, Biorad, USA) at 150 V in a gel electrophoresis system. After electrophoresis, the resolved protein bands were stained with coomassie brilliant blue (0.25% (W/V) in 40% methanol and 10% glacial acetic acid solution) for 1 h. The stained gel was destained in 40% methanol and 10% glacial acetic acid till the background is clear. Differential protein expression between the biofilm and free cell was determined by comparing with standard protein markers.

Mass spectrometry analysis

Differentially expressed biofilm proteins were excised followed by gel digested by alkylation and reduction method standardized at NCBS facility. Tryptic digested peptides were subjected to standard nano-RP-LC (70 min gradient is required due to the complexity of the sample) run, followed by LTQ-Orbitrap MS/MS analysis. A separate 12.5fmol of standard BSA digest was injection as a quality control. Results were analyzed on MASCOT using proteome discoverer 1.3 by having Swissprot as database. High confident peptides with the prerequisite of minimum two peptides leading into the identification of proteins were selected and the list was generated.

RESULTS AND DISCUSSION

Standardization of nutrient requirement for optimum biofilm production of *V. anguillarum*

The *Vibrio anguillarum* isolate was confirmed by PCR, which showed an amplified DNA fragment of the expected size of 429bp (Fig 1).

Among the various concentrations studied, the highest CFU of BF cells was obtained with 0.1% TSB supplemented with 2% NaCl and 0.3% chitin flakes (Fig 2). Generally, it is known that bacteria form biofilm in nutrient-deficient conditions (Morita, 1997; Hall-Stoodley and Stoodley, 2005). Our present study confirms that among various concentrations, low concentration of TSB (0.1%) forms a better growth of biofilm. In *A. hydrophila* (Azad *et al*., 1997) and *V. alginolyticus* (Sharma *et al*., 2010), it was observed that favorable concentration for the development of biofilm was 0.225% and 0.15% TSB respectively.

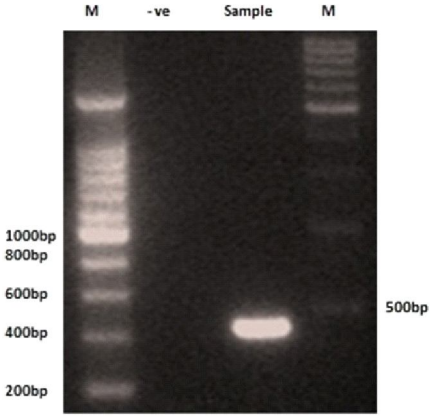

Fig 1: Confirmation of *V. anguillarum* isolate by PCR: Lane M: 200bp Marker, Lane -Ve: Negative Control, Lane sample: V. anguillarum isolate, Lane M: 500bp marker.

Growth kinetics of biofilm and free cells of *V. anguillarum*

Total plate count of biofilm on chitin and free cells was estimated for 6 days. Biofilm density increased from 0 hours reaching a peak of 72 hours and then decreased gradually. On the other hand, planktonic cells increased for 0 hours to 48 hours (peak) followed by decreasing.The highest CFU g^{-1} of chitin flakes was obtained on the third day of incubation that was significantly higher than the CFU on 1, 2, 4, 5, 6^{th} day. The highest CFU ml^{-1} of free cells was obtained on the second day of incubation (Fig 3). Growth kinetics of *V. anguillarum* with time indicates an increase in biofilm population with a corresponding decrease in the population of free cells. Similar observations were made in case of *Staphylococcus aureus* (Anwar *et al.,* 1992), *A. hydrophila* (Azad *et al.,* 1999), *V. alginolyticus* (Sharma *et al.,* 2010) and *Escherichia coli* (Divya and Masiamoni, 2011). The purpose of nutrient standardization and growth kinetics of

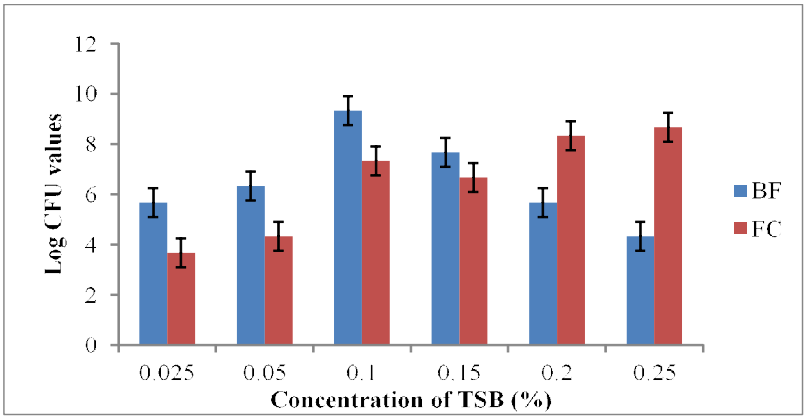

Fig 2: Growth of BF and FC of *V. anguillarum* in different nutrient concentrations (vertical bar indicates SD).

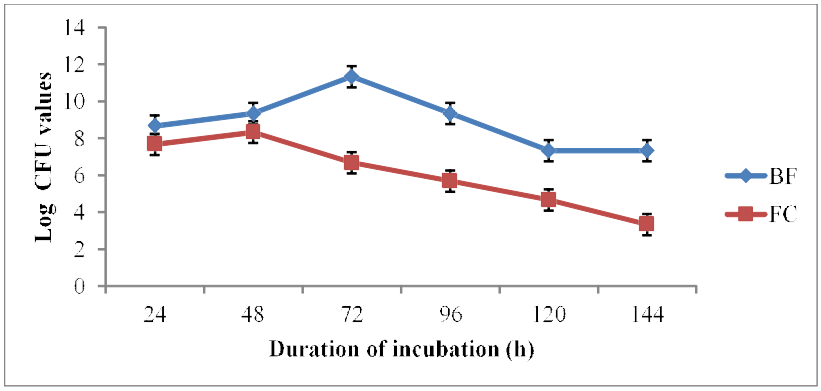

Fig 3: Growth kinetics of *V. anguillarum* BF and FC in 0.1% TSB (vertical bar indicates SD).

V. anguillarum is to optimize the *in vitro* culture conditions for biofilm production.

Protein profile of biofilm and free cells of *V. anguillarum*

The SDS PAGE analysis of biofilm and free cell of *V. anguillarum* revealed expression of 3 new novel proteins

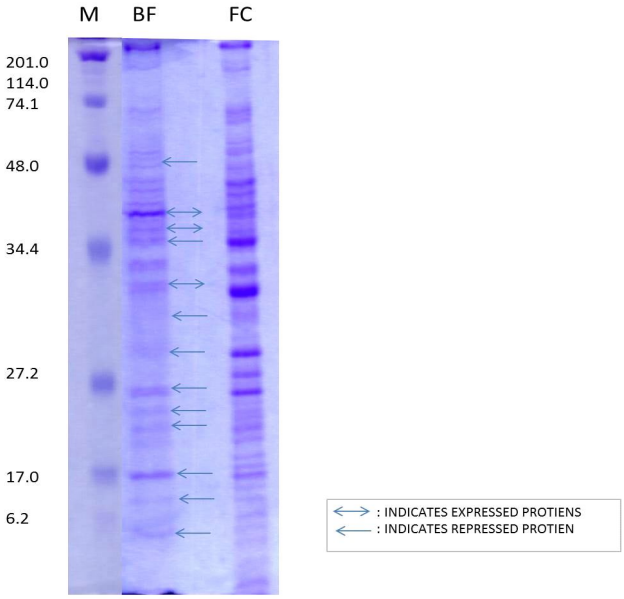

Fig 4: SDS-PAGE of free cell and biofilm cell of *V. anguillarum*.: Lane M: Markers (broad range, Biorad), Lane BF: Biofilm cell, Lane FC: Free cell.

(32, 35, 38kda) represent the highest confidence score for the outer membrane protein A (Omp A) observed in 32 and 35 kDa bands (Fig 4) showed a protein sequence coverage of 70% and 60% coverage for 38 kDa band represent Elongation factor Tu 1 (Table 1). The repression of 10 proteins (4, 5, 14, 19, 21, 28, 29, 31, 34, 48kda) of biofilm cells compared that from free cell counterpart (Fig 4). Expression of extra proteins in biofilm cells could be a stress response which might help the bacteria to survive in the new mode of life (Anwar *et al.,* 1992) or variation in physiological and phenotypical difference (Reisner *et al.,* 2003). Earlier studies have shown that as many as 30-40% of the proteins present in bacterial cell walls differ between sessile and planktonic bacteria (Coghlan, 1996). Furthermore, BF of *P. aeruginosa* developed on glass wool 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, it was observed that starved cells of *Vibrio sp.* lost many cellular proteins and synthesized new proteins called starvation proteins (Amy and Morita, 1983; Nelson *et al.,* 1997). Similarly in our lab, *in-vitro* culture of biofilm of *A. hydrophila* had found to be expression of 3 new protein and repression of 9 proteins (Amy and Morita, 1983) and biofilm of *V. alginolyticus* had found to be expression of 3 new proteins and repression of 4 proteins (Sharma *et al*., 2010), biofilm of *Pseudomonas putida* with additional expression of 15 new proteins (Sauer and Camper, 2001)

Table 1: Identified peak spectra of expressed protein in Nano-LC/MS/MS using Proteome Discover 1.3 software.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	Area	# AAs	MW [kDa]	calc. pI
P24016	Outer membrane protein A (Fragment) OS=Citrobacterfreundii GN=ompA PE=3 SV=1 - [OMPA_CITFR]	1787.81	70.17	1	5	12	53	6.365E8	238	25.6	5.11
P24754	Outer membrane protein A (Fragment) OS=Escherichia hermannii GN=ompA PE=3 SV=1 - [OMPA_ESCHE]	1770.32	60.49	1	4	11	54	6.443E8	243	26.2	5.49
A7ZSL4	Elongation factor Tu 1 OS=Escherichia coli O139:H28 (strain E24377A / ETEC)	1980.47	57.61	1	0	15	49	1.008E9	394	43.3	5.45
A8A779	Elongation factor Tu 2 OS=Escherichia coli O9:H4 (strain HS) GN=tuf2 PE=3 SV=1 - [EFTU2_ECOHS]	1980.47	57.61	1	0	15	49	1.008E9	394	43.3	5.45
A9MHG0	Elongation factor Tu OS=Salmonella arizonae (strain ATCC BAA-731 / CDC346-86 / RSK2980) GN=tuf1 PE=3 SV=1 - [EFTU_SALAR]	1980.47	57.61	1	0	15	49	1.008E9	394	43.3	5.45

and biofilm of *P.aeruginosa*with additional expression of 2 new proteins (Anwar *et al.,* 1984). The proteins expressed in a biofilm mode or *in vivo* could be a mirror image for vaccine development (Asha *et al.,* 2004). In general, biofilms are resistant to antibiotics, antibodies, heat, surfactants, biocides and phagocytic cells due to the presence of glycocalyx coat. This resistance nature of biofilm will be helpful to overcome poor and inconsistent performance of free cell vaccine, due to the destruction of vaccine in stomach or foregut before vaccine antigen reaching immune responsive areas of hindgut and lymphoid organs like spleen and kidney (Azad *et al.,* 1999). Biofilm of *A. hydrophila* in fish (Azad *et al.,* 1999; Nayak *et al.,* 2004) and *V. alginolyticus* in shrimp (Sharma *et al.,* 2010) as oral vaccines have been evaluated successfully with good antibody response and protection. This concept of biofilm oral vaccine was also tried in veterinary to successfully vaccinate broiler chicks against *E. coli* (Shivaraj and Krishnappa, 2002).

In *Vibrio anguillarum* OM2 is an OMP of 86 kDa is induced under iron limitation. The gene responsible for coding this protein is present on the virulence plasmid pJM (Actis *et al.,* 1999). Studies have emphasized the role of the OMPs in protective antigenicity as they are highly immunogenic, with exposed epitopes on the cell surface that are conserved in different serovars. The 38 kDa major Outer membrane porin (OMP) protein of *Vibrio anguillarum* is bile resistant and stimulates biofilm formation (Wang, 2003). Resistance to bile is important for the bacterium to colonize the intestine of the fish host and biofilm formation provides an adaptive and survival advantage for bacteria in the aquatic environment. It is a highly immunodominant bacterial antigen. Therefore OMPs considered ideal candidates in vaccine development (Koebnik *et al*., 2000; Rahman and Kawai, 2000). In this study, we have observed expression nearly 38Kda protein in biofilm mode which can be considered as a major candidate antigen for development of vaccine.

Further, mass spectrometry used to detect the proteins which were expressed in the SDS-PAGE and in two samples identified proteins with their unique peptides belonged to OmpA and one elongation factor Tu 1 without any unique peptide which showed similarity to OMPA_CITFR and *E. coli* (Table 1). The expressed proteins can be useful for further expression study using quantitative proteomics.

The present study determined 0.1% TSB as appropriate nutrient concentration for the development of biofilm of *V. anguillarum*. At this concentration, the highest biofilm concentration was achieved at 72 hrs post-inoculation. The biofilm cells had a different protein profile, with 3 or 4 new expressed proteins and repression of 10 proteins. The additional proteins may be suitable antigen candidates for vaccine development.

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