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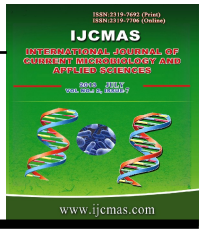

Original Research Article

Immunomostimulation effects of *Sargassum whitti* on *Mugil cephalus* against *Pseudomonas fluorescence*

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K e y w o r d s

Mugil cephalus; Immunomostimulation; *Sargassum whitti; Pseudomonas fluorescence*; non specific immune parameters.

A B S T R A C T

The purpose of this study was to determine the effect of water extract of seaweeds *Sargassum whitti* on the nonspecific immune mechanisms and disease resistance in *Mugil cephalus*. *Sargassum whitti* was collected from Pudumadam (Rameshwaram district). The Seaweeds was extracted by using hot water extract method. After extraction it was mix with feed at different concentration. Fishes were feed with different doses of 0.5, 1.0 and 1.5.0 % (kg diet)$^{-1}$ body weight of water extract of seaweed. The nonspecific immune mechanisms were assessed in terms of Counting of white blood cells (WBC), Lysozyme assay and Respiratory burst activity. The functional immunity in terms of percentage mortality and Relative Percent Survival (RPS) was assessed by a challenge with live *P.fluorescence*. In our present study 1% Seaweed shows a significantly increased WBC, higher lysozyme activities and respiratory burst activities when compare with other diet 0.5, 1.5% Seaweed died & control feed. Cumulative survival rate after 24h of challenge test, experiment group showed 80% survival and control 70% survival were observed. After 120 h of experimental studies 70%, 80%, 65 %, and 30% survival was observed in G1, G2, G3 and Control group respectively. This preliminary study indicates that *Sargassum whitti* could be used to promote the health status of fish in intensive finfish aquaculture.

Introduction

Mugil cephalus (striped mullet or sea mullet) is an extremely widespread fish species. This species is found in temperate and tropical waters throughout the world. Sea mullet is an object of both commercial fishery and game angling and it is not considered as a threatened or endangered fish species. The mullet caught on ocean beaches are mostly spawning run fish and growing market for sea mullet roe, considered as a highly popular delicatessen fish product. Because of this, mullet is successfully cultivated in several countries Ramireza *et al*., (2003).

Bacterial disease outbreaks impose a significant constraint on the production of fish and shellfish (Verschuere *et al*.,

2000). The aquatic environment contains a plethora of obligate and opportunistic bacterial pathogens as well as beneficial and neutral bacterial strains. In hatchery facilities, the environmental conditions (availability of iron, osmotic strength, oxygen levels, pH, water quality and temperature) and sometimes poor management practices (inadequate nutrition, overcrowding and overfeeding) can cause stress to the organisms being cultured and thus make them more susceptible to disease outbreaks (Winton, 2001).

Pseudomonas has also been reported to cause disease in a number of fish species, including goldfish (*Carassius auratus*) Bullock (1965). *Pseudomonas* have been isolated from Gilthead sea bream (*Sparus aurata*), European seabass (*Dicentrachus labrax*) Berthe *et al.,* (1995) and the challenges have caused mortalitiesin carp (*Cypinus carpio*) and loach (*Misgurnusanguilli caudatus*) Muroga *et al.,* (1975). The pathogenic microorganisms isolated from wild population also belong to those unknown Gram-negative bacteria and Pseudomonadaceae. Clinical signs of the disease appear to be the same in all species affected namely petechial hemorrhages of the skin, peritoneum and liver (Wiklund and Bylund 1993). Moreover Altinok *et al.,* (2007) also isolated *Pseudomonas luteola* from rainbow trout, *Oncorhynchus mykiss* during the outbreak occurred in spring of 2004 and the strain was resistant to most commonly used antibiotics.

The World Health Organization (WHO) recommends that preventative (prophylactic) approaches to disease management are preferred over costly post-effect treatments (WHO, 2002). The increasing political and environmental pressure to decrease the use of antibiotics and other therapeutic chemicals in agriculture and aquaculture has stimulated research into more environmentally friendly approaches to disease control (Verschuere *et al*., 2000).

Immunotherapy is an approach that has been actively investigated in recent years as a method for disease prevention. It does not involve recognition of a specific antigen or targeting the immune response towards a specific pathogen, but causes an overall immune response that hastens recognition of foreign proteins (Sordello *et al.*, 1997). So the use of immunostimulants for prevention of diseases in fish is considered an alternative and promising area (Sakai, 1999).

Immunostimulants are agents/factors that trigger the non-specific immune response and result in enhanced disease resistance. Several compounds have been reported to have Immunostimulation properties. Many of these are derivatives or cellular components of bacterial, fungal or animal origin. Laminarin, barley, glucan, lactoferrin, levamisole, lipopolysaccharides, curdlan, scleroglucan, zymosan, inulin, chitosan, β-glucans, dextran, lentinan, saponins, herbal extracts, peptidoglycans and so forth, are some of the examples of immunostimulants used in shrimp/fish aquaculture (Rajasekar *et al.,* 2011).

Over the past 20 years, various chemotherapeutics have been used to treat bacterial infections in cultured fish but the emergence of drug-resistant has become a major problem Aoki (1992). Vaccination may prevent fish disease out breaks, but the development of vaccines against many intracellular pathogens has not yet been successful. A more effective approach to

controlling disease in aquaculture seems to be through the enhancement of natural disease resistance, using immunostimulants (Sakai, 1999).

The ability of seaweeds to produce secondary metabolites of antimicrobial value, such as volatile components (phenols, terpenes) (Awad, 2000, 2004), steroids, phlorotannins (Nagayama *et al*., 2002) and lipids Freile-Pelegriniand (Morales, 2004) has already been studied. In contrast to the brown and green algae, the red algae are more known to produce halogenated metabolites, particularly bromine and iodine (Konig *et al.*, 1999).

Materials and Methods

Seaweed collection and Extraction

Seaweeds *Sargassum whitii* was collected from Pudumadam costal region (Rameswaram District, Tamil Nadu, India). Seaweed was shadow dried and grinned by using mixer grinder.

Isolation pathogens

Pseudomonas fluorescence was collected from CAS in Marine Biology, Annamalai University, Parangipettai. The strain was subculture into a fresh Nutrient broth (50 % of Salt water) and stored in -20ºC.

Fish

Mugel cephlus (mullet) weighing 100 g were obtained from a local fish farm. The fish were kept in 250L tanks with recirculation, aerated sea water at 14ºC, and fed daily with commercial pellets. Fish were acclimatized to laboratory conditions for 2 weeks before starting the experiments.

Feed preparation

Seaweed was washed and dried. The dried sample was ground to flour with a mixer grinder. The dried seaweed (100 g) was treated with 5 of distilled water and boiled (100°C) for 30 min. The extracts were centrifuged at 4,500 rpm for 20 min, and the supernatant was lyophilized under reduced pressure under as following condition: The temperature was about -20°C, the pressure was about 2 mm Hg, and the hot-water extract was then kept at -20°C for the following tests. The test solution was prepared of the freeze-dried hot-water extract dissolving in distilled water.

Experimental design

Four diets containing different levels of seaweed were prepared as described in Table 1. Artificial feed was served as the control diet. Proximate analysis of the basal diet was 41.7% crude protein, 8.5% crude lipid, 12.1% ash, and 9.5% moisture. Seaweed was added to the test diets at levels of 0.5, 1.0 and 1.5 % (kg diet) ¯1. The ingredients were ground up in a Hammer mill to pass through a 60-mesh screen.

Experimental diets were prepared by mixing the dry ingredients with fish oil and then adding water until a stiff dough resulted. Each diet was then passed through a mincer with a die, and the resulting spaghetti-like strings were dried in a drying cabinet using an air blower at 40 ºC until the moisture levels were at around 10%. After drying, the finished pellets were stored in plastic bins at 4 ºC until use**.**

Immunological Analysis- Non Specific Immune response

Blood collection and serum separation

Fish were bled from common cardinal vein using 1 ml tuberculin syringe fitted with 24-gauge needle with the whole procedure being completed within 20 s. This would cause a minimal handling stress and the cortisol secreted if any, due to handling stress will enter blood stream only after 4 min of handling. For serum separation, about 200 ml of blood was drawn and collected in serological tubes and stored in a refrigerator overnight. The clot was separated from the wall of the tubes and spun down at 400 g for 10 min. The collected serum was stored at -20 ºC until used for assays Michael *et al.*, (1994).

Counting of white blood cells (WBC)

To determine the total white blood cell count (WBC), a 1 in100 dilution of the blood was made in phosphate saline buffer (PBS, 0.02 M, pH 7.3). Counts were carried out using a Neubauer haemocytometer and were expressed as cells ml^{-1}.

Lysozyme assay

Lysozyme activity was measured according to Parry et al., (1965) using a turbidity assay in which 0.2 mg ml^{-1} lyophilised Micrococcus lysodekticus in 0.04 M sodium phosphate buffer (pH 5.75) was used as substrate. Forty microlitre of fish serum was added to 3 ml of the bacterial suspension and the reduction in absorbance at 540 nm determined after 0.5 and 4.5 min incubation at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min.

Respiratory burst activity

Production of intracellular superoxide anion (O^{2-}) was evaluated using Nitroblue tetrazolium (NBT) (Himedia, India) reduction following the method of Secombes (1990) with some modifications. A 100 µl cell suspension was stained with 100 µl 0.3% NBT and 100 µl Phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) (1 mg ml^{-1}) for 40 min. Absolute methanol was added to terminate the staining. Each tube was washed three times with 70% methanol and air-dried. Then 120 µl 2 M KOH and 140 µl dimethyl sulfoxide (DMSO, Sigma, USA) were added and the color was subsequently measured at 630 nm with a spectrophotometer using KOH/DMSO as a blank (Qinghui *et al*., 2011).

Challenge with *Pseudomonas fluorescence*

In the present study, the bacterial pathogen *P. fluorescence* was used as a test organism. The bacterial strain *P. fluorescence* subculture was centrifuged at 1000 g for 10 minutes at -4 °C. The supernatant were discarded and the bacterial pellet was washed three times and resuspended in phosphate buffered saline (PBS) at pH 7.4. The OD of the solution was adjusted to 0.5 at 456 nm which corresponded to 1×10^{7} cells ml^{-1}. After seaweed extract treatment, fish were injected (50 µl) with *P. fluorescence* (1×10^{7} cells ml^{-1}) on day 15.

Cumulative survival rate

After the infection of bacterial pathogen *P. fluorescence* survival rate of the fish were recorded from 24 hours to 144 hours. The clinical symptoms were noted including hemorrhagic septicemia, distended

abdomen and lesions on the ventral surface of the body. Relative percentage survival rate (RPS) was calculated by the following formula (Divyagnaneswari *et al.*, 2007).

$$RPS = 1 = \frac{\text{Present mortality in treated group}}{\text{Precent mortality in control group}} \times 100$$

Results and Discussion

The use of high doses of vitamin C results in proliferation of rainbow trout lymphocytes. Furthermore, the white blood cells were increased in the grouper *Epinephelus malabaricus* fish fed with lipid containing diet than control fish (Hung and Shaiu, 2003). In our present study 3rd Group WBC was significantly increased in the fish feed with 1 % seaweed extract than control and other group of fish. In 3rd group WBC count was reached maximum 6.8×10^6 ml^{-1} in 9th day but in 12th day WBC amount reduced. In the 2nd and 3rd group fish WBC count reach maximum 5.2×10^6 ml^{-1} in the 9th day and 5.1×10^6 ml^{-1} in the 6th day respectively . Several studies showed that the white blood cells can be increased in infected or damaged animals. WBC and neutrophil quantities in infected samples were accepted as a response of cellular immune system to fungal infection (Palikova and Navratil, 2001) concluded that the immune system of fish displays similar responses to unfavorable conditions. Sahan *et al*., (2007) *also* reported an increased observed in leukocyte cells of fish infected with the parasite in a European feel, *Anguilla anguila*.

In our present study the serum lysozyme activities of fish fed with seaweeds extract-containing diets at 1 and 1.5% were significantly higher than that of fish fed with control diet after 3rd days of feeding. Fish fed with seaweed-containing diets feed at 1 and 1.5% lysozyme activities increased than control group after 3rd day. It was reach maximum 1500 and 1300 lysozyme Activity (U min^{-1} ml^{-1}) graph 2. In our present also supported Ann *et al*., (2007). The lysozyme activity of grouper that received sodium alginate or *i*-carrageenan at 20 and 30 mg kg^{-1}, respectively, increased significantly after 24 and 72 h, but thereafter slightly decreased or returned to the original level after 120 h (Ann *et al*., 2007). Lysozyme is a fish defense element, which causes lysis of bacteria and activation of the complement system and phagocytes by acting as opsonin (Magnadottir, 2006).

Phagocytes produce toxic oxygen forms during a process called respiratory burst (Neumann *et al*., 2001). In the present study respiratory bursts activities of fish fed the 0.5%, 1 % and 1.5% hotwater extract of Seaweed containing diets were significantly higher than those of fish fed the control diet. In the 6th day 4th group respiratory bursts activities increased than group 2 and group3. In 9th and 12th day group2 activity was increased than group1 and group4. Since superoxide anion was the first product to be released from the respiratory burst, the measurement of O^-_2 has been accepted as a precise way of measuring respiratory burst (Secombes and Olivier, 1997). In another study, groupers injected with sodium alginate at 20 mg kg^{-1} or *i*-carrageenan at 30 mg kg^{-1} showed increased respiratory burst activity Cheng *et al*., (2007). However in this study, the fed contain polysaccharide extract injected through i.p showed decreased respiratory burst activity after 10 days in both 50 and 100 mg kg^{-1} of fish. This difference is considered due to the source of polysaccharide extract, concentration of polysaccharide, administration method and exposure time.

Table.1 Antibacterial activity of different Seaweeds extracts against the Fish pathogen *Pseudomonas fluorescence*

S. No	Different solvent –(disc size 3cm)																			
	Methanolic				Isopronal				Acetone				Choloroform				Diethyla ether			
	Zone 1 (cm)	Zone 2 (cm)	Zone 3 (cm)	Mean ± S.D	Zone 1 (cm)	Zone 2 (cm)	Zone 3 (cm)	Mean ±S.D	Zone 1 (cm)	Zone 2 (cm)	Zone3 (cm)	Mean ±S.D	Zone1 (cm)	Zone 2 (cm)	Zone3 (cm)	Mean ±S.D	Zone 1 (cm)	Zone2 (cm)	Zone3 (cm)	Mean ±S.D
1	1.2	1.4	1.3	1.33 ±0.05	0	0	0	0	0	0	0	0	0	0	0	0	1.4	1.2	1	1.13 ±0.23
2	1.8	2	2	1.93 ±0.11	1.6	.16	1.4	1.05 ±0.78	1	1	1.2	1.06 ±0.11	1.3	1.6	1.8	1.56 ±0.25	2	1.8	1.8	1.86 ±0.11
3	1.3	1.2	1	1.16 ±0.15	1.4	1.2	1.1	1.33 ±0.20	0	0	0	0	1	1	1.2	1.06 ±0.11	1.3	1.3	1.5	1.36 ±0.23
4	1.2	1.1	1	1.1 ±0.1	0	0	0	0	1.2	1.2	1.3	1.23 ±0.05	1	1.3	1.4	1.23 ±0.20	0	0	0	0
5	0.9	0.8	0.9	0.86 ±0.05	0	0	0	0	1	1.4	1	1.36 ±0.05	0	0	0	0	1	1.3	1.3	1.2 ±0.17

No1: Kappaphycus alvarezii
No2: *Sargassum wightii*
No 3: Ulva lactuca
No 4: *Gracilaria edulis*
No 5: *Gracilaria corticata*

Table.2 MIC values of Sargassum *wightii* -methanol extracts against PF microorganisms

S.No	Solvent	MIC values (µg/ml)			Mean ±SD
		MIC1	MIC2	MIC	
1.	D-Methanol	40	60	40	46.66 ±11.54
2.	E- Di ethyl ether	80	80	40	66.66 ±23.09
3.	F=Choloroform	160	80	80	106 .66 46.18
4.	G- Isopropanol	160	80	160	133 .33±46.18
5.	H- Acetone	320	160	160	213.333 ±92.37

Figure.1 Total white blood cell counts

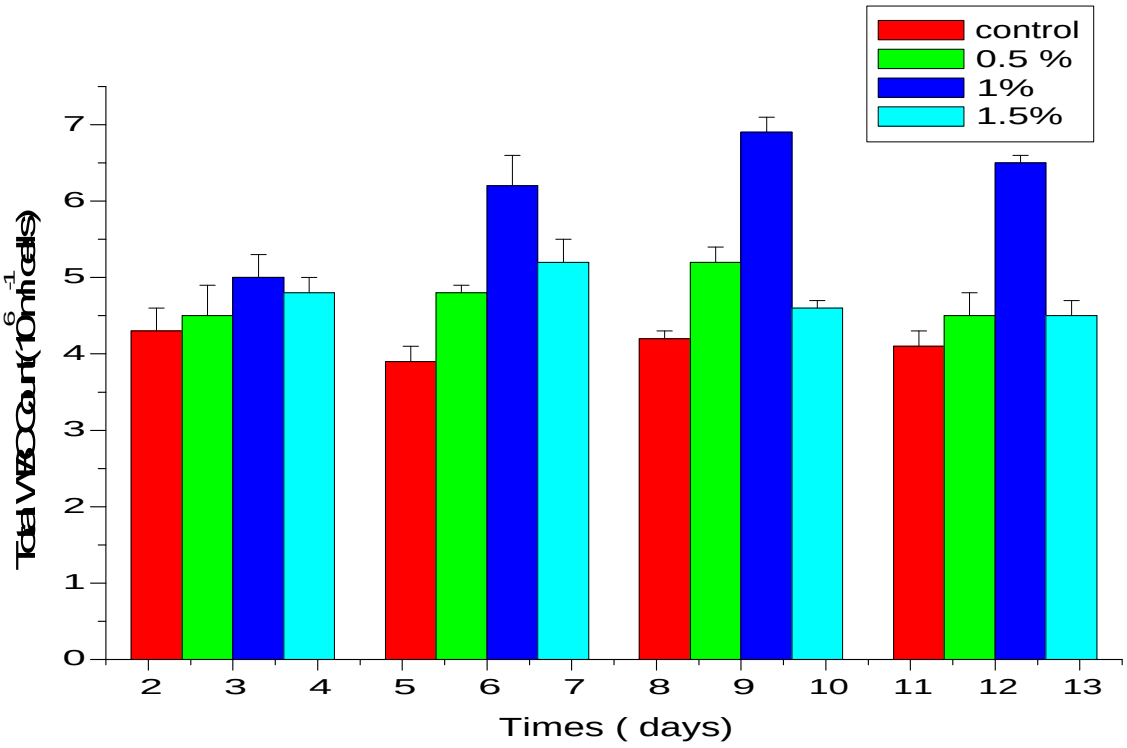


Figure.2 Lysozyme activity

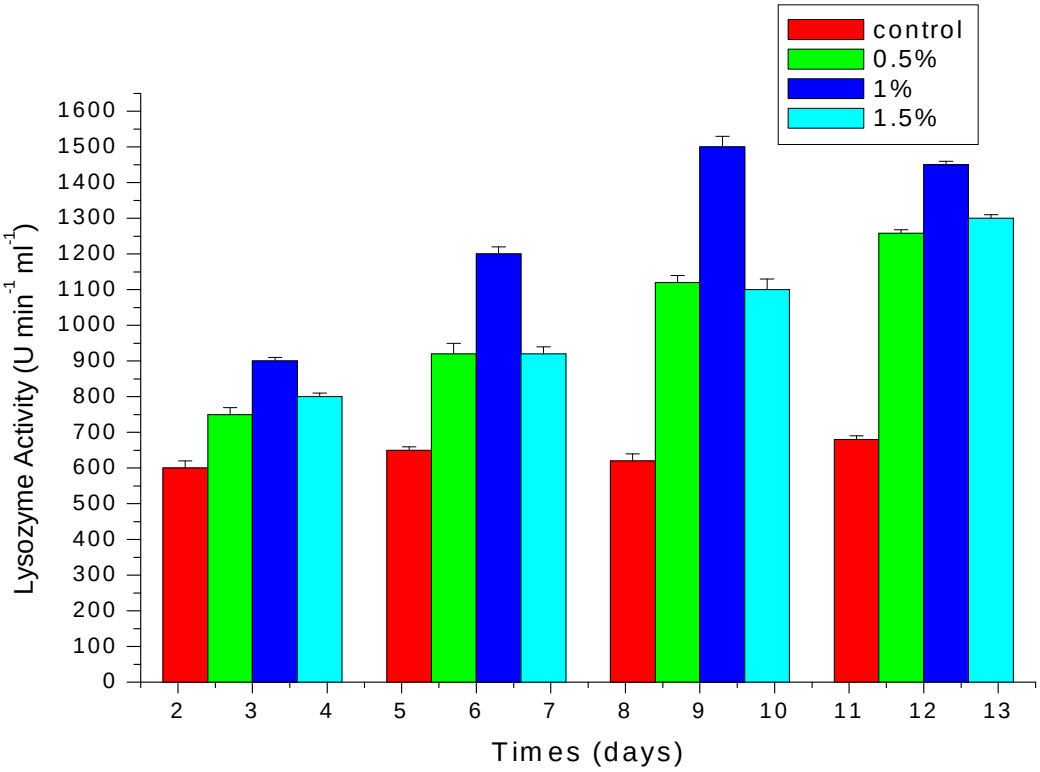


Figure. 3 The respiratory burst activity

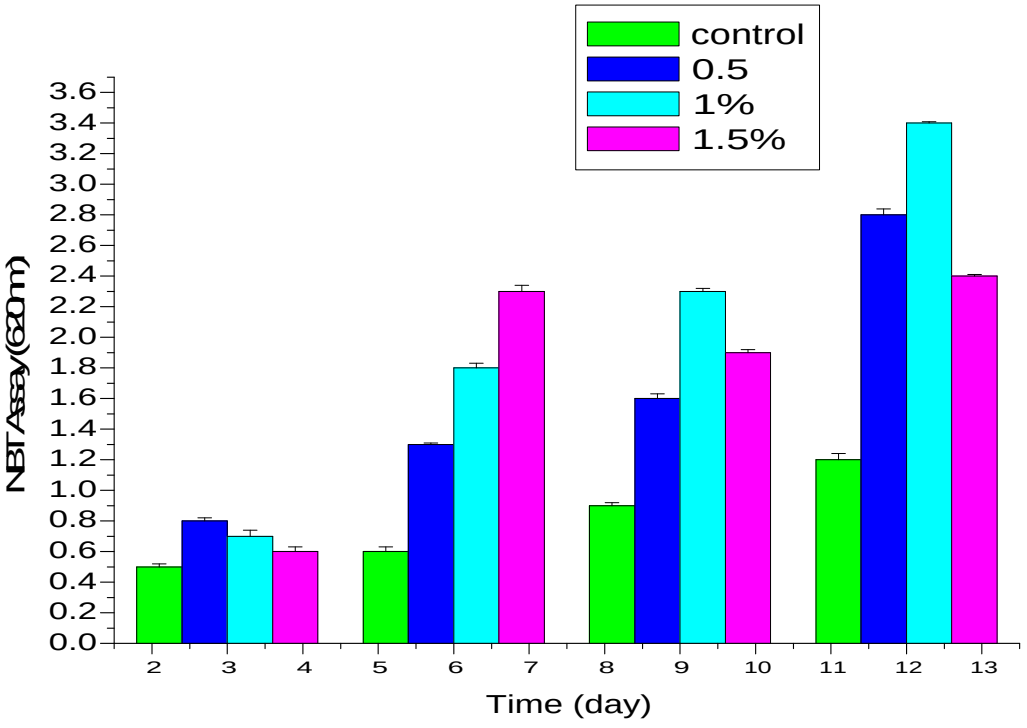
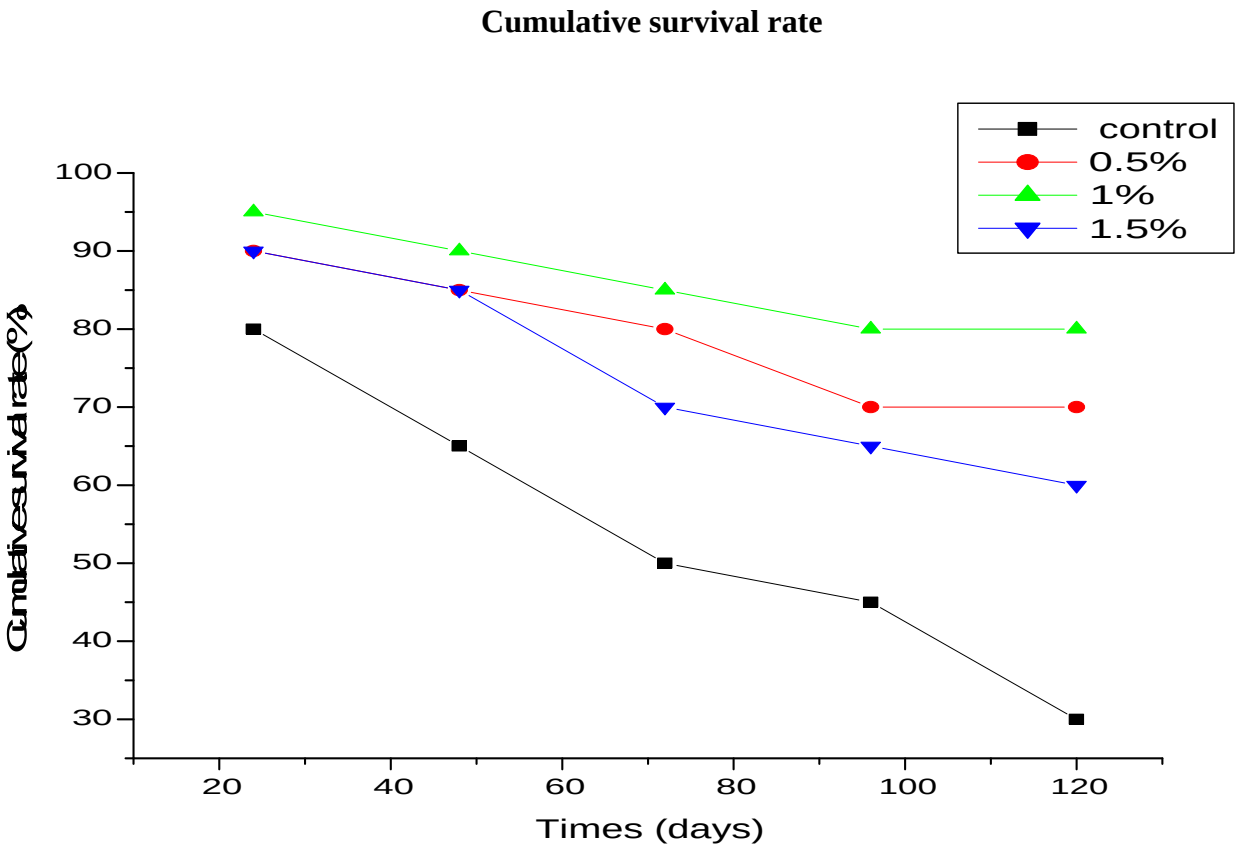


Figure.4 The



Generally the highest respiratory activity can be achieved by occurrence of highest reduction in NBT (Sajid *et al.*, 2009).

After fed diet experiment shrimp were challenge with *P.fluorescence* and observed for the survival rate and after 24h of challenge test, experiment group showed 80% survival and control 70% survival were observed. After 120 h of experimental studies 70% of survival was observed in G1 Group and 80% of survival was observed in G2 and 65 % of Survival was observed in G3, control fish 30% of survival was observed. In conclusion, the administration of water extract of some of the nonspecific immune parameters and disease resistance against *P.fluorescence* in *Sargassum whitti*. The different parameters of the present work revealed that the seaweeds immunostimulants are very effective against the bacterial (*P.fluorescence*) infection and further works are to be done on the isolation and characterization of the active compounds from these seaweeds. This preliminary study indicates that *Sargassum whitti* could be used to promote the health status of fish in intensive fish aquaculture.

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