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Short Communication

Immune enhancement assessment of dietry incorporated marine alga *Sargassum wightii* (Phaeophyceae/Punctariales) in tiger shrimp *Penaeus monodon* (Crustacia/Penaeidae) through prophenoloxidase (proPO) systems

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An experiment of 30 days duration was conducted to test the efficacy of the seaweed *Sargassum wightii*, as immunostimulant in tiger shrimp *Penaeus monodon*. The shrimps were fed with the experimental diets coated with different concentrations of the brown algae *S. wightii* viz. 10, 20 and 30 g/kg. They were challenged with a marine pathogen *Vibrio parahaemolyticus* to assess the increase in survival rate if any due to immune enhancement. Another group of shrimp was used for drawing the haemolymph to estimate the increase in the level of prophenoloxidase activity. Samplings for the above said analysis were carried out at regular intervals of 3 days viz. 1st, 3rd, 6th, 9th and 12th days. The highest prophenoloxidase activity (0.62) and the highest survival rate (83%) were recorded on the 12th day with the experimental diet (10 g/kg) . Hence the 10 g/kg of *S. wightii* added to the diet could be an eco-friendly and economically viable immunostimulant for penaeid shrimps.

[**Key word**s: Pro phenol oxidase activity, challenge, immunostimulant, marine alga, *Sargassum wightii*]
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The success of modern aquaculture has been the direct result of better management, balanced nutrition and appropriate disease control strategies. Vaccination and treatment have been able to control many diseases that affect farmed fish and shellfish. Crustacean group of organisms lack the specific immune system found in mammals and fish[1]. However, they posses a non-specific immune response system that includes agglutinins, killing factors, lysins, precipitins and clotting agents[2]. These factors have all been associated with the removal of invading organisms, phagocytosis or haemocyte encapsulation. The pro-Phenoloxidase (pro-PO) mechanism is one of the main defensive mechanisms as a non-self recognition system in crustaceans[3].

Immunostimulants have been proven to be safer than chemotherapeutics and their range of efficacy is wider than vaccination[4]. Immunostimulants are particularly suitable for boosting immature immune systems and effective against a number of opportunistic and secondary pathogens. With a detailed understanding of the efficacy and limitations of immunostimulants, they may become powerful tools to control shrimp diseases. Use of marine resources such as marine macroalgae, which are abundantly available particularly in the Gulf of Mannar region, if could be used as a source of immunostimulants, will be an added advantage to the aquaculture practices. The bio-activity of incorporated seaweed in shrimp feed could help to enhance the immunity and thereby the productivity can be increased. Further, this study was also designed to assess the immune enhancement level due to seaweed using proPO assay in penaeid shrimps.

Marine brown alga, *Sargassum wightii* (Phaeophyceae/Punctariales) collected from the Gulf of Mannar coast was dried under room temperature and powdered. The basal shrimp feed was prepared[5] and the feed was given a coating of seaweed powder, using egg albumin as binder. The inclusion level of the seaweed was at three different concentrations viz. 10, 20, and 30 g/kg (experimental feeds). The shrimps were tested PCR negative (Nested PCR). Hatchery produced tiger shrimp (*Penaeus monodon*) seeds (PL-

20) were reared in nursery tanks (2 ton capacity cement tanks) till they attain 3-4 g size. They were distributed to 70 numbers of experimental tanks (6 each) of 50 liter capacity, kept on the water recirculation system (WRS) and acclimitized. WRS ensured adequate dissolved oxygen level (>3.0 ppm) and the ammonia within the safer limits (< 0.1 ppm) for the shrimps. While four tanks were maintained as control, six more tanks were used for the estimation of LD_{50} (pathogenicity test) by injecting bacterial strain *Vibrio parahaemolyticus*. The other 60 tanks were fed with the experimental feeds and 30 each for haemolymph collection and challenge studies. The seaweed (*Sargassum wightii*) coated feed was fed at their maximum feeding level for 5 days to the treatment shrimps followed by control feed for the next 12 days. Haemolymph was drawn from the experimental shrimps at regular intervals viz. 1st, 3rd, 6th, 9th and 12th day after feeding with the treatment feed for quantitating the prophenol oxidase activity (proPO) level.

On the same day of the haemolymph collection, immune enhancement in treated shrimps (10, 20, and 30 g/kg of seaweed) was detected by challenging them with *Vibrio parahaemolyticus* (LD_{50} - 2.5×10^7 cfu / shrimp). For challenge studies, bacterial culture of *Vibrio parahaemolyticus* obtained from the Institute of Microbial Technology (IMTECH, Chandigarh) was maintained by subculturing after testing them on healthy shrimps to reproduce the specific pathogen. After incubation at 37°C for 24 h in Tryptic Soya Broth (TSB), the *Vibrio parahaemolyticus* culture were harvested in sterile saline solution (2% NaCl) and diluted by tenfold serial dilution. The LD_{50} value was determined by administering the lower concentration of cultures ($10°–10^{-4}$) to the juveniles of *Penaeus monodon* by intra muscular injection between fourth and fifth abdominal segment with 0.05 ml from different suspensions ($10^0– 10^{-4}$). Parallel controls with sterile, saline injection (2% NaCl) and no injection were also maintained. The LD_{50} was determined by recording the mortality for 5 days. The experimental shrimp that is immunostimulant treated shrimps were challenged with pathogenic strain of *Vibrio parahaemolyticus*. Bacterial cell counts approximately to that of LD_{50} values, were injected into the experimental shrimps. Parallel controls with no immunostimulant treatment and saline control were also maintained. The survival pattern was also observed for the period of 5 days after challenging.

ProPO activity in haemolymph was measured[3]. For proPO assay, haemolymph was collected from experimental shrimps (after giving 10 days of immunostimulant-incorporated shrimp feed and 5 days of control feed) using a 26 gauge needle of 1 ml syringe by inserting it into the ventral sinus located at the base of the abdominal segment. Haemolymph was collected in anticoagulant (in 1:1 - trisodium citrate-30 mmol, sodium chloride-338 mmol, glucose-115 mmol, EDTA-10 mmol, *p*H. 7) for the proPO assay.

Phenoloxidase activity in haemocytes was measured[3] using ELISA reader. Haemocyte suspension was separately incubated with zymosan and transferred to microtitre plate (ELISA plate) in duplicate (60 µL each). L-DOPA was added to haemocyte suspension. After 10 min, optical density was measured at 492 nm using ELISA reader (Lab Systems, Finland). Protein

Table 1—Pro phenol oxidase activity in haemocytes of *Penaeus monodon*

Sl. No.	Treatment g/kg of feed	Pro phenol oxidase activity (u/min/mg of protein × 10^{-5})				
		1st day	3rd day	6th day	9th day	12th day
1	*Sargassum* 10	0.37	0.29	0.22	0.52	0.62
2	*Sargassum* 20	0.29	0.29	0.46	0.26	0.42
3	*Sargassum* 30	0.39	0.34	0.57	0.27	0.42
4	Control	0.38	0.38	0.38	0.38	0.38

Table 2—Challenge studies in *Penaeus monodon* by using *Vibrio parahaemolyticus* (dose 2.5×10^7 cfu/shrimp)

Sl. No.	Treatment g/kg of feed	Survival (%)				
		1st day	3rd day	6th day	9th day	12th day
1	*Sargassum* 10	58	42	42	75	83
2	*Sargassum* 20	42	50	67	42	67
3	*Sargassum* 30	58	50	75	50	67
4	Control	50	50	50	50	50

content of haemocyte suspension was measured [6] to estimate the proPO activity for 0.001 μ/min/mg of protein. Significant difference in prophenoloxidase and challenge test between treatments depending on diets and duration, using Statistical Package for Social Studies (SPSS) was established. The significant level used was $P < 0.05$.

The highest prophenoloxidase (Table 1) activity in the haemolymph of *Penaeus monodon* (0.62 μ/min/mg of protein) was recorded on the 12th day with the experimental feed 10 g/kg. It was much higher than the control which recorded only 0.38 U/min/mg of protein proPO activity. The other relatively higher result was observed on the 9th day of 10 g/kg and 6th day of 30 g/kg of experimental feeds respectively. Challenge study (Table 2) using *Vibrio parahaemolyticus* in *Penaeus monodon* showed relatively a higher survival rate on the 12th day. The highest survival level and relative percent survival were observed at the concentration of 10 g/kg of experimental feed on the 12th day after treatment feed were 83% and 66 respectively. The other higher results observed were on the 9th day of 10 g/kg and on the 6th day of 30 g/kg of experimental feeds respectively (Table 2). Even though the percentage of increment was conspicuous in both proPO assay and challenged shrimps, there was no significant difference exhibited in immunostimulant treated shrimps.

The highest (0.62 μ/min/mg of protein) proPO activity was recorded on the 12th day with the seaweed incorporated (10 g/kg) shrimp feed, explained that the resistance level to pathogen gradually increased in shrimps to attain its peak on the 12th day. Whereas for the other two concentrations (20 and 30 g /kg) the peak level attained during the 6th day and subsequently reduced with the extended duration. The control value however did not show any changes and remained at the mean level of 0.38, confirmed that the incorporation of seaweed did alter the proPO system of shrimps.

The conventional challenge tests conducted for the shrimps exposed to the similar concentrations of seaweeds viz. 10, 20 and 30 g/kg recorded a similar pattern with respect to the proPO activity. The maximum survival (83%) was witnessed for 10g/kg concentration at 12th day, followed by the other two concentrations viz. 20 and 30 g/kg which recorded the maximum survival at 6th day, proved that proPO assay can be an effective tool to assess the immune enhancement or depression pattern in shrimps. As the accuracy level of challenge test is nowadays put under more scrutiny, tests like proPO assay can throw more light on the immune system of shrimps if adopted along with challenge tests to derive meaningful conclusions.

By evaluating the efficiency of the abundant marine resources such as seaweeds as marine immunostimulant will be handy to the aquaculture industry. Since seaweeds are known to contain carbohydrates, protein, vitamin, minerals and micronutrients and they are the potential sources of food, fertilizer and renewable energy.

The proPhenol Oxidase (proPO) activity of haemolymph, an important enzyme linked mediator of crustacean immunity has been demonstrated to be an effective tool that can be used independently or along with challenge studies for confirmation of immune enhancement pattern in penaeid shrimps. The effectiveness of the seaweed *Sargassum wightii* as immunostimulant in penaeid shrimps is comparable to those of other immunostimulants (LPS, chitin and β-Glucan) tested with proPO effects (8-10). Seaweed *Sargassum wightii* would prove to be an eco-friendly and economically viable source of marine immunostimulant for penaeid shrimps due to their abundant availability as marine resources all along Indian coast in general and Gulf of Mannar in particular. Further, the scope for farming seaweeds also has been standardized in India, to fulfill the demand of the shrimp aquaculture sector.

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