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Immunostimulation by rhamnolipid biosurfactant from *Pseudomonas putida* in *Labeo rohita*

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Abstract

The immunostimulatory effect of phospholipopeptide biosurfactant from *Pseudomonas putida* was assessed with *Labeo rohita*. Haematological and survival rates were recorded at the end of the experiment. The immunostimlatory effects, of biosurfactants, were determined from differences between treatment and control groups in survival rate, Heamoglobulin rate, total leukocytic counts (TLC), Total RBC, and packed cell volume values. A challenge test was conducted using 25 *Labeo rohita* from each group (5 fishes) by I/P inoculation with 0.5 ml suspension culture of the pathogen *Aeromonas hydrophila* (10^8 bacteria ml^{-1}). The mortality rate was recorded for 7 days post-challenge. Both normal and cured cells showed a significant increase in hematocrit values and leucotrit values. The survival rate was significantly increased in both, with challenge, when compared with control. It may be concluded that, biosurfactants can be used as a growth enhancer, immunostimulant and a disease control agent in fish. It is recommended as a means of improving the tilapia aquaculture production under certain conditions.

Keywords: *Pseudomonas putida*, biosurfactant, haematological values, survival of infection.

Introduction

In aquaculture, infectious diseases are a major problem, causing heavy loss to fish farmers. The recent expansion of intensive aquaculture practices has led to a growing interest in understanding fish diseases, so that they can be treated or prevented. Bacterial fish diseases have been studied extensively (Austin and Austin, 1987; Jhingran, 1990; Logothetis and Austin, 1996). One of the major bacterial pathogens in India, *Aeromonas hydrophila*, is known to cause a variety of diseases in fish such as haemorrhagic septicaemia, infectious dropsy, tropical ulcerative disease and fin rot leading to heavy mortality in aquaculture farms (Kumar and Dey, 1988; Rath, 1993; Karunasagar et al., 1997). Various synthetic chemicals and antibiotics have been used to prevent or treat fish diseases with a partial success.

Recently, the use of immunostimulants was introduced as a prophylactic measure (Mulero *et al*., 1998). Since such uses have so far not shown any of the negative side effects that antibiotics and live vaccines may have on the fish and on the environment, they are an attractive alternative way of controlling bacterial infections (Siwicki et al., 1994; Mulero et al., 1998).

So far, a number of immunostimulants that include a very heterogenous group of substances like levamisole, lipopolysaccharide, glucans, peptidoglycan and muramyl dipeptide on the immune response have been tested in a variety of fish species (Anderson, 1992). But knowledge on the use of rhamnolipid biosurfactant as immunostimulants is

limited (Rajeswari *et al*, 2015). Arijo *et al*., 2008, also reported that subcellular components of *Vibrio harveyi* were successful for the stimulation of immunity and the prevention of V. harveyi infections in rainbow trout, Oncorhynchus mykiss (Walbaum). Hence, screening of new immunostimulants from the secondary metabolites (biosurfactants) of microorganisms could be advantageous to strengthen fish immune system and to reduce the quantity of antibiotics required to control infectious diseases. Hence the current study focus on the evaluation of immunostimulatory property of extracellular secondary metabolite rhamnolipid biosurfactant isolated from *P. putida* to fish (*Labeo rohita)*.

Materials and Methods

Oil contaminated soil and water samples from Ennore horbour were collected and enriched by inoculating into sterile mineral salt medium (MSM), individually. one gram/ml of each soil and water sample were inoculated into 50 mL of minimal salt medium (Tahzibi *et al.,* 2004) containing (g/L); 15 g $NaNO_3$, 1.1 g KCl, 1.1 g NaCl, 0.00028 g $FeSO_4.7H_2O$, 3.4 g KH_2PO_4, 4.4 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g yeast extract at 37°C in shaker incubator (100 rpm). After 48 h of incubation, the samples were serially diluted using sterile saline (0.85% NaCl) and different bacterial isolates were selected based on the colony morphology on nutrient agar. The role of plasmids analysis in the biosurfactants production was confirmed by curing the plasmid with acridine orange at a concentration of 500 µg /ml which was added to the culture broth and incubated for 12hrs (Fujji *et al*., 1997). The normal biosurfactants and plasmid cured strains were screened for immunostimulant studies.

Specimens of *Labeo rohita* were obtained from a private fish farm, Chidambaram, Tamil Nadu, India and acclimated to the laboratory conditions for 15 days in fish tank (4 X 3 X 3). During acclimization period, fish were fed ad libitum with rice bran and ground oil cake in the form of dough once daily. Water was replaced every 24 hours after feeding in order to maintain a healthy environment for the fish during both acclimization and experimental period. This ensures sufficient oxygen supply for the fish and the environment is devoid of any accumulated metabolic waste. After acclimization, fish with an average length 8.5 cm and average weight of 7.0 g were selected for the study.

Fish were divided into 5 groups (six fishes/group). Fish diet it contained 30% crude protein, 3.7 Kcal/g of metabolizable energy, 3.4% fiber and 7.03% fat as well as vitamins and minerals in the form of dry pellets. Two different concentration (50 and 100 ppm of normal BS and Cured BS) containing biosurfctant were used and mixed thoroughly with the prepared basal fish diet during its preparation, one control is maintained (Marzouk, et al., 2008).

Survival rate was recorded during the course of the feeding experiment for all treatment replicates. The Heamoglobulin rate, total leukocytic counts (TLC), Total RBC, and packed cell volume were carried out according to the method of Stoskoph (1993). These parameters were determined from blood samples, collected after the first and second phases from the caudal vein of 20 fish from each treatment group (5 from each replicate) using sterile syringes with saturated EDTA.

Challenge of infections was carried out three times on the treatment groups: after feeding on the test diets for one and two months and at the end of the experiment (month 8). Twenty fish from each treatment group and from the control (5 from each replicate), were clinically examined and blood samples bacteriologically tested and determined to be free from bacterial infection, were then artificially infected by intraperitoneal injection with 0.5 ml of culture suspension of pathogenic *Aeromonas hydrophila* containing 10^8 bacteria ml^{-1} that were previously isolated from moribund fish and studied for pathogenicity. A culture suspension of *Aeromonas hydrophila* was prepared by culturing in agar for 24 h, washed and suspended in saline (0.85%) and counted using MacFirland standard tubes (No.1). The relative level of protection (RLP), among the challenged fish was determined according to Ruangroupan *et al*., (1986) using the following equation.

RLP = 100 - percentage of immunized mortality ÷ percentage of control mortality x100.

Results and Discussion

Several molecules of bacterial origin, such as lipopolysaccharides, lipoproteins and glycoproteins, as well as by enzymes produced by immune cells, such as cytokines, transferrin, lysozyme and interleukins (Magnadottir, 2006; Magnadottir *et al*., 2005; Arancibia *et al*., 2007 and Palti, 2011) are involved in the activation of innate immune system in fish. Microbial secondary metabolites, a protective subcellular component has received more attention in disease control. Newaj-Fyzul *et al*., (2007) has demonstrated that feeding rainbow trout with cell-free

supernatant of *Bacillus subtilis* AB1 significantly reduced cumulative mortalities after challenge with Aeromonas sp. Arijo et al. 2008, also reported that subcellular components of Vibrio harveyi were successful for the stimulation of immunity and the prevention of V. harveyi infections in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Hence, screening of new immunostimulants from the secondary metabolites (biosurfactants) of microorganisms could be advantageous to strengthen fish immune system and to reduce the quantity of antibiotics required to control infectious diseases. The present study demonstrated that the rhamnolipid biosurfactant produced by *Pseudomonas putida* strain was able to influence haematological components in *Labeo rohita*.

The blood parameters of the fish showed a significant increase in mean Hb, RBC, WBC and PCV and that indicated improved health and immune status of the biosurfactants treated group. These could be attributed to the fact that, the biosurfactant used increased the blood parameter values as a result of hemopiotic stimulation. These results supported the results of Sarma *et al*. (2003) and Rajesh *et al*. (2006).

Several investigations have shown the immumomodulatory or immunostimulatory activity from biosurfactants in fishes. This result is supported by another study (Choudhury *et al*., 2005; Sahu *et al*., 2007), which found that there was an increase in the WBC count when *L. rohita* juveniles were treated with immunostimulants like levamisole and ascorbic acid.

The increase in total white blood cells, neutrophils, lymphocytes and monocytes following feeding of algal and herbal diets supports the notion of antimicrobial properties of the algae *Euglena viridis* (Das *et al*., 2005) and traditional herbal medicines (Sahu *et al*., 2007).

The fish blood packed cell volume is an indicator of the health status and can be helpful in detecting any abnormal changes including improvement through the use of immunostimulants. Anemic fish may have hematocrit values as low as 10%. The reduced hematocrit values may indicate that the fish are not eating properly or were suffering from infections (Blaxhall, 1972*).*

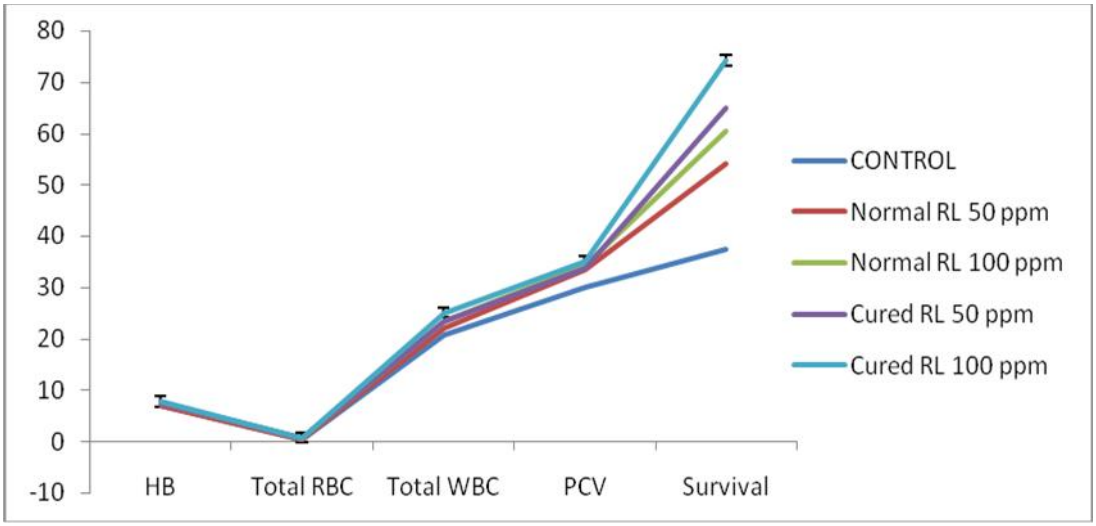
The biosurfactants treated group in the current work, exhibited significantly higher survival throughout the experimental period and a non-statistically significant lower mortality rate post challenge infection when compared with the control group. Earlier studies also revealed that secondary metabolite produced from *Bacillus* genus namely, 4-trans-hydroxy-L-proline and cyclo-(L-Pro-Gly)2 treatment increased the survival rate of the fish infected with A. hydrophila (Tada et al., Also a peptide FK-565 (heptanoyl-g-D-glutamyl-(L)-meso-diaminopimelyl-(D)-alanine) isolated from the culture supernatant of *Streptomyces olivaceogriseus* into rainbow trout (*Salmo gairdneri*) increased their resistance to *Aeromonas salmonicida*, following the activation of phagocytic cells (Nikl et al., 1993)

It may be concluded that, echinacea acts as both an immunostimulant and a disease control agent in fish. It may be recommended as a dietary supplement in order to improve aquaculture production, after further studies are running to evaluate cost-benefits.

Table 1: Effect of biosurfactants on immunostimulatory activity.

Groups	Hb (%)	Total RBC (10^6/cu.mm)	Total WBC (10^3/cu.mm)	PCV (%)	% of survival
Control	7.0±0.3	0.38±0.04	20.8±0.3	30.0±0.3	37.4±1.69
Normal RL 50 ppm	7.2±0.3	0.47±0.02	22.2±0.7	33.4±0.2	54.12±3.33
Normal RL 100 ppm	7.5±0.4	0.64±0.03	23.3±0.4	34.3±0.3	60.37±1.12
Cured RL 50 ppm	7.6±0.5	0.69±0.05	23.5±0.6	33.8±0.4	65.09±1.25
Cured RL 100 ppm	7.8±0.3	0.71±0.02	25.1±0.5	35.2±0.5	74±28±2.28

Fig 1: Effect of biosurfactants on immunostimulatory activity.



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