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Effect of dietary supplementation of periphyton on growth performance and digestive enzyme activities in *Penaeus monodon*

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ABSTRACT

A 60-day indoor growth trial was conducted to study the effect of dietary supplementation of periphyton on growth performance and digestive enzyme activities in *Penaeus monodon* juvenile. Periphyton developed over bamboo substrate in outdoor cistern tanks (15 m^2) was used as a dietary supplement in *P. monodon* (2.02 ± 0.04 g) reared in 1000 l fiberglass reinforced plastic tank. Graded level of dried periphyton was included in shrimp basal diets; 0% (control), 3% (T1), 6% (T2), 9% (T3) and a control diet with natural bamboo substrate for periphyton growth (T4) served as a positive control. Significantly lower ($p<0.01$) level of NO_3-N, and PO_4-P were noticed in T4 compared to other experimental groups, while no significant difference in other water quality parameters were noticed among the treatments. At the end of the feeding trial, significantly higher ($p<0.01$) body weight was noticed in T2 (5.50 ± 0.21 g) and T4 (5.33 ± 0.13 g) compared with control (4.44 ± 0.17 g). Similarly, significantly better ($p<0.01$) feed conversion ratio (FCR), 2.01 ± 0.08 and protein efficiency ratio (PER), 2.70 ± 0.12 was noticed in T2 compared to control (FCR 2.81 ± 0.19 and PER 1.94 ± 0.14) and T3 (FCR 3.58 ± 0.14 and PER 1.51 ± 0.06), while no significant difference was noticed between T4 and T1. At the end of the feeding trial, there was 75.45, 128.3, 178.5 and 76.6% significant increase ($p<0.05$) in gut digestive enzymes amylase, cellulase, protease and trypsin respectively in T2 compared with control. Similarly, most of the digestive enzyme activities in T1 and T4 were superior compared to control and T3. However, treatment with 9% level of periphyton inclusion (T3) did not differ significantly ($p>0.05$) for most of the digestive enzyme activities, growth, FCR and PER compared with control indicating inherent upper limit of periphyton inclusion. The present study elucidates the suitability of periphyton as a novel dietary supplement and its supplementation up to 6% in shrimp for enhancing growth and digestive enzyme activities in tiger shrimp juvenile.

1. Introduction

Commercial shrimp feed constitutes 50–60% operational cost of intensive shrimp farming (Wasielesky et al., 2006). So, formulation of nutritionally balanced feed with quality dietary supplement is an imperative area of research to meet the nutritional demand and maximize the growth of shrimp. Dietary supplements are widely used in shrimp aquaculture to enhance growth, immune response, digestive enzyme activity and nutrient absorption in shrimp. Most commonly used dietary supplements in penaeid shrimp diets are microalgae (Ju et al., 2009; Supamattaya et al., 2005), seaweed (Yeh et al., 2006), herbal extract (Sankar et al., 2011), yeast (Yang et al., 2010), probiotics (Wang, 2007; Ziaei-Nejad et al., 2006), carotenoids (Boonyaratpalin et al., 2001) and prebiotics (Zhang et al., 2012). Recently, manipulation of carbon nitrogen ratio for development of biofloc and applications of immobile substrate for development of autotrophic and heterotrophic community have shown promise as the environmentally-friendly alternatives to feed additives or growth promoters in aquaculture (Anand et al., 2012; Asaduzzaman et al., 2010; Avnimelech, 1999).

Periphyton refers to the entire complex of attached aquatic biota on submerged substrates comprise phytoplankton, zooplankton, benthic organisms and detritus (Azim et al., 2005; van Dam et al., 2002) and forms an additional food in aquatic production systems. Many trials in fish culture ponds have demonstrated the utility of submerged substrate in enhancing the fish production (Asaduzzaman et al., 2009; Azim et al., 2005; Jana et al., 2004; Keshavanath et al., 2002). In penaeid shrimp culture, consumption of periphyton developed over submerged substrate significantly improves growth of penaeid shrimp, *Fenneropenaeus paulensis* (Ballester et al., 2007; Thompson

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et al., 2002), *Penaeus esculentus* (Burford et al., 2004), *Litopenaeus vannamei* (Audelo-Naranjo et al., 2011; Moss and Moss, 2004) and *Penaeus monodon* (Anand et al., 2012; Khatoon et al., 2007).

In general, most research makes use of *in situ* developed periphyton for growth enhancement of shrimp (Audelo-Naranjo et al., 2011; Khatoon et al., 2009). However, it has been noticed that consumption pattern and feeding efficiency of periphyton depend on the grazing efficacy of cultured species and, fishes are found to be fast grazers than crustaceans (Asaduzzaman et al., 2010). Moreover, this *in situ* based technique needs additional oxygen demands for algal respiration, in addition to the oxygen demand of shrimp (Tacon et al., 2002).

Although reports are available about beneficial effects of single species algae or microbial products as a dietary ingredient in penaeid shrimp (Ju et al., 2009; Kuhn et al., 2010), no information is available to support the dietary role of periphyton, a complex mixture of diverse group of autotrophic and heterotrophic community. Hence, to investigate the suitability of periphyton as a dietary supplement, whole periphyton was included in shrimp diets at different level and fed to *P. monodon* in clear water in the absence of periphyton. In this context, the present study aims to evaluate the effect of dietary supplementation of periphyton biomass on growth, survival and digestive enzyme activities in *P. monodon* juveniles.

2. Materials and methods

2.1. Experimental design

Periphyton, a complex mixture of autotrophic and heterotrophic community produced in outdoor tanks was used as dietary supplement in shrimp feed, over a 60-day indoor growth trial. A control diet without periphyton was compared against 3 experimental diets with graded level of periphyton inclusion. Experimental groups were also compared with a positive control, provided with bamboo substrate for natural growth of periphyton. The experiment was conducted at Kakdwip Research Centre, Central Institute of Brackishwater Aquaculture, Kakdwip (21° 51′N and 88° 11′E), West Bengal, India.

2.2. Production of periphyton

Periphyton production trial was carried out in four cement cistern outdoor tanks ($5\times3\times1.7$ m; bottom area 15 m^2) for a period of 45 days during March to May, 2011. Tanks were filled with water from the nearby brackishwater source. Fine meshed filter bag was used to prevent entry of unwanted materials in to the tank and kept for 2 days to settle the suspended particles. Bamboo sticks (165 cm × 2.5 cm) were suspended vertically in the tank at the rate of 100 numbers per tank. Round the clock aeration was provided to meet the demand of oxygen especially at night hours. Agricultural lime ($CaCO_3$) was applied to all the tanks at 200 kg ha^{-1}. Tanks were fertilized with vermicompost made up of 70% cow dung and 30% vegetable waste (3000 kg ha^{-1}), inorganic fertilizers like urea (150 kg ha^{-1}) and single super phosphate (150 kg ha^{-1}) for development of periphytic algae on the submerged substrate. Inorganic fertilizers were applied at fortnightly intervals. Periphyton biomass was manually collected at 15 days interval by scrapping out of periphyton from each bamboo poles and reinstalled them at their position. Due care was taken to remove the periphytic algae and to minimize the chance of scraping out bamboo surface. Collected samples were dried under shade followed by drying in hot air oven at 45 °C. The dried samples were grind in to fine powder (less than 200 μm) and kept in airtight containers in refrigerator until experimental diets were made.

2.3. Estimation of periphyton biomass and chlorophyll pigments

The periphyton biomass in terms of dry mater (DM), ash free dry matter (AFDM) and chlorophyll pigments were determined by collecting periphyton samples from 2×2 cm^2 area of bamboo substrate. The DM and AFDM were determined by weight differences (APHA, 1998). Chlorophyll concentration was calculated using the trichromatic equation given in APHA (1998). For the microscopic examination of periphyton, the scraped periphyton was suspended in 10% buffered formalin and vortexed for 3 min to detach the attached algae and subsequently examined under binocular microscope (Olympus, India) as described by Asaduzzaman et al. (2010). Autotrophic index (AI), the ratio of ash free dry matter with respect to chlorophyll a concentration, was calculated as per the formula described by Asaduzzaman et al. (2008).

$$\text{Autotrophic Index} = \text{AFDM}\left(\mu g\ cm^{-2}\right)/\text{Chlorophyll a}\left(\mu g\ cm^{-2}\right)$$

2.4. Experimental diets

Four isonitrogenous and isoenergetic experimental diets were formulated and its compositions are presented in Table 1. A control diet without periphyton was compared against three experimental diets formulated with graded level of periphyton at 3 (T1), 6 (T2) and 9% (T3). All the ingredients except periphyton powder, cholesterol, butylated hydroxytoluene (BHT), oil and vitamin–mineral mixture were mixed with water to make dough. The dough was steam cooked for 20 min in a pressure cooker at 15 psi. Periphyton powder and other additives were mixed after cooling and dough was pressed through a pelletizer with 2 mm die and then dried at 60 °C till the desired moisture level (<10%) was reached. The feed were stored at 4 °C until use.

2.5. Experimental system and feeding

Healthy juvenile shrimp, *P. monodon* tested negative for white spot syndrome virus was obtained from a scientific shrimp farm (South 24 Parganas, West Bengal, India). Shrimp were acclimatized for 14 days and fed with control diet (40% crude protein) three times daily before start of experiment. The experiment was conducted in triplicate in Fiberglass Reinforced Plastic (FRP) tanks (1000 l; bottom area of 2 m^2) filled with chlorine free brackishwater. Three hundred and sixty *P. monodon* juveniles (2.2 ± 0.04 g) were randomly distributed in the five experimental groups @ 12 nos m^{-2} in each FRP tank following a completely randomized design (CRD). In treatment T4, natural split bamboos ($5\times2\times1$ cm) were suspended in the water column at the rate of 27 nos $tank^{-1}$ for periphyton growth and arranged in three horizontal rows and three vertical columns at 10 cm apart.

Table 1
Composition of experimental diets on dry matter basis (g/kg).

Ingredients	Experimental diets			
	T1	T2	T3	Control
Fish meal	380	380	380	380
Shrimp meal	150	150	150	150
Soyabean meal	195.3	183	170.7	207.6
Wheat flour	155.2	137.5	119.8	172.9
Dried periphyton powder	30	60	90	0
Soya oil	15	15	15	15
Cod liver oil	20	20	20	20
Lecithin	10	10	10	10
Cholesterol	1	1	1	1
Vitamin and mineral mix[a]	23	23	23	23
Butylated hydroxytoluene	0.5	0.5	0.5	0.5
Guar gum	20	20	20	20
Total	1000	1000	1000	1000

[a] Composition of vitamin mineral mix (Supplevite-M) (quantity/kg): Vitamin A, 20,00,000 IU; Vitamin D3, 400,000 IU; Vitamin B2, 800 mg; Vitamin E, 300 unit; Vitamin K, 400 mg; Vitamin B6, 400 mg; Vitamin B12, 2.4 mg; Calcium Pantothenate, 1000 mg; Nicotinamide, 4 g; Choline Chloride, 60 g; Mn, 10,800 mg; Iodine, 400 mg; Fe, 3000 mg; Zn,6 g; Cu,800 mg; Co,180 mg; Vitamin C,1000 mg.

The daily feeding was done at 8% of the body weight at the start of experiment, and declined gradually to 4% of body weight at the end of the experiment. The daily ration was divided in two parts, 40% feed was given in the morning and 60% in the evening. Equal amount of feed was fed to shrimps in all the experimental tanks, twice daily at 10:00 and 18.00 h for 60 days. Left over feed and fecal matter were removed daily and 25% water was exchanged every 3rd day.

2.6. Proximate composition of periphyton and experimental diets

The proximate composition of the periphyton and experimental diets were determined following the standard methods of AOAC (1995). The moisture content was determined by drying at 105 °C to a constant weight and, the difference in weight of the sample indicated the moisture content. Nitrogen content was estimated by Kjeldahl (Kelplus, DXVA, Pelican Equipments, India) method and crude protein was calculated by multiplying nitrogen percentage by 6.25. Crude lipid was determined by the solvent extraction method by Soxtec system (Soxtec system, SCS-6, Pelican Equipments, India) using diethyl ether (boiling point, 40–60 °C) as a solvent. Ash content was determined by incinerating the samples in a muffle furnace at 600 °C for 6 h. Crude fiber was determined based on the weight loss on ignition of the oven dried residue remaining after sequential digestion of a sample with H_2SO_4 and NaOH solution using Fibretec (Foss Tecator 2022, Sweden). Gross energy was determined as per the N.R.C. formula (1993). Total nitrogen free extract (NFE) was determined as per the formula described in Hastings and Dupree (1969).

$$\mathrm{NFE}(\%) = 100 - (\text{Crude protein} + \text{Ether extract} + \text{Ash} + \text{Fiber} + \text{Moisture})$$

$$\text{Gross energy}\left(\text{kcal } 100^{-1}\text{g}\right) = \text{Protein}(\%) \times 5.6 + \text{Lipid}(\%) \times 9.44 + \text{Crude fiber}(\%) \times 4.1 + \text{NFE}(\%) \times 4.1$$

2.7. Determination of water quality parameters

The water quality parameters were measured at fortnightly intervals between 09:00 and 10:00 h. Salinity, temperature and pH were measured using an ATAGO hand refractometer, thermometer and pH meter respectively. Total alkalinity, dissolved oxygen, total ammonia-N (TAN), nitrite-N (NO_2-N), nitrate-N (NO_3-N) and phosphate-P (PO_4-P) were analyzed immediately after sample collection following the procedures described in APHA (1998).

2.8. Growth performance

The growth performance was assessed in terms of percentage weight gain, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival using the following formulae

$$\text{Weight gain}(\%) = (\text{Final weight} - \text{Initial weight}/\text{Initial weight}) \times 100$$

$$\mathrm{SGR}(\%) = (\mathrm{Log_e}\text{final weight} - \mathrm{Log_e}\text{initial weight}/\text{Experimental days}) \times 100$$

$$\mathrm{FCR} = \text{Feed applied}/\text{Body weight gain}$$

$$\mathrm{PER} = \text{Net weight gain}/\text{Protein in feed applied}$$

$$\text{Survival} = \text{Total number of shrimps survived}/\text{Total number of shrimp stocked}) \times 100.$$

2.9. Analysis of digestive enzymes

After completion of the feeding experiment, 18 inter-molt shrimp from each treatment group (6 from each replicate) were sacrificed for digestive enzyme analysis. The molt stage was determined by the setal development of the uropod using stereomicroscope (Dall et al., 1990). The samples were collected 1 h after the last feeding to guarantee maximum activities of digestive enzymes. The hepatopancreas and gut of the shrimp were dissected out, weighed and separately homogenized with 0.25 M chilled sucrose on wet basis (pH 7, 1:10 w/v) in a hand-held glass homogenizer in ice cooled condition. The homogenate was centrifuged at 6000 rpm (2400 × g) for 20 min at 4 °C (Centrifuge 5417R, Eppendorf, Germany). After centrifugation the floating top lipid layer was removed and the supernatant solution was divided as aliquots in 1.5 ml Eppendorf tubes. The samples were stored at −40 °C until analysis.

Total soluble protein of the homogenate was measured using folin-phenol reagent (Lowry et al., 1951). Amylase activity was measured by 3,5-dinitrosalicylic acid (DNS) method (Rick and Stegbauer, 1974). The reaction mixture consisted 0.1 ml of 1% (w/v) starch solution as substrate, 1.8 ml phosphate buffer (0.1 M, pH 7) and 0.1 ml tissue homogenate. The mixture was incubated at 37 °C for 30 min. Later, the reaction was stopped by adding 2 ml DNS reagent and kept in boiling water bath for 5 min. After cooling, the reaction mixture was diluted with distilled water and recorded the absorbance at 540 nm. Activity was determined from the maltose standard curve and expressed as mole of maltose released from starch/min/mg protein at 37 °C.

Cellulase activity was determined based on Miller (1959). The reaction mixture consisted 0.5 ml of 1% carboxymethyl cellulose (CMC) as substrate in 1 ml phosphate buffer (0.1 M, pH 6.8) with 0.5 ml tissue homogenate. The reaction mixture was incubated at 37 °C for 1 h. After incubation the reaction was stopped by adding 3 ml of DNS reagent and the mixture was kept in boiling water bath for 10 min. After cooling the reaction mixture was diluted with distilled water and absorbance was recorded at 574 nm. One unit of cellulase activity was defined as number of molecules of glucose released from cellulose mg^{-1} protein min^{-1} at 37 °C.

Protease activity was determined by the casein digestion method of Drapeau (1976). The reaction mixture consisted 2.5 ml of 1% (w/v) casein prepared in 0.01 N NAOH and 0.05 M tris phosphate buffer (pH 7.8) and 0.1 ml tissue homogenate. The reaction mixture was incubated at 37 °C for 15 min. Later, the reaction was stopped by adding 2.5 ml, 10% trichloroacetic acid (TCA) and the whole content were filtered. The reagent blank was made by adding tissue homogenate just before stopping the reaction and without incubation. The absorbance was recorded at 280 nm. The protease activity was determined from the tyrosine standard curve and expressed as micromole of tyrosine released min^{-1} mg^{-1} protein at 37 °C.

Trypsin and chymotrypsin activities were determined by casein digestion method of Kunitz (1947). For trypsin, the reaction mixture consisted 2 ml of 1% casein as substrate prepared in 0.1 M phosphate buffer (pH 7.6) with 0.2 ml tissue homogenate. The mixture was incubated at 37 °C for 20 min. The reaction was stopped by 2 ml of 5% TCA, filtered and OD of supernatant was recorded at 280 nm. Enzyme activity was expressed as micromole of tyrosine released/min/mg protein at 37 °C. Chymotrypsin activity was assayed by same procedure as for trypsin except that it contained borate buffer (0.1 M, pH 8) in place of phosphate buffer and CaCl2 was added at a final concentration of 0.005 M in enzyme–substrate mixture (Laskowski, 1955).

Lipase activity was determined based on Cherry and Crandall (1932). The reaction mixture consisted of distilled water, tissue homogenate, phosphate buffer (0.1 M pH 7) and olive oil emulsion as substrate and incubated at 27 °C for 24 h. Then 95% alcohol and two drops of phenolphthalein indicator were added and titrated against 0.05 N NaOH until the appearance of permanent pink color. A control was taken using enzyme source and inactivated by keeping for 15 min in boiling water bath prior to addition of buffer and olive oil emulsion. The milliequivalent of alkali consumed was taken as lipase activity.

Table 2
Periphyton biomass (dry matter and ash free dry matter), autotrophic index and chlorophyll concentrations (μg cm^{-2}) during periphyton production cycles (mean ± SE).

Periphyton biomass	Mean ± SE	Range
Dry matter (mg cm^{-2})	3.79 ± 0.27	2.09–6.35
Ash free Dry matter (mg cm^{-2})	1.88 ± 0.15	0.85–3.46
Chlorophyll a (μg cm^{-2})	12.42 ± 0.76	7.13–16.79
Chlorophyll b (μg cm^{-2})	2.23 ± 0.27	0.76–3.57
Chlorophyll c (μg cm^{-2})	19.37 ± 1.15	10.69–25.01
Autotrophic index	121.63 ± 9.67	75.14–184.84

Values are presented as mean of three production cycles at the time of harvest of each cycle.

2.10. Statistical analysis

The data were statistically analyzed by statistical package SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Before all analysis data were analyzed for normality by probability plots and Kolmogorov–Smirnov test and for homogeneity of variances by Levene's test. One way ANOVA was used to determine the significance of each parameter among different treatments. If a main effect was significant, the ANOVA was followed by Tukey's test. Level of significance was made at 99 and 95% probability levels.

3. Results

3.1. Periphyton biomass and chlorophyll pigments

The periphyton biomass in terms of dry matter (DM), ash free dry matter (AFDM), autotrophic index (AI), and chlorophyll (a, b and c) content per unit surface area of the harvested periphyton is given in Table 2. The average value of harvested periphyton DM and AFDM was 3.79 ± 0.27 and 1.88 ± 0.15 mg cm^{-2} respectively. The mean autotrophic index in the harvested periphyton biomass was 121.63 ± 9.67. Chlorophyll analysis of the harvested periphyton showed an average value of 12.42 ± 0.76 μ g cm^{-2} Chl a, 2.23 ± 0.27 μ g cm^{-2} Chl b and 19.37 ± 1.15 μ g cm^{-2} Chl c. Qualitative analysis of periphyton indicated the harvested periphyton was composed of 37 genera of algae belonging to Bacillariophyceae (13 genera), Cyanophyceae (10), Chlorophyceae (11) and Euglenophyceae (3), and 5 genera of zooplankton belonging to Rotifer (3) and Crustacea (2).

3.2. Proximate composition of periphyton biomass and experimental diets

Proximate compositions of periphyton and experimental diets are given in Tables 3 & 4. The dried periphyton contained 25.96 ± 0.51% crude protein, 2.65 ± 0.21% crude lipid and 26.04 ± 1.50% nitrogen free extract (NFE). The mean ash content and acid insoluble ash content was 32.75 ± 0.64% and 16.08 ± 0.40% of the dried periphyton biomass respectively. Experimental diets did not show significant difference ($p > 0.05$) in crude protein, lipid, crude fiber and nitrogen free extract. However, the total ash content was significantly higher ($p < 0.01$) in experimental diets T2 (6% periphyton) and T3 (9% periphyton) compared to control (0% periphyton) and T1 (3% periphyton). The crude protein content ranged from 37.97 ± 0.25 to 38.52 ± 0.18% and crude lipid from 7.88 ± 0.03 to 8.50 ± 0.06%. The calculated gross energy (kcal 100 g^{-1}) of the diets was in the range of 400.98 ± 0.13 to 402.36 ± 0.46.

Table 3
Proximate composition (%) of periphyton (mean ± SD).

Nutrients	Proximate composition (%)
Organic matter[a]	67.25 ± 0.64
Crude protein	25.96 ± 0.51
Crude lipid	2.65 ± 0.21
Ash	32.75 ± 0.64
Acid insoluble ash	16.08 ± 0.40
Crude fiber	5.25 ± 0.35
Moisture	7.35 ± 0.21
Total NFE[b]	26.04 ± 1.50
Gross energy (kcal/100 g)[c]	298.58 ± 3.84

[a] Organic matter = 100 − Ash (%).
[b] NFE = 100 − (CP + EE + CF + Ash + moisture).
[c] Gross energy (GE) = (CP × 5.6) + (EE × 9.44) + (CF × 4.1) + (NFE × 4.1) kcal/100 g (NRC, 1993).

3.3. Physicochemical parameters of water in experimental tanks

The water quality parameters of experimental groups during the study period are presented in Table 5. Physicochemical parameters except phosphate-P and nitrate-N did not show significant variation ($p > 0.05$) among the treatments, during the experimental period. The lowest level of total ammonia-N (83.70 ± 4.62 μg l^{-1}) and nitrite-N (41.55 ± 3.48 μg l^{-1}) was noticed in T4, treatment with submerged bamboo substrate compared with control (99.88 ± 7.44 μg l^{-1} TAN and 50.57 ± 4.53 μg l^{-1} nitrite-N). Similarly, T4 showed significantly lower ($p < 0.01$) level of NO_3-N, 61.27 ± 5.58 μg l^{-1} and PO_4-P, 45.69 ± 1.59 μg l^{-1} compared to other treatments. However, water quality parameters in control did not show significant difference with the treatments T1, T2 and T3.

3.4. Growth parameters

Growth performance of *P. monodon* juveniles over the time period are presented in Table 6. Final body weight among treatments differed significantly with 23.9% and 20% significantly higher ($p < 0.01$) final body weight in T2 and T4 respectively compared with control. Also, T1, treatment with 3% periphyton inclusion showed 14.4% non-significant higher growth compared with control. However, the body weight of shrimp fed with 9% inclusion level of periphyton (4.31 ± 0.23 g) did not differ significantly with control (4.44 ± 0.17 g). Significantly higher SGR ($p < 0.01$) was recorded in T2 (1.54 ± 0.06) and T4 (1.49 ± 0.04) and were significantly higher from control (1.18 ± 0.06) and T3 (1.13 ± 0.09). Feed conversion ratio (FCR), protein efficiency ratio (PER) and survival rate showed a highly significant difference among the treatments ($p < 0.01$). A better FCR, 2.01 ± 0.08 was observed in T2 which were significantly lower from control (2.81 ± 0.19) and T3 (3.5 ± 0.14). Similarly, significantly higher PER, 2.70 ± 0.12 was registered in T2 compared to 1.94 ± 0.14 and 1.51 ± 0.06 in control and T3 respectively. However, no significant difference in FCR and PER was observed between T1 and T4. Significant difference ($p < 0.05$) in survival was noticed among the experimental groups. The highest survival was recorded in T2 (77.78 ± 1.39%) which was significantly 12% higher compared with control (69.44 ± 6.05%). However, no significant difference ($p > 0.05$) in survival was observed among control, T1 and T4 treatments. Overall, treatment with 6% dietary supplementation of periphyton had significant effect ($p < 0.01$) over SGR, FCR, PER and survival compared to other treatment and, treatment with higher supplementation of periphyton biomass did not differ significantly from control.

3.5. Digestive enzymes

As for gut, 75.45% and 29.8% significant increase ($p < 0.05$) in gut amylase activity was observed in T2 and T4 treatment respectively compared with control (Fig. 1). Specific activity of cellulase (Fig. 2) in gut differed significantly ($p < 0.05$) with 128.3 and 126% increase in T2 and T4 respectively compared with control. Similarly, in hepatopancreas nonsignificantly higher amylase and cellulase activity was noticed in T2 compared to other treatments.

Table 4
Proximate composition (%) of experimental diets supplemented with graded level of periphyton (mean ± SD).

Nutrients	Control	T1	T2	T3	Level of significance
Organic matter*	81.79 ± 0.03[a]	81.50 ± 0.07[a]	80.75 ± 0.14[b]	80.55 ± 0.10[b]	**
Moisture	8.30 ± 0.14[a]	8.05 ± 0.30[a]	7.90 ± 0.19[a]	7.82 ± 0.18[a]	NS
Crude protein	37.97 ± 0.25[a]	38.25 ± 0.42[a]	38.52 ± 0.18[a]	38.29 ± 0.09[a]	NS
Crude lipid	7.88 ± 0.03[a]	8.15 ± 0.28[a]	8.39 ± 0.04[a]	8.50 ± 0.06[a]	NS
Ash	18.21 ± 0.03[a]	18.50 ± 0.07[a]	19.25 ± 0.14[b]	19.45 ± 0.10[b]	**
Crude fiber	2.60 ± 0.08[a]	2.80 ± 0.07[a]	2.90 ± 0.04[a]	2.93 ± 0.04[a]	NS
NFE**	25.05 ± 0.47[a]	24.25 ± 1.15[a]	23.07 ± 0.23[a]	23.03 ± 0.28[a]	NS
Gross energy (kcal/100 g) ***	402.36 ± 0.46[a]	402.04 ± 0.64[a]	401.33 ± 0.15[a]	400.98 ± 0.13[a]	NS

The means with no superscript letter in common per factor indicate significant difference. If the effects were significant, ANOVA was followed by Tukey's test. **p<0.01; NS—Non-significant.
* Organic matter = 100 − Ash (%).
** NFE = 100 − (CP + EE + CF + ash + moisture).
*** Gross energy (GE) = (CP × 5.6) + (EE × 9.44) + (CF × 4.1) + (NFE × 4.1) kcal/100 g (NRC, 1993).

Table 5
Water quality parameters of experimental groups fed with graded level of periphyton supplemented diets (mean ± SE).

Parameters	Control	T1	T2	T3	T4	Level of significance
Temperature (°C)	30.18 ± 0.19 (29.50–31.00)	30.41 ± 0.28 (29.00–31.50)	29.90 ± 0.32 (28.00–31.00)	29.98 ± 0.24 (29.00–31.00)	29.92 ± 0.25 (28.80–30.80)	NS
pH	7.99 ± 0.08 (7.60–8.30)	8.04 ± 0.09 (7.60–8.40)	8.13 ± 0.08 (7.90–8.50)	8.12 ± 0.09 (7.80–8.50)	8.12 ± 0.06 (7.80–8.40)	NS
Salinity (ppt)	9.37 ± 0.30 (8.20–10.50)	9.36 ± 0.33 (8.00–10.60)	9.39 ± 0.29 (8.20–10.50)	9.34 ± 0.26 (8.30–10.40)	9.32 ± 0.26 (8.40–10.40)	NS
Alkalinity (mg $CaCO_3$ l^{-1})	130.11 ± 2.19 (121.0–142.0)	126.29 ± 2.69 (112.0–140.0)	128.44 ± 1.83 (120.0–137.0)	127.55 ± 2.40 (115.0–136.0)	132.78 ± 2.22 (124.0–146.0)	NS
Nitrate-N (μg l^{-1})	112.43 ± 11.6 (50.50–138.40)	104.97 ± 10.34 (55.40–128.80)	105.44 ± 12.05 (52.44–136.00)	114.14 ± 11.78 (56.80–139.50)	61.27 ± 5.58 (47.00–88.60)	NS
Total ammonia-N (μg l^{-1})	99.88 ± 7.44 (69.70–128.26)	99.50 ± 8.61 (65.30–131.00)	101.34 ± 7.67 (70.55–124.60)	104.84 ± 8.27 (68.80–129.80)	83.70 ± 4.62 (66.70–101.10)	NS
Nitrite-N (μg l^{-1})	50.57 ± 4.53[a] (34.56–71.05)	47.86 ± 4.87[a] (31.10–68.54)	48.53 ± 4.12[a] (33.30–68.80)	50.42 ± 4.43[a] (34.24–73.40)	41.55 ± 3.48[b] (26.78–53.56)	**
Phosphate-P (μg l^{-1})	69.52 ± 5.11[a] (40.56–89.00)	63.42 ± 4.34[a] (45.40–84.20)	65.44 ± 4.43[a] (42.54–82.00)	66.33 ± 5.16[a] (40.34–89.00)	45.69 ± 1.59[b] (41.10–53.40)	**
Dissolved oxygen (ppm)	5.93 ± 0.13 (5.28–6.50)	6.03 ± 0.11 (5.70–6.65)	6.18 ± 0.15 (5.50–6.77)	6.06 ± 0.16 (5.40–6.88)	5.95 ± 0.16 (5.30–6.70)	NS

The means with no superscript letter in common per factor indicate significant difference. If the effects were significant, ANOVA was followed by Tukey's test. **p<0.01; NS—Non-significant.
The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

Gut protease activity (Fig. 3) showed 178.5, 99.2 and 74.3% increases in T2, T4 and T3 treatments respectively compared with control. Similar trend was observed in hepatopancreas protease activity with the highest specific activity, 0.39 ± 0.03 U mg^{-1} in the treatment T2 followed by 0.37 ± 0.03 U mg^{-1} in T4 and the lowest value 0.29 ± 0.05 in control.

Trypsin activity (Fig. 4) showed significant difference in both gut (p<0.01) and hepatopancreas (p<0.05). There were 76.6, 61.5 and 46.7% higher specific activity of trypsin in the gut of T2, T3 and T4 respectively compared with control. Similarly, trypsin activity in the hepatopancreas was 56.83 and 29.5% higher in T2 and T1 respectively compared with control. Treatment T2 showed significantly higher (p<0.05) chymotrypsin activity (Fig. 5) in both gut and hepatopancreas compared with control while no significant difference was observed among other treatments.

There was a significant difference in the specific activity of lipase (Fig. 6) in both gut (p<0.05) and hepatopancreas (p<0.01) among the treatments. As for gut, the highest specific activity of lipase was observed in T4 (9.23 ± 0.59 U mg^{-1}) followed by T2 (7.68 ± 0.79 U mg^{-1}) and T1 (7.58 ± 1.33 U mg^{-1}) and the lowest in T3 (3.43 ± 0.01 U mg^{-1}). In

Table 6
Growth performance of *Penaeus monodon* juveniles fed experimental diets with graded level of periphyton supplementation (mean ± SE).

Parameters	Control	T1	T2	T3	T4	Level of significance
Initial wt(g)	2.18 ± 0.16[a]	2.17 ± 0.17[a]	2.18 ± 0.20[a]	2.19 ± 0.19[a]	2.19 ± 0.17[a]	NS
Final wt(g)	4.44 ± 0.17[a]	5.08 ± 0.18[ab]	5.50 ± 0.21[b]	4.31 ± 0.23[a]	5.33 ± 0.13[b]	**
Weight gain (%)	103.64 ± 7.63[a]	133.04 ± 8.18[ab]	152.09 ± 9.58[b]	97.74 ± 10.68[a]	144.44 ± 5.98[b]	**
FCR[1]	2.81 ± 0.19[b]	2.43 ± 0.05[ab]	2.01 ± 0.08[a]	3.58 ± 0.14[c]	2.28 ± 0.17[ab]	**
PER[2]	1.94 ± 0.14[ab]	2.23 ± 0.04[bc]	2.70 ± 0.12[c]	1.51 ± 0.06[a]	2.39 ± 0.18[bc]	**
SGR[3]	1.18 ± 0.06[a]	1.41 ± 0.05[ab]	1.54 ± 0.06[b]	1.13 ± 0.09[a]	1.49 ± 0.04[b]	**
Survival	69.44 ± 6.05[ab]	69.44 ± 1.39[ab]	77.78 ± 1.39[b]	55.56 ± 1.40[a]	70.83 ± 4.17[ab]	*

FCR[1] = Feed conversion ratio; PER[2] = Protein efficiency ratio; SGR[3] = Specific growth rate.
The means with no superscript letter in common per factor indicate significant difference. If the effects were significant, ANOVA was followed by Tukey's test. *p<0.05; **p<0.01; NS, not significant.
The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

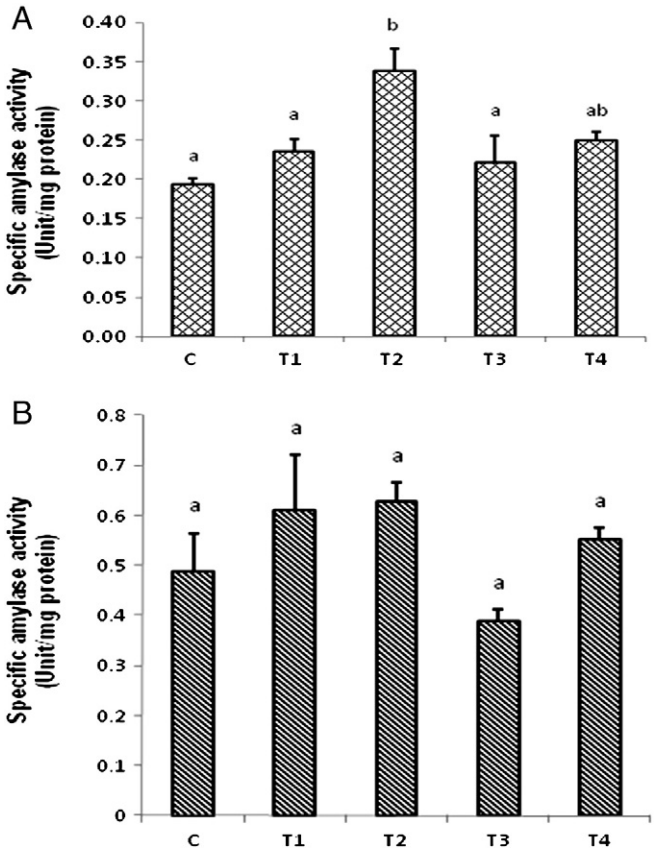

Fig. 1. Specific amylase activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of periphyton in A) Gut and B) Hepatopancreas. Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p<0.05$). The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

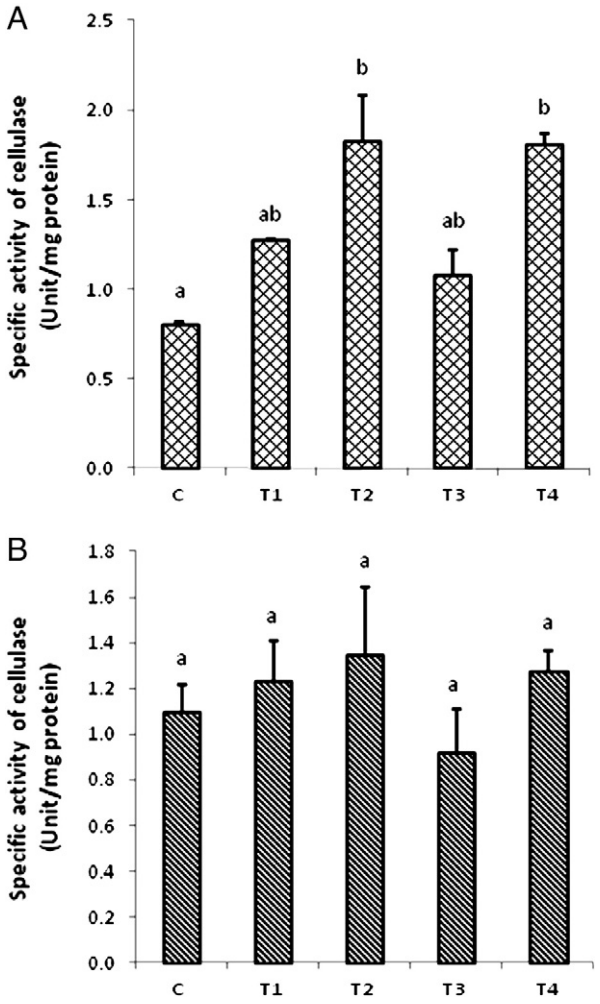

Fig. 2. Specific cellulase activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of periphyton in A) Gut and B) Hepatopancreas. Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p<0.05$). The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

hepatopancreas, there was 76.8, 39.9 and 17.4% increase in specific lipase activity in T2, T1 and T4 respectively compared with control.

4. Discussion

It is well documented that *P. monodon* which grow over periphytic microalgae attain significant improvement in body weight compared to control (Anand et al., 2012; Arnold et al., 2009). Microalgae and probiotic bacterial products are well-known for their nutritional benefits (Ju et al., 2009; Van Der Meeren et al., 2007) and widely used as dietary stimulants to shrimp juvenile (Ju et al., 2009; Wang, 2007). The present study elucidates for the first time the role of periphyton as dietary supplement in shrimp culture.

4.1. Periphyton biomass and chlorophyll pigments

In the present study, moderate dose of fertilization was applied for periphyton growth over the bamboo substrate as lower and higher dose of fertilization results either in competition of algae for nutrients and light or shading of plankton, resulting in hampered periphyton growth (Azim et al., 2003; Keshavanath et al., 2002). The autotrophic index (AI) of the harvested periphyton biomass (75.14–184.84) indicates the dominance of autotrophic community. In general, AI value, 100 to 200 is considered algae dominated and, above 300 as heterotrophs dominated periphytic system (APHA, 2005) or ungrazed condition (Azim et al., 2005). The periodic collection of periphyton at 15 days interval have promoted the formation of new algal cells, because increased standing biomass in the absence of grazers can lead to self-shading and death of algal community (Ballester et al., 2007; Keshavanath et al., 2001). Lower level of AI and better Chl a across the collection period indicate that the harvested periphyton was rich in fresh periphytic algal biomass.

4.2. Physicochemical parameters of water

The recorded water quality parameters across the treatments were within desired range (Van Wyk et al., 1999) and were optimum for growth of cultured shrimp. No significant difference in water quality parameters was observed in periphyton incorporated dietary treatments compared with control. However, treatment with natural substratum, T4 showed significantly lower level of PO_4-P and NO_3-N and non-significantly lower level of total NH_3-N compared to other treatments. This supports the earlier findings that periphyton developed over the submerged substratum helps to maintain water nutrient parameters like total NH_3-N and NO_3-N within the optimum level (Anand et al., 2012; Khatoon et al., 2009; Ramesh et al., 1999).

4.3. Periphyton as a feed ingredient

It has been documented that nutritional composition of periphyton can be considered as broadly appropriate to fish dietary needs (Azim et al., 2002; Dempster et al., 1993; Makarevich et al., 1993). Proximate analysis of the periphyton in the present study is in agreement with

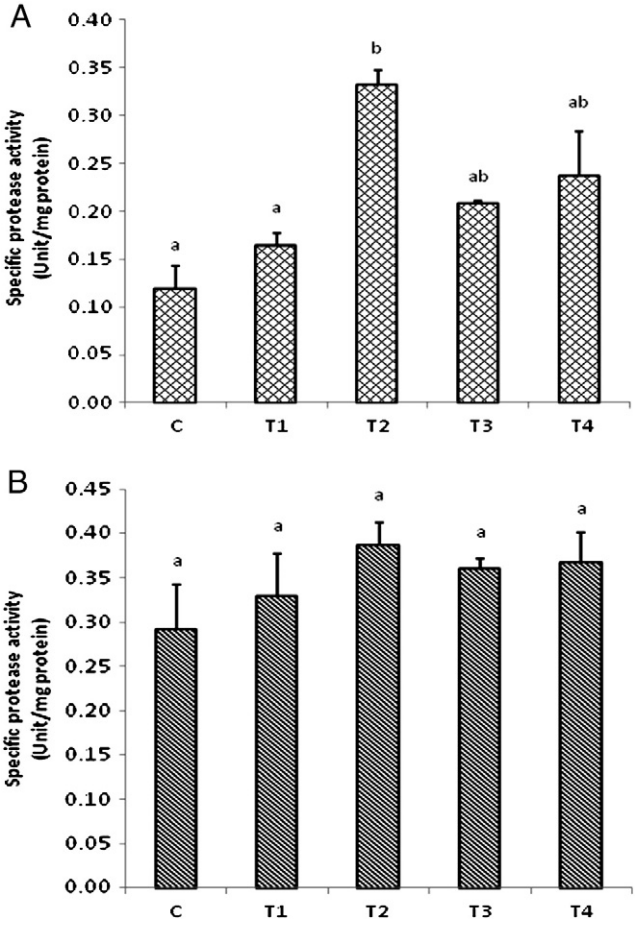

Fig. 3. Specific protease activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of periphyton in A) Gut and B) Hepatopancreas. Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p<0.05$). The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

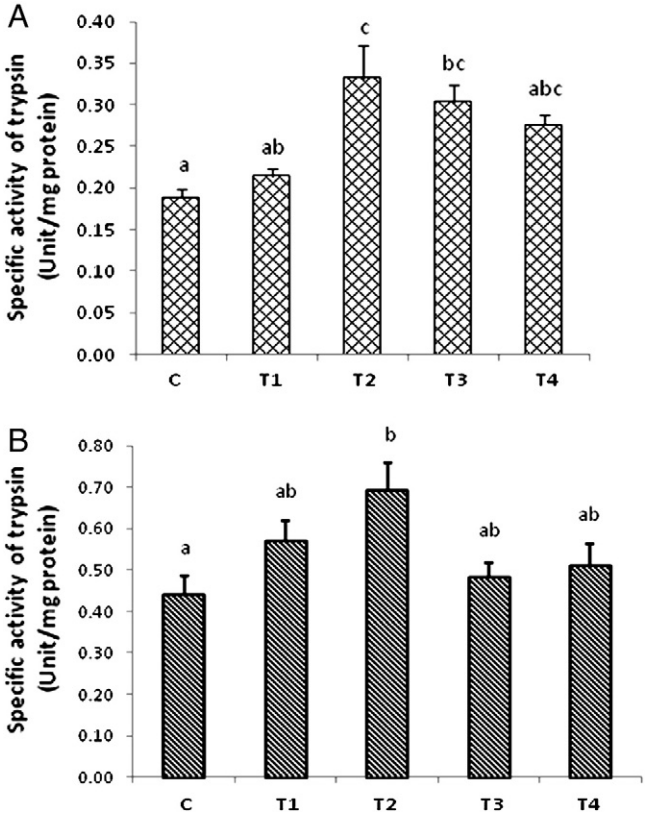

Fig. 4. Specific trypsin activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of periphyton in A) Gut and B) Hepatopancreas. Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p<0.01$ in gut and $p<0.05$ in hepatopancreas). The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

the earlier findings which documented that proximate composition of periphyton varies from 23 to 30% protein, 2–9% lipid, 25–28% NFE and 16–42% ash (Azim et al., 2005; Thompson et al., 2002; van Dam et al., 2002). This indicates that nutritional quality of periphyton observed in the present study qualifies it to consider as dietary supplement in penaeid shrimps. *P. monodon* juvenile need 35 to 40% protein (Alava and Lim, 1983; Shiau, 1998), minimum 20% starch (Alava and Pascual, 1987) and up to 10% lipid (Akiyama et al., 1992). All the test and control diets had the macronutrient level within this normal range.

4.4. Growth parameters

Consumption of periphytic algae improves the nutritional quality of penaeid shrimp (Audelo-Naranjo et al., 2011). In the present study, dietary periphyton supplementation at 6% level (T2) significantly enhanced ($p<0.01$) the growth, FCR, PER and survival of tiger shrimp compared with control diet. Our results are in accordance with Ju et al. (2009) who recorded significantly higher growth and survival in *L. vannamei* diet supplemented with microalgae. Even though, algal proteins are considered as good quality protein source (Oser, 1959), all the test diets used in the present study had similar macronutrient level to that of control. Therefore, better growth might be attributed to growth promoting nutritional factors in the periphyton. Apart from being a source of macronutrients, microalgae and heterotrophic bacteria are rich source of immune enhancers (Supamattaya et al., 2005), growth promoters (Kuhn et al., 2010), bioactive compounds (Ju et al., 2008) and dietary stimulants (Xu et al., 2012) which can enhance growth performance of cultured shrimp. Hence, it can be inferred that these beneficial effects of algae and microbes in the periphyton might have attributed to improved growth response in tiger shrimp juvenile.

Administration of periphyton at higher level, 9% (T3) could not show significant increase in body weight compared to other treatments. This is in line with the findings of Liao et al. (1993) who reported supplementation of higher level of *Spirulina* in shrimp diets caused lower growth rate and poor FCR of *P. monodon*. This may be due to imbalance in amino acid composition or higher inclusion level of a particular macro or micro minerals (Supamattaya et al., 2005). Further, ash and acid soluble ash level noticed in the periphyton up to 6% inclusion level was within the limit of shrimp assimilation capacity as up to 30% detritus was noticed in the gut content analysis of shrimp juvenile without having negative effect on growth and physiology (Bombeo-Tuburan et al., 1993). However, as dietary supplementation level of periphyton increased, acid insoluble ash content also increased which might have resulted into lower feed intake resulting in non-significantly lower growth rate and higher FCR in T3 as compared to control.

Even though, we have not used any pretreatment methods, our results are comparable to the findings of Ju et al. (2009) who reported shrimp fed with whole algal incorporated meals showed significantly higher growth rate compared to carotenoid-fraction diet. However, higher growth rate was observed in *P. japonicus* when fed with cell wall disrupted algal cells compared with intact cell supplemented diet (Boonyaratpalin et al., 2001). This is due to lack of enzyme to degrade the algal cell wall components (Tangeras and Slinde, 1994), thus their higher level in aquafeed can lead to poor digestibility

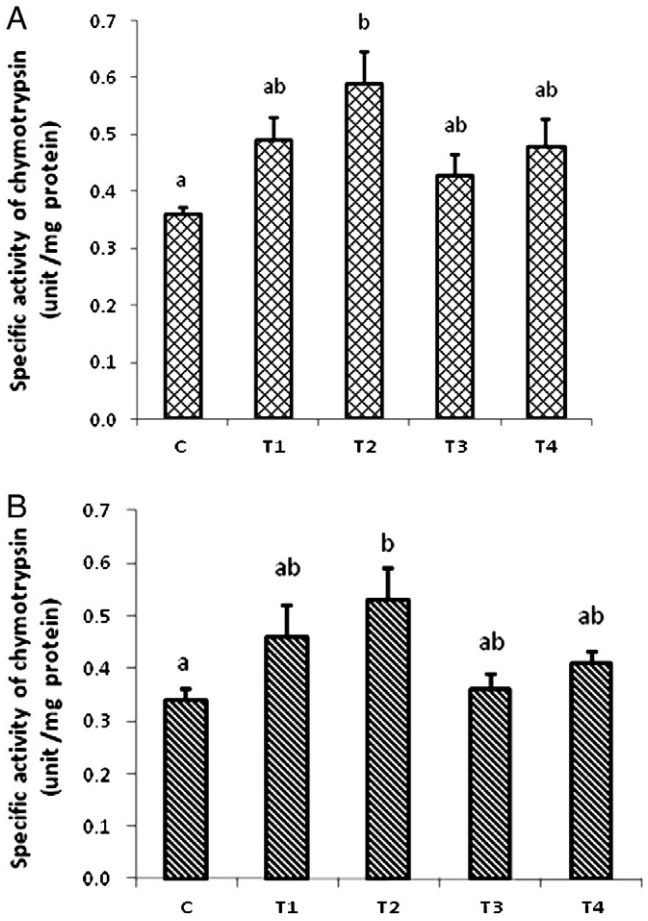

Fig. 5. Specific chymotrypsing activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of periphyton in A) Gut and B) Hepatopancreas. Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p<0.05$). The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

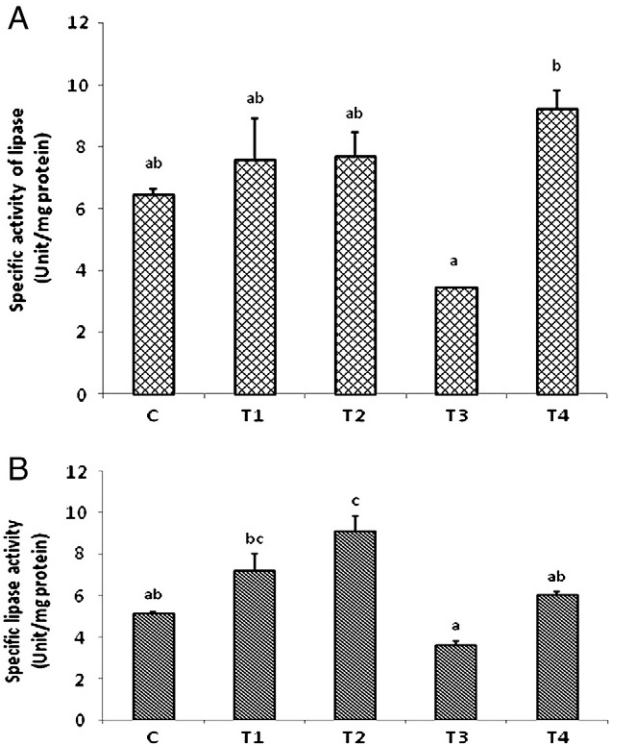

Fig. 6. Specific lipase activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of periphyton in A) Gut and B) Hepatopancreas. Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p<0.05$ in gut and $p<0.01$ in hepatopancreas). The level of periphyton supplementation was control (0%), T1 (3%), T2 (6%), T3 (9%), T4 (0% with bamboo substrate).

(Halver and Hardy, 2002). Therefore, mechanical disruptions might have better viable pretreatment option at higher periphyton inclusion level to enhance the nutrient utilization by shrimp in better ways.

4.5. Digestive enzymes

Macronutrients like dietary carbohydrate and protein influence the digestive enzyme activities in shrimps (Gamboa-Delgado et al., 2003; Guzman et al., 2001; Huang et al., 2003). However, formulation of isonitrogenous and isocaloric diets allowed the direct comparison of periphyton supplemented and control diets. The presence of microalgae and its components even at low concentration in the gut can trigger the production of digestive enzymes (Reitan et al., 1993; Sheele, 1993). It has been demonstrated that the consumption of the biota present in the farming systems improves the activity of digestive enzymes and physiological status of farmed aquatic animals (Brito et al., 2004).

The higher level of specific activity of digestive enzymes like amylase, cellulase, protease, lipase, trypsin and chymotrypsin recorded in T1, T2 and T4 might have improved the digestion of protein, starch and fat. This resulted in enhanced growth and better feed conversion ratios compared with control. Similarly, elevated level of digestive enzymes resulted in better growth performance of shrimps (Wang, 2007; Ziaei-Nejad et al., 2006) and fishes (Lara-flores et al., 2003) when fed with probiotic and herbal extract (Sankar et al., 2011) supplemented diets compared to control. This suggests that diversified form of autotrophic algal and heterotrophic microbial community present in the periphyton have the capacity to stimulate digestive enzyme activities in shrimp.

Significant increase in cellulase activity in the gut of the treatment groups (T2 and T4) support the findings of Moss et al. (2001) who observed a six-fold increase in cellulase activity in pond reared *L. vannamei* compared to well water-reared shrimp. Similarly, it has been noticed that microalgae enhances trypsin activity (Le Vay et al., 1993) as it contain large amounts of free amino acids (Admiral et al., 1986). However, it is important to make distinction between enzymes produced endogenously by the shrimp and exogenous enzymes synthesized by resident gut flora (Harris, 1993). Enzyme activity noticed in the shrimp fed with periphyton supplemented group (T1 and T2) did not differ significantly with T4 where shrimp were grazing over the live periphytic algal cells. On the contrary, T2 recorded significantly higher digestive enzyme activities compared with T4. The presence of the cell wall components and other unknown growth promoters stimulate the production of endogenous digestive enzymes by the shrimp (Moss and Moss, 2004). This further confirms the fact that presence of beneficial microbial components or unknown growth promoters in the periphyton supplemented diet might have stimulated the production of endogenous enzymes by the shrimp and performed equally well or better compared with T4.

Shrimp fed with the highest concentration of periphyton incorporated diet (T3) was not significantly higher than that of other treatment groups. This supports the findings of Wang (2007) who reported that increase in dietary supplementation of probiotics do not increase proportionately the digestive enzymes and growth of the animal due to its inherent limit. In contrast, it showed a negative effect on growth and survival, suggesting that the increased activity of the digestive enzymes in juvenile shrimp induced by periphyton biomass has an inherent limit.

5. Conclusion

It was clear from our study that dietary supplementation of periphyton had beneficial effects on growth and digestive enzyme activities compared to control. The inclusion of periphytic biomass up to 6% level in shrimp *P. monodon* diet has the stimulatory effect on the growth and digestive enzyme activities of juvenile tiger shrimp. Current method of periphyton production is cheaper and easier compared to mass scale production of microalgae and may encourage feed manufacturers to consider periphyton as a viable alternative dietary supplement. Use of pre treatment methods for maximum utilization of nutrient at higher level is a matter of further research. Future studies will focus on determining the effect of periphyton biomass on nutritional profile of shrimp.

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