IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 7 Issue 11, November 2020
ISSN (Online) 2348 – 7968 | Impact Factor (2020) – 6.72
www.ijiset.com

Immunology of Rohu Grown in Biofloc

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Abstract

Rohu was grown in (laboratory conditions) biofloc system for 120 days with continuous aeration and high stocking densities(40, 50, 60 per m ^{3}) in 100 lit. capacity reinforced fiber tanks. Water quality parameters checked every day. Animals were sampled every week throughout the period. Three treatments (40, 50, 60 per m ^{3}) in 100 liters tanks made up of reinforced polyvinyl chloride, fishes after experiment collected and infected with 0.5 mL, 1.0 mL and 1.5 mL of *Aeromonas hydrophila* bacteria to the fish. In each ml. of broth 1.5X10^5 bacteria were present. After the experiment blood and serum were collected from the experimental and control fishes and immunological parameters were observed three days after infecting with bacteria. Serum protein and plasma protein have increased along with increased dose of bacteria compared to control. Serum lysozyme and plasma lysozyme also increased at 450 NM of optical density. Super oxide dismutase, alternate complementary activity and myleoperoxidase activity have increased from control to 0.5 mL, 1.0 mL and 1.5 mL *Aeromonas hydrophila* infected fishes. The fishes grown in biofloc have proved better immune response towards *Aeromonas hydrophila* bacterial infection compared to the control group.

Key words: *Aeromonas hydrophyla*, myeloperoxidase, rohu, , serum lysozyme

1. Introduction

Immunity of an aquatic organism depends on the environment which it grows, feed availability and also gets from its parents. Some times it may acquire immunity by it's conditions which it might be crossing across the difficulties in facing from disease causative agents. Weaker fishes die if they are unable to tolerate the problems created by pathogens and stronger ones withstand. Normally rohu fish will have additional genes (six specific genes) they are: TRL 22- like, lysozyme G, β2 – macroglobulin genes in kidney regulating argulus infected rohu and TNF -∞ and complement component 3 (C3), CXCa are the immune related genes in rohu (Sailesh *et al*., 2011). They will be responsible to overcome the pathogenic activity. The immunity of rohu is assessed by estimating serum protein, plasma protein, super oxide dismutase, alternate complementary activity, myeloperxide activity etc. Other than this if we use antimicrobials and indiscriminate use of the commercial antibiotics in disease prevention can bring about the emergence of drug-resistant microorganisms and leave antibiotic residues in the body and in the environment; chemotherapy may kill or inhibit the normal micro flora in the digestive tract which is beneficial to the health.

2.0 Materials and methods

The experimental fishes along with control group were inoculated with 0.5 ml, 1.0 ml and 1.5 ml of broth containing $1.5X10^5$ bacteria per ml. For all the immunological assays blood of *Labeo rohita* from different experimental groups was collected at the end of the experiment. Three fish from each treatment were anaesthetized using 30 ppm MS 222 for serum assays. The blood was collected without anticoagulant from the caudal vein using No. 24 gauge syringe. The blood was kept undisturbed at room temperature for 2 hr and then centrifuged at 1500 rpm for 10 min followed by collection of supernatant serum. The collected serum was stored at -80 ºC until further use.

The Time Period (Hrs.)	Optical Density	Total Plate Count
2	0.1	$1X10^2$
4	0.2	$3X10^3$
6	0.25	$5X10^3$
8	0.3	$4X10^4$
10	0.4	$7X10^4$
12	0.45	$1X10^5$
16	0.5	$6X10^6$
24	0.6	$3X10^7$

Table 1. Time period, the values and Optical Densities and bacterial count

2.1 Serum and Lysozyme Activity

The serum lysozyme activity level was determined by a turbidimetric method (Ellis, 1990). The chicken egg white lysozyme (Sigma) was diluted with 0.04 M phosphate buffer (PB) for various concentrations served as the standard solution. The substratum used was *Micrococcus lysodeikticus* (0.25 mg/mL of 0.05 M PB; pH 7.4). Diluted lysozyme (15 µL; standard solution) or serum with 250 µL *M. lysodeikticus* was added to every well of a 96-well microtiter plate for 10 min at 37°C. The absorbance at 450 nm was read at 1 min and then at 1-min intervals. The lysozyme content was defined as the amount of lysozyme that caused a decrease in absorbance of levels per min. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

2.2 Myeloperoxidase Activity

In the MPO assay protocol, myeloperoxidase produces HClO from H_2O_2 and Cl^-. The HClO reacts with taurine to generate the taurine chloramine, which subsequently reacts with the DTNB probe to eliminate color (absorbance at 412 nm). The absorbance is inversely proportional to the amount of MPO enzyme.This MPO assay kit can be used to detect myeloperoxidase as low as 0.05 mU per well.

2.3 Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical-dependent reactions using the Ransod Kit (Randox, Crumlin, UK). Briefly, the reaction mixture (1.7 ml) containing xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) was dissolved in CAPS 50 mM (pH 10.2) and EDTA (0.94 mM).

2.4 Serum Protein

The concentration of total protein in serum samples was analyzed using Bradford (1976) method. The serum samples were normally assayed in triplicate. Two ml of Bradford reagent was added to 2 µl of serum sample in each tube and well mixed, followed by incubation at room temperature for 20 min. Finally the absorbance was measured at 595 nm using spectrophotometer. Total serum protein was expressed in mg/mL.

2.5 Alternate Complementary Activity

The alternative pathway of the complement system is an innate component of the immune system's natural defense against infections. The alternative pathway is one of the three complement pathways that opsonize and kill pathogens. The pathway is triggered when the C3b protein directly binds a microbe. It can also be triggered by foreign materials and damaged tissues.

3.0 Results

Serum protein 85 mg/mL and plasma protein 70 mg/mL were found in control while in T1, 120 mg/mL and 95 mg/mL, in T2, 100 and 98 mg/mL and in T3 145 and 98 mg/mL were observed serum protein and plasma protein alternatively as shown in the Fig 1.

Serum lysozyme and plasma lysozymes were 1.7 and 2.4 OD at 450 NM in control and T1, 2.0 2.4, T2, 1.7, 2.4, and 2.0 and 2.5 were recorded (Fig 2).

Superoxide dismutase values from control to T3 were; 18, 24, 29, and 30 (Fig 3) were recorded. Myeloperoxidase activity in control 1.9, T1; 2.2 T2; 2.4 and T3; 2.1(Fig5) . Alternate complementary activity in control was 125, T1; 145, T2; 160, and T3; 180(Fig 4). All the immunological parameters have shown gradual increment with the increase of dose of the bacteria.

4.0 Discussion

Baidya *et al*. (2015) conducted a 60-day feeding trial to evaluate the effect of dietary nucleotide on the growth, survival, immunity and resistance of *A. hydrophila* infection in rohu (*Labeo rohita*). The nucleotide was supplemented at 0, 5, 10 and 15 g/kg diet. The test diets were fed to fish for 60 days in triplicate groups of fish, which had initial weight of 1.3 g. At the end of the feeding trial, the growth was recorded and non-specific immune parameters, such as superoxide anion production and total serum protein were studied in blood samples. Total serum protein and superoxide anion production were significantly ($P<0.05$) higher in fish fed nucleotide-based diets. The relative percent survival of fish after the challenge test against *A. hydrophila* disease was significantly ($P<0.05$) higher in fish fed nucleotide-incorporated diets. Jayasree, (2016) reported that total serum protein was significantly affected by various dietary treatments and the highest total protein of 44mg/ml was recorded in T_2, followed by 42.3 mg/ml, in T_3 and T_1 was recorded 41.3 mg/ml, and in T_0 33.33 mg/ml in rohu with threonine supplemented diets.

In the present study, serum protein was increased in T1, slightly decreased in T2 and increased in T3 compared to control. According to Dharmakar (2017) in rohu, Anusha (2017) in *Litopenaeus vannamei*, it was increased from T1 to T4 when compared to control. Sahoo K. *et al*. (2016) concluded that superoxide dismutase activity in Golden Mahaseer was significantly higher in test samples as

compared to control group. Present experiment also showed significant increase in the treated animals according to the dosage of *A. hydrophila* infected. Myeloperoxidase activity also increased with higher dose of bacteria but decreased slightly to 1.5 ml ($1.5X10^5$ bacteria per ml) compared to the lesser doses.

Xia *et al*. (2017) proved in their experiment that in *Megalobroma ambycephala* the superoxide dismutase activity in the treatment group showed a significant decrease at 1 d, 3 d, 5 d, 14 d, 21 d, and 28 d compared to the control group. In the treatment group, superoxide dismutase activity was decreased gradually after *A.hydrophila* challenge. In the present study, superoxide dismutase activity was increased according to the dosage of *A. hydrophila* and decreased in control. Lysozyme activity in the treatment group showed significant increase (p <0.01) at 4 h, 1 d, 3 d, 5 d, 14 d, and 21 d compared to the control group. In the treatment group, lysozyme activity was increased gradually after *A. hydrophila* challenge and reached the peak at 1 day, and finally there was a tendency to recover in control group after 1 day. Serum lysozyme activity has been increased compared to the control but a slight decrease has been observed in the treatments from T1 to T3 but there was no significant difference between the treatments at 0.05% and 1% level of significance. In the plasma lysozyme activity gradual increase has been observed in the treatments compared to the control. It is considered that lysozyme activity is an important defense mechanism in vertebrates and fish serum lysozyme is believed to be of leukocyte origin. Lysozyme plays an important role in innate immunity by lysis of bacterial cell wall, and thus stimulates the phagocytosis of bacteria. Its ability to disrupt the cell walls of certain pathogens makes lysozyme a natural antagonist to harmful invaders like parasites, bacteria, and viruses. Lysozyme occurs prominently in fish serum and mucus. Verma *et al*. (2016) disclosed that the biofloc based system significantly enhanced non specific innate immune parameters i.e. NBT, MPO activity and lysozyme activity. Free radicals were formed for the destruction of bacterial invaders and the highest NBT assay was observed in tapioca based biofloc treatment. Thus the

immunostimulatory effect of biofloc is influenced by the organic carbon source used to produce it. Borgia *et al*. (2018) in their studies on common carp showed that chronic exposure of fish to 0.004, 0.007, 0.010 and 0.013 % EIE, dose-dependently decreased the non-specific and specific immune responses on all the days tested when compared to control fish whereas statistically significant suppressive effects were observed in fish exposed to 0.013 % of EIE on all activities tested.

Kheti *et al*. (2017) proved in their challenge studies in an experiment with *Edwardsiella tarda* against rohu the lysozyme activity of serum was significantly higher in the fish group fed with 4% microbial floc meal supplemented diet compared to control. Further proved that statistically they did not observe any difference in myeloperoxidase content of serum in all the dietary groups as compared to control groups.

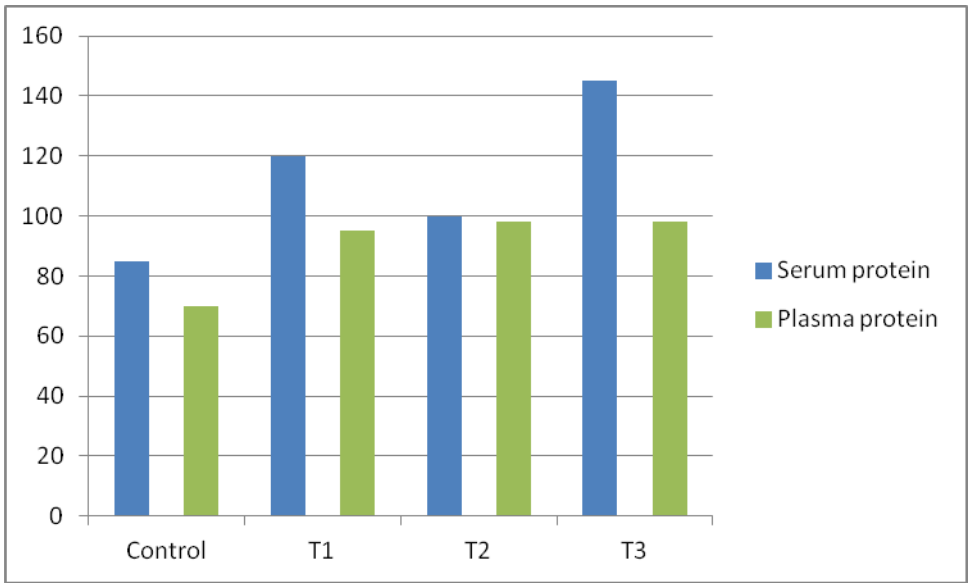

Fig. 1. Graph showing the results of lysozyme activity in different treatments

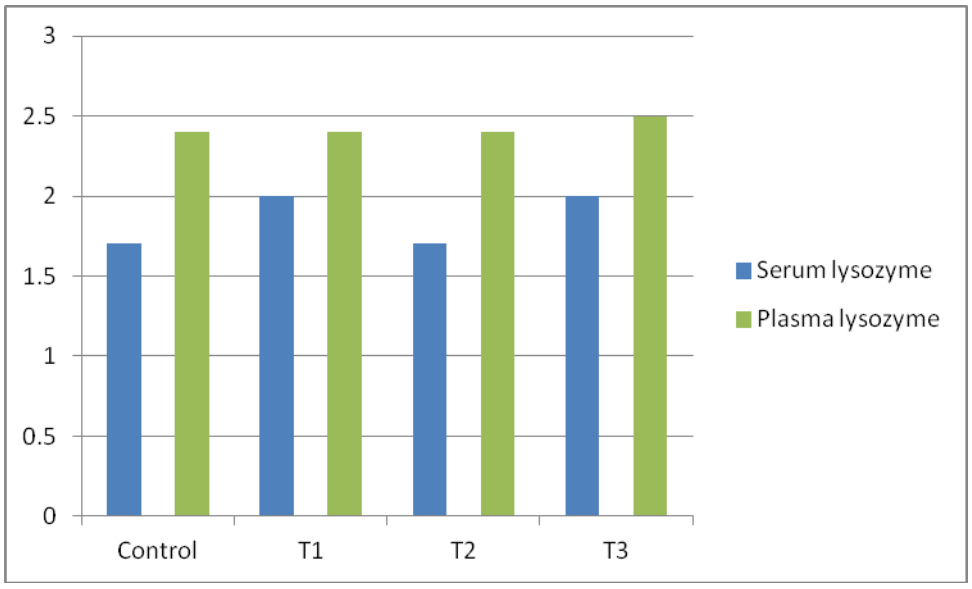

Fig. 2 Graph showing serum and plasma lysozyme activity at 450NM

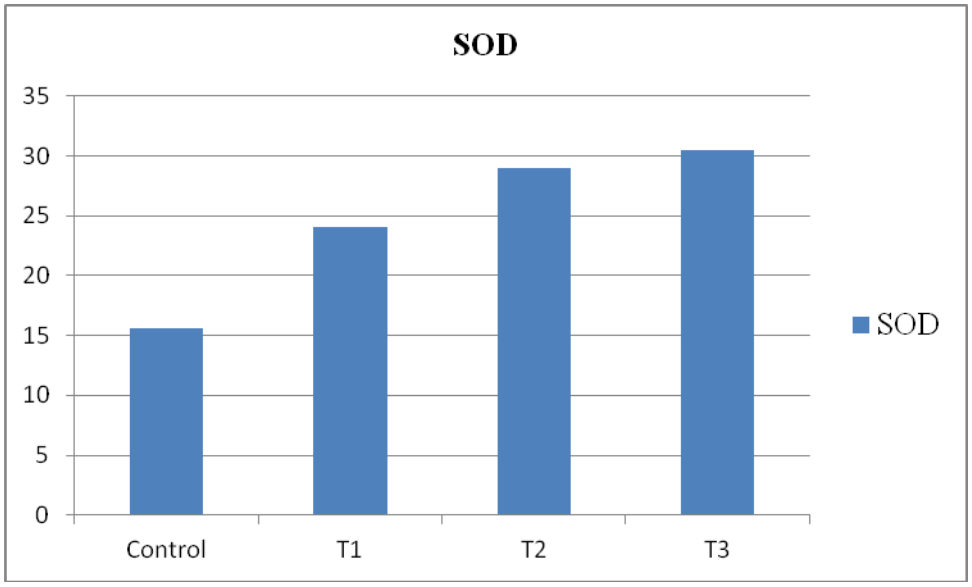

Fig. 3. Graph showing average superoxide dismutase activity

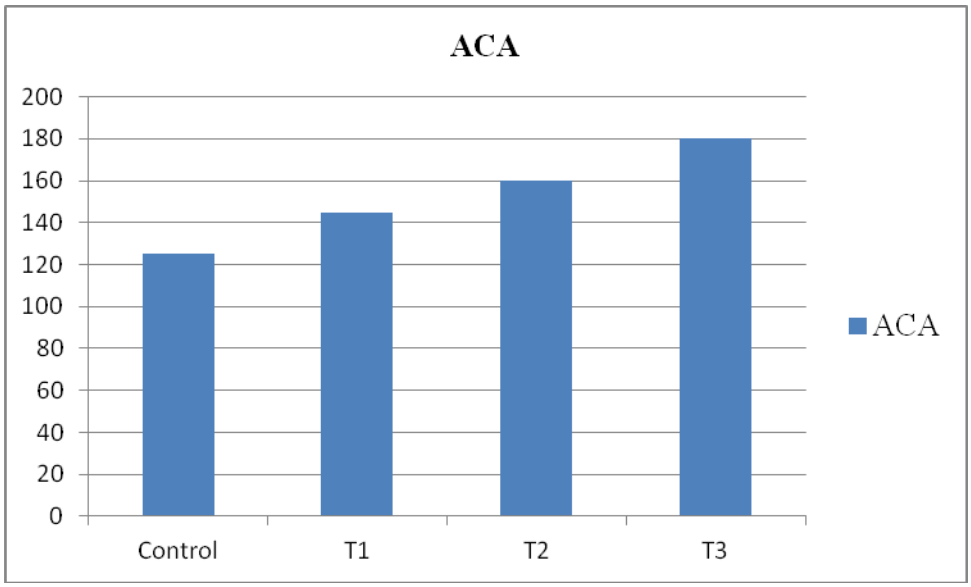

Fig. 4. Showing Alternate Complementary Activity.

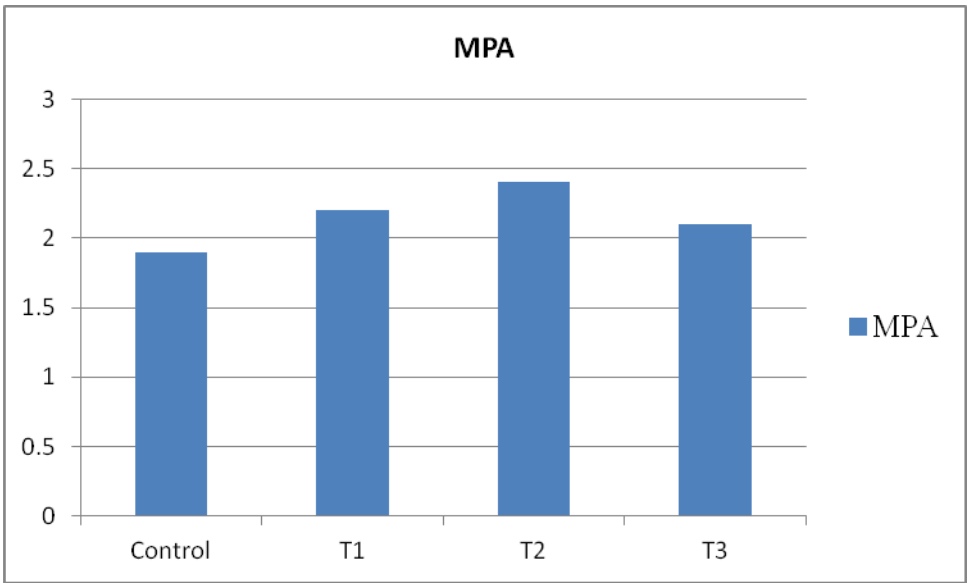

Fig.5. Graph showing Myeloperoxidase activity

Acknowledgements

We are thankful to the office subordinates who ever helped to complete the experiment and we are grateful to the Head of the institute College of Fishery Science, Muthukur and all the Department heads who helped in the study of immunology of rohu. I am also grateful to the committee members who allowed to do the work in the College of Fishery Science, Muthukur.

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