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Effect of periphyton on microbial dynamics, immune responses and growth performance in black tiger shrimp *Penaeus monodon* Fabricius, 1798

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ABSTRACT

To evaluate the effect of periphyton, an on-station grow out trial was conducted for 130 days with tiger shrimp, *Penaeus monodon* @ 8 nos. m^{-2} in zero water exchange ponds. Bamboo poles (1.8×0.06 m) were fixed @ 2000 nos. ha^{-1} in treatment ponds for periphyton development. Total bacterial count (TBC) in water was significantly lower ($p<0.05$) in periphyton based ponds ($83.50\pm11.86\times10^3$ CFU ml^{-1}) compared with control ($288.00\pm90.15\times10^3$ CFU ml^{-1}). The total *Vibrio* count (TVC) in water had similar pattern which was 35.3% lower in treatment group compared to control. The TBC and TVC in periphyton biomass over the submerged substrate were $333.13\pm114.14\times10^6$ CFU g^{-1} and $151.88\pm26.37\times10^3$ CFU g^{-1} respectively. At the end of the culture period, higher haemocyte count ($p>0.05$) ($10.83\pm0.71\times10^7$ cells ml^{-1}) and prophenol oxidase activity ($p<0.05$) (8.65±0.47 U) was recorded in treatment ponds compared to control ($9.38\pm0.47\times10^7$ cells ml^{-1} and 5.18±0.51 U). In pathogen clearance test, treatment group recorded significant reduction ($p<0.05$) of *Vibrio harveyi,* 3 h post-inoculation. At the end of the trial, 17.90% gain in production and 22.29% reduction in FCR was observed in periphyton group compared to control.

Keywords: Black tiger shrimp, Immune response, Microbial dynamics, *Penaeus monodon,* Periphyton, Substrate

Introduction

Aquaculture is the fastest growing food producing sectors in the last three decades with an average annual growth rate of 8% since 1981 (FAO, 2012). Black tiger shrimp, *Penaeus monodon* is one among the commercially important penaeid shrimps in India due to its fast growth rate, high unit price and increase in return on investment. However, frequent disease outbreaks, environmental degradation and escalating price of commercial feeds and probiotics for shrimp culture necessitate exploring sustainable and environmentally sound farming methods. Periphyton based shrimp culture is an eco-friendly approach in aquaculture. Periphyton refers to the entire complex of microalgae, heterotrophic bacteria, benthic organisms and detritus developed over submerged substrate in aquatic systems (Azim and Azaeda, *et al.,* 2005). Consumption of microorganisms present in submerged biofilms significantly improve the growth of post-larval stages of penaeid shrimps like pink shrimp *Farfantepenaeus paulensis* (Ballester *et al.,* 2007), brown tiger shrimp *Penaeus esculentus* (Burford *et al.,* 2004), black tiger shrimp *Penaeus monodon* (Khatoon *et al.,* 2009; Anand *et al.,* 2013a) and western white leg shrimp *Litopenaeus vannamei* (Moss and Moss, 2004). Apart from providing the high quality natural food, microbial community developed over submerged substrate improves water quality through nitrification (Ramesh *et al.,* 1999; Thompson *et al.,* 2002; Anand *et al.,* 2013a).

Disease defense mechanism in fishes and other vertebrates depends on highly evolved adaptive immunity and non-specific innate immune system (Uribe *et al.,* 2011). However, invertebrates like shrimp mainly rely upon the non-specific innate immune mechanism (Bachere, 2000). This comprises structural barriers such as cuticle and tegumental glands, and cellular and humoral responses (Amparyup *et al.,* 2013). Circulating haemocyte population indicates the cellular immunological response in crustaceans (Bachere, 2000). These cells demonstrate a variety of activities like phagocytosis, encapsulation, nodular aggregation (Rodriguez and Le Moullac, 2000) and release of prophenoloxidase system (Hernandez-Lopez *et al.*, 1996). The diverse array of humoral response includes prophenoloxidase (proPO) system and antioxidant enzymes such as superoxide dismutase

(SOD), catalase and peroxidase (Amparyup *et al.,* 2013). The proPO system has been associated with synthesis of antimicrobial peptides (Fagutao *et al.,* 2009), activation of reactive oxygen and nitrogen intermediates (Cerenius *et al.,* 2008; Zhao *et al.,* 2011) and synthesis and deposition of melanin at the site of infection (Cerenius and Soderhall, 2004).

Algal products and their cell wall components are widely used to elicit non-specific defence mechanism in fishes and shrimp (Promya and Chitmanat, 2011). It is speculated that microbial biofilms also play a positive role in immune mechanism of aquatic animals (Sharma *et al.,* 2010). Periphyton, being a complex of autotrophic algae and heterotrophic bacteria enhances immune responses in shrimps like *L. vannamei* (Zhang *et al.,* 2010). Recently, it has been reported that periphyton powder as dietary supplement enhances immune response and disease resistance in *P. monodon* (Anand *et al.,* 2014). However, there is dearth of information with regard to culture of shrimp in periphyton based pond ecosystem and its role on immune responses. Therefore, the present work evaluates the effect of periphyton on pond microbial dynamics and immune responses in black tiger shrimp in periphyton based culture system.

Materials and methods

Experimental site and pond management

The experiment was carried out for a period of 130 days during July to November 2010 in the brackishwater farm of Kakdwip Research Centre of ICAR-Central Institute of Brackishwater Aquaculture **(**ICAR-CIBA), Kakdwip (21^0 51'N and 88^0 11' E), South 24 Parganas, West Bengal, India. Four earthen ponds (0.18-37 ha) were selected for grow out culture. Before start of the experiment, all ponds were allowed to dry until cracks developed and top soil was removed. Ponds were filled with strained brackishwater from a nearby Muriganga Creek to a depth of 150 cm and kept for 5-6 days. Bleaching powder ($CaOCl_3$) was applied @ 600 kg ha^{-1} to reduce the risk of disease outbreak from pathogenic bacteria, virus and unwanted seed of other organisms. After two weeks, agricultural lime ($CaCO_3$) was applied at the rate of 100-200 kg ha^{-1} based on the pond pH. On the 18^{th} day, ponds were fertilised with semi-decomposed cattle manure, urea and triple super phosphate (TSP) at a dose of 1000, 100 and 100 kg ha^{-1}, respectively. On day 20, bamboo poles (whole and split bamboo @ 1:1) were installed at a rate of 2000 numbers ha^{-1} in treatment ponds. After bamboo pole installation, the ponds were left for 15 days to facilitate development of periphyton over bamboo substrate. Hatchery reared *P. monodon* PL 20 (Chennai, India) was stocked at a density of 8 nos. m^{-2} in the treatment ponds with bamboo substrate (T_S) and control ponds without substrate (T_C). Culture was carried out during monsoon season as zero water exchange system which relies mainly on rain to compensate evaporation and seepage loss.

Shrimp stocking and feeding management

Formulated shrimp feed with 38% protein content was used in the culture pond (Table 1). During the first month of the culture period [0–30 days of culture (DOC)], "blind feeding" was adopted in both treatment and control groups. This consisted of 1 kg feed per 100,000 post-larvae (PL) (Corre, 1999). Subsequently, feed quantity was adjusted based on shrimp body weight calculated from weekly sampling and assumed survival percentage from cast net sampling and check tray observation. Feed was given at 5–2% of the body weight of shrimps from second to final month of culture. Daily ration was distributed four times per day, 40% in the morning (06:00 and 11:00 h) and 60% in the evening (18:00 and 22:00 h). Proximate composition of feed is presented in the Table 1. Check tray observations were monitored to keep strict feeding regime. Yeast based probiotic preparation was periodically applied as nutrient supplement, and for overall improvement of pond environment. For this purpose, yeast (2 kg), molasses (30 kg) and paddy flour (60 kg) were soaked in water for 48 h and applied at fortnightly intervals.

Table 1. Proximate composition of the feed used in *P. monodon* culture

Composition	Percentage
[a]Crude protein	38.35
Crude fat	7.75
Crude fiber	3.36
Total ash	14.25
[b]Nitrogen free extract	27.80
Organic matter	85.75
Moisture	8.49

[a]Organic matter = 100-Ash %.
[b]Nitrogen free extract = 100 - (Crude protein% + Lipid % +Crude fiber % +Ash % + Moisture)

Water quality parameters

Water samples were collected between 09:00 and 10:00 hrs at fortnightly intervals and analysed immediately after return to the laboratory. Physical parameters like salinity, temperature and pH were analysed using an Atago hand refractometer (Atago, Japan), thermometer and pH meter (model 10E; Deluxe), respectively. Water quality parameters like total ammonia-N (TAN), nitrite-N (NO_2-N), nitrate–N (NO_3-N) and PO_4-P were determined spectrophotometrically following APHA (1998).

Bacterial load estimation

Total bacterial count (TBC) and total *Vibrio* counts (TVC) of water, sediment and periphyton were determined at monthly intervals as per the method of Kumar *et al.* (2014). In brief, water samples were collected from 5–10 cm depth at two different sites, mixed and homogenised in a kitchen blender (12000 rpm, 30 sec). Sediment samples were processed by homogenising (12000 rpm, 1 min) 20 g of soil in 180 ml of normal saline solution (NSS). Periphyton sample was collected by gently scrapping the three bamboo substrates with sterile scalpel and processed by homogenising (12000 rpm, 1 min) 2 g of sample in 198 ml of NSS. These bamboo substrates were marked and restored in the pond and not used for further sampling. Subsequently, ten-fold serial dilutions of water, sediment and periphyton samples were made in NSS. Appropriate dilutions (0.1 ml each) were plated in duplicates on tryptone soya agar (1.0% w/v NaCl) for total bacteral count (TBC) and thiosulfate citrate bile salt sucrose (TCBS) agar for total *Vibrio* count (TVC). Plates were incubated at room temperature for 48 h and those with colonies in the range of 30 to 300 were counted and expressed as bacterial colony forming unit (CFU).

Collection of haemolymph and serum

After completion of the experiment, 10 inter-moult shrimps from each replicate of each treatment group were anesthetised with clove oil (50 µl of clove oil l^{-1} brackishwater) before collecting hemolymph (Anand *et al.,* 2014). The inter–moult stage was determined by the setal development of the uropod using stereomicroscope (Dall *et al.,* 1990). Hemolymph was collected from the ventral sinus of each shrimp with 26 gauge 1ml tuberculin syringe and mixed with cooled anticoagulant (30 mM tri-sodium citrate, 388 mM sodium chloride, 0.12 M glucose, 10 mM EDTA; 780 mOsm kg^{-1} osmolality, and pH 7.55). To collect serum, hemolymph without anti-coagulant was allowed to clot at 4°C overnight in refrigerator, and centrifuged at 600 *g* for 5 min. The supernatant was collected as serum in aliquots and stored immediately at -20°C.

Total haemocyte count

Immediately after haemolymph collection, 50 µl haemolymph–anticoagulant solution (1:10) was mixed with 50 µl of Rose Bengal solution (1.2% Rose Bengal in 50% ethanol). The stained haemolymph was counted in improved Neubauer bright-line chamber under 40X objective in binocular microscope and expressed as total hemocyte count (Ananda Raja *et al.*, 2012).

Immunological and biochemical parameters

The prophenoloxidase activity (proPO activity) was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as substrate (Hernandez–Lopez, 1996). Briefly, 50 µl serum was incubated with 50 µl trypsin (0.1% in cacodylate-citrate (CAC) buffer; 0.45 M sodium chloride, 0.10 M trisodium citrate, 0.01 M sodium cacodylate, pH 7.0) in Eppendorf tube at 25°C for 10 min in water bath. Subsequently, 50 µl L-DOPA (0.3% in CAC buffer) was added and incubated at 25°C. After 5 min, 800 µl CAC buffer was added and further incubated at 25°C for 3 min. The OD was recorded at 490 nm against blank (50 µL of L-DOPA, 50 µL 0.1% trypsin, and 850 µl CAC buffer). One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1}.

Pathogen clearance test was conducted at the end of pond trial as per the method described by Sritunyalucksana *et al.* (2005) with minor modifications. In brief, to determine bacterial clearance rate, 16 shrimp from each of the 2 treatment groups were injected intramuscularly at the 6th abdominal segment with 20 µl of a suspension of *Vibrio harveyi* containing 1.13×10^8 cells ml^{-1}. Three shrimps from each treatment groups were removed immediately after injection and at 3 h post-injection another three, to determine the number of bacterial cells ml^{-1} in hemolymph. Hemolymph (50 µl) was collected from the ventral sinus of the first abdominal segment into a syringe containing 50 µl of anticoagulant solution. This was serially diluted 10 fold in NSS. Quadruplicate aliquots of 20 µl each were dropped onto plates of thiosulphate citrate bile salt sucrose (TCBS) agar. Plates were counted after incubation at 28°C for 18 h.

Serum protein was estimated by Lowry's method (Lowry *et al.,* 1951) using bovine serum albumin as standard. Serum glucose was quantified by 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959).

Growth performance

After 130 days of culture, growth performance was evaluated in terms of final average body weight (ABW), feed conversion ratio (FCR) and total production (kg ha^{-1}) (Anand *et al.,* 2013b).

Statistical analysis

Student's t test was used to statistically analyse the data on microbial, immune response and water quality parameters between substrate and without substrate based systems. Statistical package SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used. Level of significance was fixed at 95% probability level.

Results and discussion

The salinity and pH during the study period ranged between 6.8-18.1 ppt and 7.75- 8.87 respectively. Dissolved

oxygen ranged between 4.8-6.6 ppm without significant difference between treatments. Water nutrient parameters like total ammonia-N (TAN), nitrite-N (NO_2-N), nitrate-N (NO_3-N) and phosphate-P (PO_4-P) during the experimental period are presented in Fig. 1. The recorded water quality parameters were within the acceptable ranges for brackishwater shrimp culture (Chakraborti *et al.,* 2002) with no significant difference ($p>0.05$) among the treatments. However, a comparatively lower mean value for TAN, NO_2-N, NO_3-N, and PO_4-P were observed in substrate based system. A marked reduction in TAN and NO_2-N were recorded from 5th and 9th week onwards respectively in treatment ponds compared to control. Earlier, it has been reported that provision of submerged substrates improves the water quality parameters in culture ponds (Ramesh *et al.,* 1999; Thompson *et al.,* 2002). This may be attributed to the utilisation of these nutrients by phytoplankton communities for periphyton production.

At the end of the grow-out trial, *P. monodon* reared in periphyton based system attained an average body weight (ABW) of 25.85±2.62 g with a total production of 1640.00±367 kg ha^{-1} as compared to 22.00±2.83 g ABW and 1390.00±28 kg ha^{-1} in control ponds (Table 2). Similarly, 22.29% reduction in FCR was noticed in substrate based ponds (1.15±0.42) compared with control (1.48±0.02). The details of growth performance of *P. monodon* grown under periphyton based culture systems are presented in Anand *et al.* (2015). This is in line with the earlier reports, where considerable production gain and improved FCR was recorded in substrate based culture of *P. monodon* (Arnold *et al.,* 2006; Anand *et al.,* 2013a), *L. vannamei* (Audelo-Naranjo *et al.,* 2011) and *F. paulensis* (Ballester *et al.,* 2007). Periphyton developed over submerged substrates provides grazing space for shrimps. The better growth performance recorded in shrimps reared in substrate based systems can be attributed to availability of natural food in the form of microalgae, zooplankton and other natural microorganisms as periphyton, over submerged substrate (Moss and Moss, 2004). Moreover, it has been reported that periphyton improves digestibility of feed by enhanced secretion of digestive enzymes (Anand *et al.,* 2013b). It further confirms that shrimp being bottom dweller, grows better by grazing on periphyton than filtering suspended algae from the water column (Kumlu *et al.,* 2001). It has been reported that presence of substrate increases the available surface area and reduces the negative effects of overcrowding (Audelo-Naranjo *et al.,* 2011).

There was significant difference ($p<0.05$) in total bacterial count (TBC) in water with lower TBC in substrate based ponds ($83.50±11.86×10^3$ CFU ml^{-1}) compared with control ($288.00±90.15×10^3$ CFU ml^{-1}) (Table 3). The total *Vibrio* count (TVC) in water had similar trends which was 35.3% lower in treatment ($29.75±9.87×10^1$ CFU ml^{-1}) compared to control ($46.00±9.09×10^1$ CFU ml^{-1}). The lower level of bacterial load in periphyton based ponds could be attributed to attachment of microbes over the substrate. This may be substantiated from the fact that microbial load in periphyton was almost 4000 times higher compared to water. There is also a possibility that periphytic community in substrate secrete some antibacterial compounds which prevents the microbial growth in pond ecosystem. In soil, comparatively lower TBC and TVC were observed in treatment groups compared to control. Total heterotrophic bacterial load and *Vibrio* in the periphyton biomass

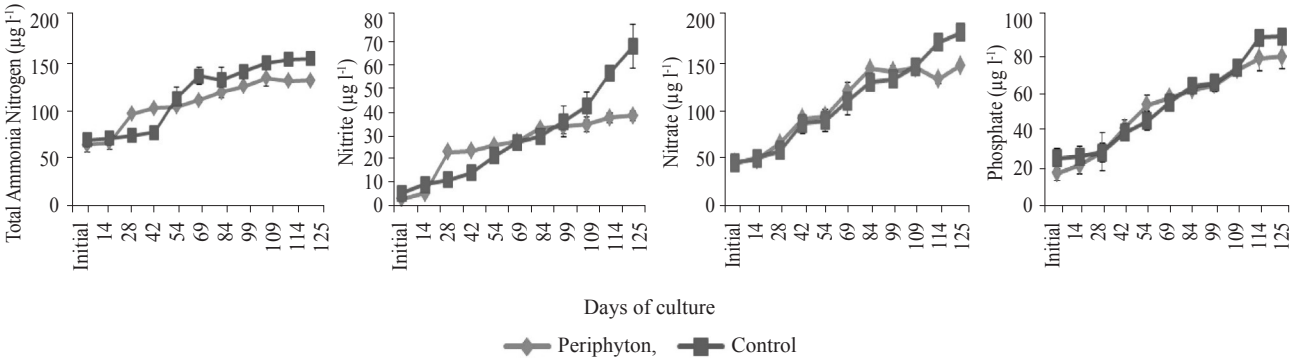

Fig. 1. Water quality parameters (Mean±SE), Total ammonia nitrogen (TAN), Nitrite-N, Nitrate-N and Phosphate-P in periphyton and control systems during the study period

Table 2. Growth performance parameters (mean ± S D) of tiger shrimp cultured in periphyton and control system*

Growth performance parameters	Periphyton	Control
Average final body weight (g)	25.85±2.62	22.00 ±2.83
Shrimp production (kg ha^{-1})	1640.00±367.00	1390.00±28.00
Feed conversion ratio	1.15± 0.42	1.48±0.02

* The details of growth performance data has been presented in Anand *et al.* (2015)

Table 3. Mean microbial load in water, sediment and periphyton between the treatment groups (mean±SE)

Sample	Microbial groups	Periphyton	Control	Level of significance
Water	Total bacterial count ($\times10^3$ CFU ml^{-1})	83.50±11.86	288.00±90.15	*
	Total *Vibrio* count ($\times10^1$ CFU ml^{-1})	29.75±9.87	46.00±9.09	NS
Soil	Total bacterial count ($\times10^6$ CFU g^{-1})	120.63±45.89	135.94±75.89	NS
	Total *Vibrio* count ($\times10^3$ CFUg^{-1})	35.94±9.84	53.75±11.85	NS
Periphyton	Total bacterial count ($\times10^6$ CFUg^{-1})	333.13±114.14	–	
	Total *Vibrio* count ($\times10^3$ CFU g^{-1})	151.88±26.37	–	

developed over the submerged bamboo substrate were 333.13±114.14x10^6 CFU g^{-1} and 151.88±26.37×10^3 CFU g^{-1} respectively. Ramesh *et al.* (1999) compared dried sugarcane bagasse, paddy straw and dried water hyacinth as substrate for biofilm production. They observed that level of biofilm production was highest with sugarcane bagasse (6.7×10^7 CFU g^{-1}) followed by paddy straw (5.2×10^7 CFU g^{-1}) and water hyacinth (4.9×10^7 CFU g^{-1}) which correlated with the fish productivity. The microbial communities over the time periods are presented in

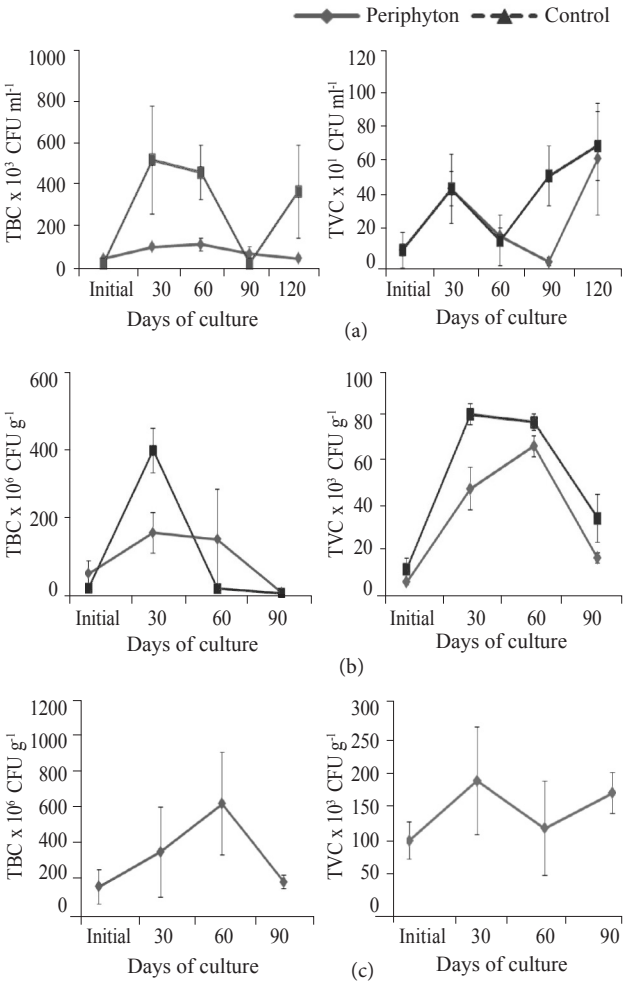

Fig. 2. Microbial composition in (a): Water, (b): Soil and (c): Periphyton (Mean±SE) at different time intervals. TBC: Total bacterial count; TVC : Total *Vibrio* count

Fig. 2. The results reflect that water bacterial load was lower throughout the culture period in periphyton based ponds compared to control. Further, presence of substrate provided the stability in microcosm with less fluctuation in microbes in water and sediment of substrate based system compared to control. In periphyton, the level of microbial load increased over the time and decreased in the last month of culture. This may be due to increased grazing pressure by shrimps during the last month of culture. Our present work was limited to the population study of total microbes and *Vibrio*. Details of microbial communities taking part in periphytic biofilm formation needs to be investigated further.

In the present study, shrimps reared in substrate based system recorded better non-specific immune factors like prophenol oxidase activity, haemocyte count and pathogen clearance parameters (Fig. 3). There was comparatively higher haemocyte count (10.83±0.71×10^7 cells ml^{-1}) in treatment groups compared to control (9.38±0.47×10^7 cells ml^{-1}). Similarly, significantly higher ($p<0.05$), prophenol oxidase activity 8.65±0.47 U was observed in the treatment group compared to control (5.18±0.51 U). Pathogen clearance test in the shrimps revealed a significantly lower pathogenic load of inoculated *V. harveyi* in haemolymph of shrimps in periphyton based farming system (0.19±0.11×10^6 CFU ml^{-1}) compared to control (0.98±0.20×10^6 CFU ml^{-1}). These indicate the positive role of periphyton in enhancing immune response in shrimps. Recently, Anand *et al.* (2014) observed that inclusion of periphyton in shrimp diets improved the non-specific immune parameters like haemocyte count, superoxide dismutase activity and survival post-challenge. It could be that probiotic bacteria may be existing in the periphytic biofilms which stimulates the shrimp immune system. There is also a possibility that continuous uptake of microbial organism by cultured shrimps at sub-infectious concentration through periphyton makes the aquatic animals resistant to the pathogens. Further research is needed to elucidate the underlying mechanism and the cause-effect relationships. It has been noticed that pathogens can be established in periphyton mats (Acs *et al.,* 2003) where it can remain for a long period in extracellular matrix of biofilm

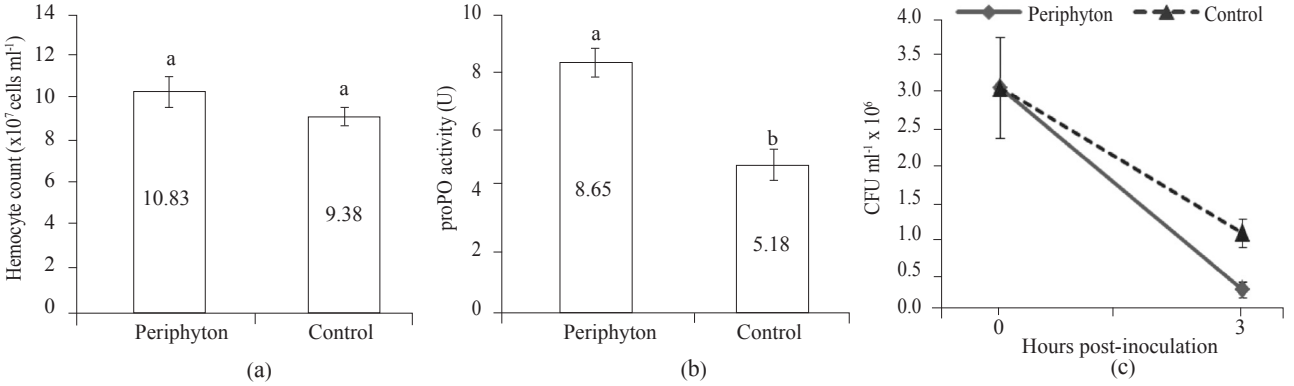

Fig. 3. Immunological parameters in *Penaeus monodon* at the end of culture (Mean±SE). (a): Total haemocyte count; (b): Prophenol oxidase (proPO) activity; (c): Pathogen clearance test.

providing protection against bacterial infection (Fux *et al.,* 2003). However, Zhang *et al.* (2010) reported that application of PVC pipes as substrate in *L. vannamei* at very high stocking density of 500 nos. m^{-2} in tank based system reduced the phenol oxidase (PO), antibacterial and lysozyme activity. The authors concluded that provision of substrate probably reduced the stress level in treatment groups leading to reduced immunological and anti-oxidative parameters. The present work was conducted in natural environment with a low stocking density of 8 nos. m^{-2} without external stress factor. These results warrant further studies with respect to species, stocking density and type of substrate in periphyton based system *vis-a-vis* shrimp immune response.

In the present study, shrimps reared in substrate based ponds recorded better serum biochemical parameters like protein and glucose (Fig. 4). A significantly higher ($p<0.01$), serum protein (8.80±0.02 mg ml^{-1}) and non-significantly higher serum glucose (0.47±0.15 mg ml^{-1}) was recorded in shrimps reared in periphyton based ponds compared to control ponds,7.35±0.07 and (0.40±0.11 mg ml^{-1}), respectively. The increase of serum glucose and protein level in periphyton based group indicates that microbial

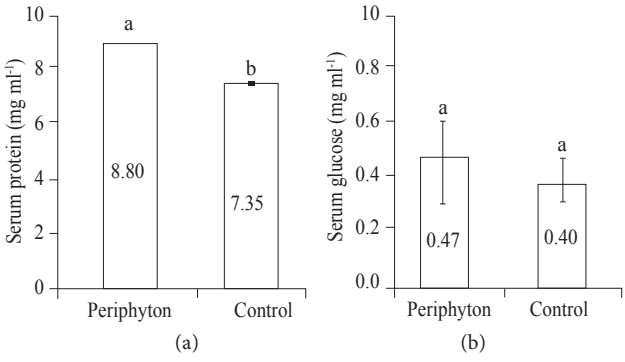

Fig. 4. Serum biochemical parameters (Mean±SE) (a): Serum protein (mg ml^{-1}); (b): Serum glucose (mg ml^{-1}) in *Penaeus monodon*

and algal communities of periphyton enhanced the digestive and assimilative ability of the shrimp (Anand *et al.,* 2013b). Higher serum glucose level will direct the glucose-6-P as a source of energy in tissues (Yu *et al.,* 2008). The increase of serum protein may result in good growth performance with protein accumulation. Overall the results indicate that shrimp in periphyton based system were healthier with better growth rate compared to shrimp of control ponds.

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