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Effective Vaccine [Outer membrane protein and Whole cell] Development from *Pseudomonas fluorescens* against Septicemic disease

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Abstract

Infectious diseases are the main cause of economic losses in aquaculture industry which is negatively impacted by various pathogenic organism (Plumb,1997). *Pseudomonas fluorescens* is a common Gram – negative bacterial pathogens associated with Septicemia / Red Skin disease in Farmed fishes and lead to mass mortality and morbidity in commercially important fishes. Fish vaccination is safe and effective method to prevent and control the infectious diseases in aquaculture industries. The present study was aimed to develop the outer membrane protein (OMP) and whole cell (WC) vaccines from ATCC 13525 *Pseudomonas fluorescens* against Bacterial septicemia /Red Skin disease. The prepared vaccines were resolved in 10% SDS –PAGE to generate Protein profiles and above proteins was quantified by following the protocol of Lowry's method. The SDS – PAGE results showed that the OMP vaccine had 8 Polypeptide bands with molecular weight of 121.80 to 3.70 KDa and WC vaccines had 11 Polypeptide bands with molecular weight of 248.00 to 9.89 KDa. The total protein concentration of OMP and WC vaccines were estimated as 100 µg/ml and 66 µg/ml respectively. The Immunoproteomic vaccines (OMP and WC) might be provide protection against bacterial pathogens in the aquaculture industries due to its safe, low cost, and long lasting immunity.

Keywords: *Pseudomonas fluorescence*, Septicemia, Vaccine, OMP, WC.

Introduction

Aquaculture in India plays an important role in the socio-economic development by contributing to the economic development, production in foods, employment opportunities and produce foreign exchange. India ranks third among the world fresh water fish producers (FAO, 2003) with Indian major carps *viz, Cirrhinus mrigala, Labeo rohita, Catla catla*. The rapid development of aquaculture allied to intensive production systems favour stress condition in captive fish, leading to disease and economic losses (Schreck, 1996). During culture, significant losses due to variety of infectious agents are reported. Among these, the bacterial pathogens belonging to the genus *Aeromonas, Pseudomonas, Edwardsiella, Flavobacterium*etc, are mainly responsible for severe mortality and morbidity of Indian major carps (Bootsma*et al.*, 1997; Kumar *et al.*, 1986; Shome *et al.*1996).

Pseudomonas fluorescens, is a Gram – negative, Obligate aerobic, motile, rod-shaped bacteria belonging to the Family of *Pseudomonadaceae*, is the recognized bacterial pathogens that commonly associated with reared aquaculture species. *Pseudomonas fluorescens* has multiple flagella and the specific name *fluorescens* refers to a microbes secretion of a soluble fluorescent pigment called pyoverdin, which is a type of siderophore (C D Cox and P Adams., 1985). *Pseudomonas fluorescens* is a ubiquitous bacterium and it has

the ability to adopt to thrive in soil and on plants and aqueous surfaces. It is considered a secondary invader of damaged fish tissues, but also be a primary pathogen of fish (Roberts and Horne,1978). It can cause Septicemic disease/Red Skin disease, which is characterized by haemorrhage, scale falling off, Fin rot and tail rot. Recently *Pseudomonas fluorescens* causing septicemia in silver carp and heavy mortality in big head has been reported in Hungary (Csaba*et al.,* 1981).

Human infection have been mainly nosocomial in immune compromised or seriously ill patients, some of which resulted in serious and occasionally fatal disease. In recent years several clinical strains of *Pseudomonas fluorescens* have been found to be able to survive at 37˚c (Chapalain *et al.,* 2007) despite being considered as psychrophile, with an optimum temperature of 25-30˚c (Balachander and Vendan, 2007) leading to belief that human physiological temperature is not a barrier for the microorganism. *Pseudomonas* bacterium is a difficult medical problem because of its high mortality and frequent occurrence of multiple antibiotic – resistant organisms. The development and sustainability of aquaculture industries depends on control the infectious disease problems without relying on antibiotics and chemotherapeutics. Thus, vaccines for fish and shell fish are being sought by the industry.

A number of different types of vaccine have been developed in fish against Gram – negative, such as whole cell (WC), outer membrane protein (OMP), extra cellular products (ECP), lipopolysaccharides (LPS) and biofilms (BF). These effective vaccines have provided varying degrees of protection in fish. Still until now no commercial vaccines are available for *Pseudomonas fluorescens* (Wang *et al.,* 2009). Therefore, the present investigation was carried out to develop the bacterial OMP and WC vaccines from *Pseudomonas fluorescens* against Septicemia / Red skin diseases in fishes.

Objectives:

To culture the virulent Bacterial strain (ATCC 13525) *Pseudomonas fluorescens*

To prepare the effective vaccines (outer membrane protein vaccine and whole cell vaccine) from the bacterial culture.

To analyse the proteins present in the vaccines by SDS-PAGE and LOWRY method.

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Materials and Methods

Bacterial Strain:

Pseudomonas fluorescens (ATCC 13525) was used as a reference strain.

Bacterial Culture:

The pure culture of the bacterial strain were grown on Nutrient agar slants for 24 to 48 hours at temperatures of 28˚C and it was maintained in -80˚C as glycerol stock for further studies. The fluorescent colonies were detected by viewing under UV light in nutrient agar medium (Fig-1). The Bacterial isolate (*Pseudomonas fluorescens* ATCC 13525) were further inoculate on Nutrient broth medium at temperatures of 28˚c overnight and pH 7.5 was maintained for the growth. After 24 hours Bacterial culture, were used for vaccine preparation following the methods of Thangaviji *et al.,* (2013).

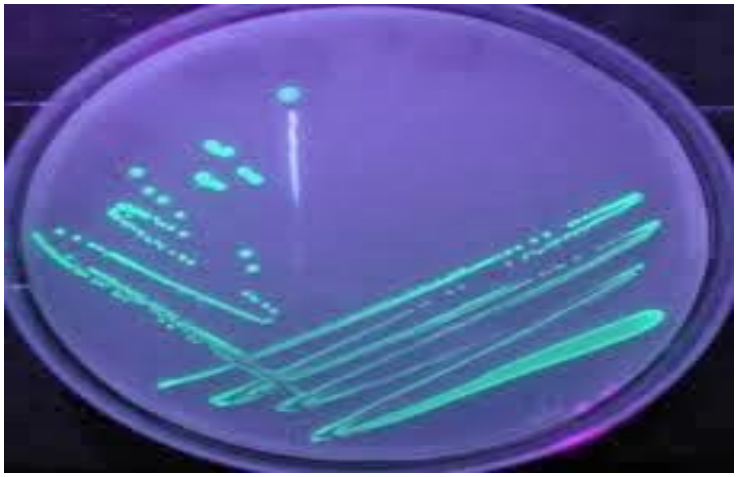

Fig-1 *Pseudomonas fluorescens* In Nutrient agar Medium under UV-transilluminator

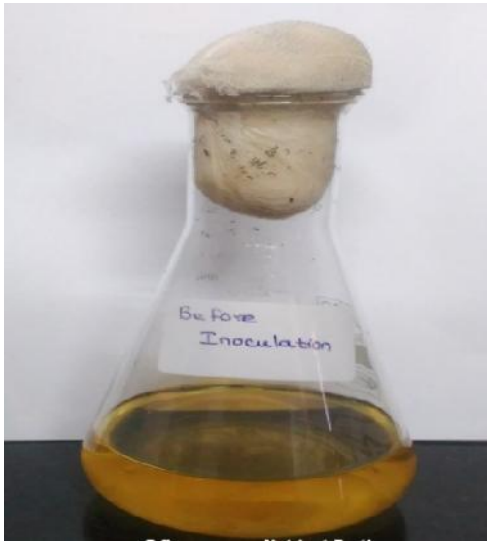

Fig-2 Nutrient broth Medium
Before *Pseudomonas fluorescens* inoculation

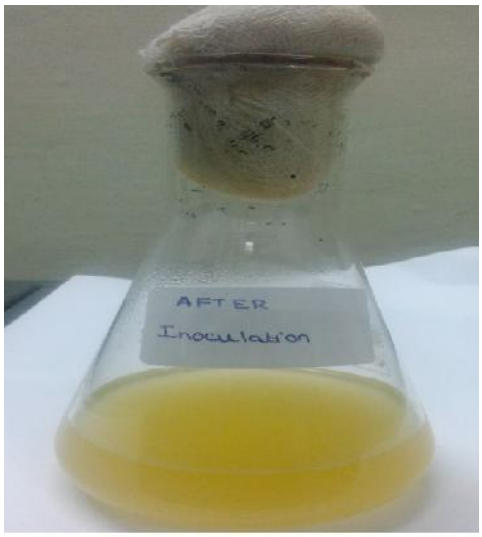

Fig-3 Nutrient broth Medium(24 hrs)
After *Pseudomonas fluorescens* inoculation

Types of Vaccine Prepared:

Whole cell vaccine (WC - Formalin inactivated)
Outer membrane protein vaccine (OMP)

Preparation of whole cell vaccine:

For the preparation of Whole cell vaccine, the bacterial isolate was inoculated separately into the Nutrient broth [NB] medium at 28^{o}C for 24 hours. 0.6% Formalin was added to the bacterial culture for inactivation and left 48 hrs at room temperature. The inactivated cells were harvested by spinning the culture at 1500 x g (20 min) at room temperature and washed twice in 0.85 % saline and finally re-suspended in saline to 35 mgml^{-1} (wet weight). This it was is collected and stored for experimental work.

Preparation of outer membrane protein vaccine:

The 24hours Bacterial culture was harvested by centrifugation from Nutrient broth at 3000 x g [20min at 25^{o}C]. The cell pellets were washed twice in Phosphate Buffer Saline [PBS] and once in 10 mMTris- Hydrochloride [pH7.5]. The cells were re-suspended in Tris-Hcl and sonicated at 50w for 30s [4 times on ice] in a sonicator to distrupt the cell wall. After sonication, the suspension was mixed with Sarkosyl for solubilization of the outer membrane protein and incubated at 25^{o}C for 30 min. After incubation, the suspension was centrifuged at 4000 x g [20min] and the supernatant was collected and again centrifuged at 45000 x g [45min] for collection of pellets and stored at 20^{o}C until it was used for experimental work. The prepared WC and OMP vaccine was quantified by Lowry's Method (1951) and the above proteins were resolved in 10% SDS-PAGE (LAEMMLI 1970) to generate profiles.

Sterility of the vaccines:

This test was done as described by Aly (1981) by cultivation of the prepared bacterins (or) vaccines on Nutrient broth to ensure that there is no growth of *Pseudomonas fluorescens* (or) other pathogens.

Results

The Qualitative analysis of prepared vaccines were done by (Fig-4) 10% SDS-PAGE (LAEMMLI) method. It reveals that the whole cell vaccines had 11 polypeptide bands with respective molecular weight of 248.00, 188.92, 142.65, 99.53, 72.56, 53.84, 40.65, 22.98, 17.05, 11.79, and 9.89 KDa and the outer membrane vaccine had 8 polypeptide bands with respective molecular weight of 121.80, 76.49, 59.30, 31.52, 25.31, 18.45, 15.08, and 3.70 KDa (Fig-4, Tab-1)

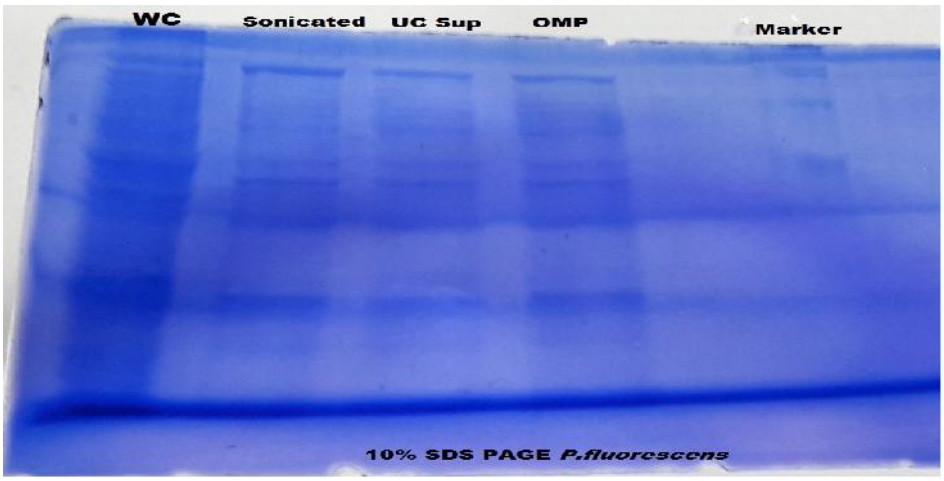

Fig-4 SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) protein profile of *Pseudomonas fluorescens* WC, Sonicated cells, UC supernatant, OMP, and Marker respectively. Molecular weights are in KDa

Tab-1 Molecular weight of *Pseudomonas fluorescens* OMP and WC proteins

Band No	WC Mol.Wt (KDa)	OMP Mol. Wt (KDa)	Marker Mol. Wt (KDa)
1	248.00	121.80	200.00
2	188.92	76.49	127.26
3	142.65	59.30	116.00
4	99.53	31.52	98.66
5	72.56	25.31	97.20
6	53.84	18.45	66.40
7	40.65	15.08	44.30
8	22.98	3.70	
9	17.05		
10	11.79		
11	9.89		

Tab-2 Total protein Quantification of Prepared Vaccines (WC and OMP).

S. No	Vaccines	Total protein/ml
1.	Whole cell vaccine [WC]	66µg
2.	Outer membrane protein vaccine[OMP]	100 µg

The Quantitative protein analysis were done by Lowry's method (Tab-2). The total protein concentration of Whole cell vaccine was estimated as 66 µg/ml and OMP vaccine was 100 µg/ml.

Discussion

The quantitative protein analysis of WC and OMP vaccine was outlined in Table-2. An increased protein concentration of 100 µg/ml was estimated in OMP vaccines and 66 µg/ml in WC vaccines. The SDS-PAGE result shows that WC vaccine had 11 polypeptide bands with the molecular weight of 248.00 to 9.89 KDa and OMP vaccine had 8 polypeptide bands with the molecular weight of 121.80 to 3.70 KDa. The increased protein concentration of OMP vaccine (100 µg/ml) was due to the high intensity polypeptide bands compare to WC vaccine where the number of polypeptide bands are more but the protein concentration was low. So that OMP and WC proteins could be used as a potential vaccine to control septicemic disease in fishes.

Atia *et al.,* (2012) studied four different prepared *Pseudomonas fluorescens* antigens to develop the best adequate strategy to control such infection in Nile tilapia. Na- Gyong Lee *et al*., 2000 studied that Opr F is a 38 KDa protein in the *Pseudomonas auroginosa*, but it runs between 31-43 KDa because of depending on denaturing and running conditions by SDS-PAGE. 30 and 45 KDa protein bands appeared in Opr H and Opr F respectively by their size, Opr F is a major porin protein of *Pseudomonas auroginosa* that has been studied most extensively as a vaccine antigen and shown to be protective against *Pseudomonas auroginosa* infection. Hancock *et al.,* (1990) studied the Opr H (30 KDa) Protein and Sandra *et al.,* 1993 observed that the 34, 45 and 55 KDa protein in *Pseudomonas fluorescens* (ATCC 13525). Li *et al.,* 2010 studied the 41 KDa protein of whole cell of *Pseudomonas fluorescens*. In the present study observed that the OMP has 31.52 and 59.30 KDa and the WC has 22.98, 40.65, 53.84 KDa polypeptide bands, so that these protein bands can use as immunogenic against the infection caused by *Pseudomonas fluorescens* in fishes.

The intensive farming of fin fishes and shell fishes has led to an imbalance of optimal culture conditions, which shows increased susceptibility to infectious disease. Increased incidence of microbial diseases in aquaculture system is the major obstacle in the success of the industry. Use of antibiotics has attracted lot of criticism due to the issues like antibiotic residues, bacterial drug resistance and toxicity. In this present scenario, vaccination would be the best alternative to combat bacterial and viral disease for the sustainable aquaculture. Vaccination is becoming an increasingly important part of aquaculture, since it is considered an easy, cost effective, and preventive method of protecting fish from diseases. Vaccines using whole cells or cell components of bacteria as immunogens can prevent outbreaks of bacterial diseases in aquaculture (Chandran *et al.,* 2002).

Most bacterial vaccines used in aquaculture to date have been inactivated vaccines obtained from a broth culture of a specific strain(s) subjected to subsequent formalin inactivation. The outer membrane proteins (OMP-components of bacteria) of bacteria function as the dynamic interface between the bacterium and its surroundings and are involved in maintenance of cell structure, binding a variety of substances, adhesion to other cells and regulation of transport of both nutrients and bactericidal agents. OMP has been considered to be the noval vaccine due to its ability to play a role as molecular adhesion molecule (Lu *et al.,* 2001, Lin *et al.,* 2002) and also their exposed epitopes on the surface. It is also responsible for stimulating the host immune system to produce strong neutralization responses in order to protect bacteria against microorganisms (Beaz-Hidalgo 2013). The present study was used to develop the WC and OMP vaccines from pathogenic strain (ATCC 13525 *Pseudomonas fluorescens*) and it might be used as potentially important vaccine for fishes against Bacterial septicemic disease.

Conclusion

The use of vaccines, combined with good health management techniques, may result in substantial disease prevention and production becomes more predictable. Vaccines stimulate the immune system of fish to produce antibodies that help to protect the fish from diseases. It was concluded that the OMP and WC protein was protective antigen of *Pseudomonas fluorescens* which had a potential to develop vaccine against *Pseudomonas fluorescens*. Therefore, the present study is used to develop the immunoproteomic vaccines to prevent the infection by *Pseudomonas fluorescens* (Septicemic disease) in fishes.

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