Aquaculture 418–419 (2014) 108–115

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

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

P.S. Shyne Anand [a,*], M.P.S. Kohli [b], Sujeet Kumar [a], J.K. Sundaray [c], S. Dam Roy [d], G. Venkateshwarlu [b], Archana Sinha [e], G.H. Pailan [f]

[a] Kakdwip Research Centre, Central Institute of Brackishwater Aquaculture (ICAR), Kakdwip, South 24 Parganas, West Bengal PIN-743 347, India
[b] Central Institute of Fisheries Education, Versova, Mumbai PIN-400061, India
[c] Central Institute of freshwater Aquaculture, Orissa, India
[d] Central Agricultural Research Institute, Portblair, India
[e] Kolkata Research Centre, Central Inland Fisheries Research Institute, Kolkata, India
[f] Kolkata Research Centre, Central Institute of Fisheries Education, Kolkata, India

ARTICLE INFO

Article history:
Received 29 April 2013
Received in revised form 14 August 2013
Accepted 30 September 2013
Available online 3 October 2013

Keywords:
Biofloc
Dietary supplement
Digestive enzyme
Fatty acid profile
Growth performance
Penaeus monodon

ABSTRACT

A 60-day indoor growth trial was conducted to study the effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon* juveniles. Biofloc developed in indoor fiberglass reinforced plastic (FRP) tanks (1000 L) was used as dietary supplement for *P. monodon* (2.90 ± 0.10 g) reared in 1000 L FRP tanks. Graded level of dried biofloc was included in shrimp basal diets; 0 (B0, control), 4 (B4), 8 (B8) and 12% (B12). The dried biofloc contained 24.30 ± 0.28% protein. Fatty acid profile of biofloc revealed palmitic acid (46.54%), cis-Vaccenic acid (15.37%), linoleic acid (10.67%) and oleic acid (9.19%) as major fatty acids. There were 16.9 and 13.9% significantly higher ($p < 0.01$) final body weights in B8 and B4 respectively compared with control, B0. Similarly, significantly better ($p < 0.05$) feed conversion ratio (FCR), 1.84 ± 0.09 and protein efficiency ratio (PER), 3.48 ± 0.17 was noticed in B4 compared to control (FCR 2.29 ± 0.11 and PER 2.80 ± 0.13). At the end of the feeding trial, B4 recorded 57.6, 45.5, 61 and 78.6% significant increases ($p < 0.01$) in hepatopancreas digestive enzyme activities for amylase, cellulase, lipase and protease respectively compared with control. However, treatment with 12% level of biofloc inclusion (B12) did not differ significantly ($p > 0.05$) for most of the growth performance parameters and digestive enzyme activities compared with control. The present study elucidates the suitability of biofloc as a dietary supplement at 4% level in shrimp feed for enhancing growth and digestive enzyme activities in tiger shrimp juvenile.

1. Introduction

Penaeid shrimps are highly valued seafood commodity in domestic and international markets. Economics of shrimp farming is largely dependent on the feed which constitutes 40–60% operational expenses (Tan et al., 2005). Dietary supplements are widely used in shrimp culture to enhance the growth, immune response and digestive enzyme activities. Commonly used dietary supplements in penaeid shrimp are microalgal products (Boonyaratpalin et al., 2001; Ju et al., 2009; Supamattaya et al., 2005), macroalgae (Yeh et al., 2006), probiotics (Wang, 2007; Yang et al., 2010; Ziaei-Nejad et al., 2006), prebiotics (Zhang et al., 2012) and periphyton (Anand et al., 2013b).

Recently, manipulation of carbon nitrogen ratio (C:N ratio) for development of biofloc has shown promise in aquaculture (Anand et al., 2013a; Avnimelech, 1999). The C:N ratio is manipulated by supplementation of external carbon source or elevated carbon level in the feed (Ballester et al., 2010; Crab et al., 2012; McIntosh, 2000). At high C:N ratio, heterotrophic bacteria immobilize the ammonium ion for production of microbial protein and maintain inorganic nitrogen level within the limit (Avnimelech, 1999). Biofloc enhances the growth performance of *Penaeus monodon* (Anand et al., 2013a; Arnold et al., 2009; Hari et al., 2006), *Litopenaeus vannamei* (Wasielesky et al., 2006; Xu and Pan, 2012), *Farfantepenaeuspaulensis* (Ballester et al., 2010) and *Marsupenaeus japonicus* (Zhao et al., 2012). Apart from being a source of quality proteins, bioflocs are rich source of growth promoters and bioactive compounds (Ju et al., 2008a) which enhance digestive enzymes (Xu and Pan, 2012) and health status of the cultured shrimps (Singh et al., 2005).

In general, most research makes use of *in situ* developed microbial floc for growth performance of shrimp (Hari et al., 2006; Xu and Pan, 2012). However, these *in situ* based techniques need additional oxygen demands for microbial respiration, in addition to the oxygen demand of shrimp (Burford et al., 2003; Tacon et al., 2002). This added oxygen demand requires additional aerators, which increases the aeration expenses in shrimp farms compared to conventional shrimp culture

* Corresponding author. Tel.: +91 3210 255072; fax: +91 3210 257030.
E-mail address: shyne.anand@gmail.com (P.S.S. Anand).

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http://dx.doi.org/10.1016/j.aquaculture.2013.09.051

systems (Tacon et al., 2002). Moreover, increased production of microbial floc particle is also a matter of challenge if production exceeds consumption by shrimp.

Recently, it has been demonstrated that dietary inclusion of biofloc enhanced the growth performance of *L. vannamei* (Bauer et al., 2012; Ju et al., 2008b; Kuhn et al., 2010). However, there is a dearth of information to support the dietary role of biofloc on growth and digestive enzyme activity in *P. monodon*. Hence, to investigate the suitability of biofloc as a dietary supplement, biofloc produced in indoor FRP tanks was included in shrimp diet at different level and fed to *P. monodon*. In this context, the present study aims to evaluate the effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *P. monodon* juveniles.

2. Materials and methods

2.1. Experimental design

Biofloc produced in indoor tanks was used as dietary supplement in shrimp feed, over a 60-day indoor growth trial. A control diet without biofloc was compared against three experimental diets with graded level of biofloc inclusion. 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 biofloc

Biofloc production was carried out in three indoor fiberglass reinforced plastic (FRP) tanks (1000 L; bottom area 2 m^2) in seven batches at 5-day interval during March to May, 2011. Tanks were filled with water from the nearby brackishwater source. The fine meshed filter bag was used to prevent entry of unwanted materials and suspended particles in to the tanks. Two aeration pipes with air stones and regulators with 7.5 m^3 air $tank^{-1}$ min^{-1} diffusing capacity were provided in each tank to meet the oxygen demand and proper mixing of floc. As an inoculum, 10 L water with bacterial floc developed in a separate tank was added to each tank. The C: N ratio was maintained at 10: 1 using ammonium sulphate as nitrogen and wheat flour as carbon source. Inorganic nitrogen source was applied up to the 3rd day (72 h) while addition of carbohydrate was continued till the 4th day. This was done for maximum utilization of left over inorganic nitrogen and to reduce the chance of occurrence of inorganic nitrogen in the form of total ammonia nitrogen in the collected biofloc.

On the 5th day, biofloc was allowed to settle by closing the aeration and subsequently harvested by passing water in nylon filter bag with 10 μm pore size. The collected floc centrifuged at 2000 rpm and discarded the supernatant water. To remove the traces of ammonia nitrogen level, bioflocs were washed twice with filtered brackish water of same salinity. Flocs were dried under shade on a polythene sheet followed by drying in a hot air oven at 45 °C. The dried flocs ground in to fine powder (less than 200 μm) and kept in airtight containers in refrigerator until experimental diets were made.

2.3. Estimation of biofloc microbial community

Total heterotrophic bacterial count, *Vibrio*, *Bacillus, Lactobacillus* and fungal count were done at the initial and final days of biofloc production cycle. To separate the flocculated microbes, sample was sonicated for 1 min using probe sonicator (PCI Analytics, India) with 20 KHz amplitude. Subsequently, 0.1 mL of appropriate dilutions were plated in duplicates on trypton soya agar with 1.0% w/v NaCl for total heterotrophic bacterial count, thiosulfate citrate bile salt sucrose agar (TCBS) for *Vibrio*, *Bacillus cereus* agar (HiMedia, India) for *Bacillus*, Lactobacillus MRS agar (HiMedia, India) for *Lactobacillus* and Sabouraud dextrose agar for fungal count. Plates were incubated at 28 °C for 7 days for fungal count and 48 h for other microbes. The colony in the range of 30 to 300 were counted and expressed as colony forming unit (cfu).

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

Ingredients	Experimental diets			
	B0	B4	B8	B12
Fish meal	380	380	380	380
Shrimp meal	150	150	150	150
Soyabean meal	207.6	194	120	166.9
Wheat flour	172.9	146.5	180.5	93.6
Dried biofloc powder	0	40	80	120
Soya oil	15	15	15	15
Cod Liver oil	20	20	20	20
Lecithin	10	10	10	10
Cholesterol	1	1	1	1
Vitamin–mineral mix[a,b]	23	23	23	23
BHT	0.5	0.5	0.5	0.5
Guar gum	20	20	20	20
Total	1000	1000	1000	1000

[a] 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, 1 g; Nicotinamide, 4 g; Choline Chloride, 60 g; Mn, 10.8 g; Iodine, 0.4 g; Fe, 3 g; Zn, 6 g; Cu, 0.8 g; Co, 0.18 g.
[b] Vitamin C, 1000 mg.

2.4. Experimental diets

The compositions of experimental diets are presented in Table 1. Four isonitrogenous and isoenergetic experimental diets were formulated *viz*., B0, B4, B8 and B12. A control diet (B0), without biofloc was compared against three experimental diets formulated with graded level of biofloc at 4 (B4), 8 (B8) and 12% (B12) by manipulating soyabean meal and wheat flour levels. All the ingredients except biofloc 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. Bioflocs 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 *P. monodon* tested negative for white spot syndrome virus were obtained from a shrimp farm (South 24 Parganas, West Bengal, India). Shrimps were acclimatized for 14 days and fed with commercial diet (40% crude protein) three times daily before start of experiment. The experiment was conducted in triplicate in FRP tanks (1000 L; bottom area 2 m^2) filled with chlorine free brackishwater. Two hundred and fifty two *P. monodon* juveniles (2.9 ± 0.10 g) were randomly distributed in the four experimental groups at 21 nos. $tank^{-1}$ following a completely randomized design (CRD).

The daily feeding was done at 6.5% of the body weight at the start of experiment and declined gradually to 4.5% of body weight at the end of the experiment. The daily ration was divided in to two parts, 40% feed

Table 2
Total and differential microbial count during biofloc production cycle.

Bacterial groups	Production day	
	Initial	Final
Total microbial count (cfu × 10^8)	0.23 ± 0.06	245.67 ± 61.32
Vibrio count (cfu × 10^4)	0.27 ± 0.03	240.83 ± 47.00
Bacillus count (cfu × 10^4)	0.17 ± 0.17	345.00 ± 37.75
Lactobacillus count (cfu × 10^1)	0.00 ± 0.00	6.67 ± 1.67
Fungal count (cfu × 10^2)	1.33 ± 0.17	526.67 ± 44.10

Microbial count is presented as colony forming unit (cfu) and expressed as mean ± SE.

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

Nutrients	Proximate composition (%)
Organic matter[a]	68.02 ± 0.98
Moisture	7.85 ± 0.10
Crude protein	24.30 ± 0.28
Crude lipid	3.53 ± 0.35
Ash	31.98 ± 1.01
Acid insoluble ash	10.75 ± 1.06
Crude fiber	3.12 ± 0.19
Total NFE[b]	29.24 ± 0.64
Gross energy (Kcal/100 g)[c]	302.01 ± 5.16

[a] OM = 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.

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 biofloc and experimental diets

The proximate composition of the biofloc 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. Crude protein was estimated by Kjeldahl method (Kelplus, Pelican equipments, India). 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. (1993). Total nitrogen free extract (NFE) was determined as per the formula described in Hastings and Dupree (1969).

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

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

2.7. Fatty acid profile of biofloc

Total lipids were extracted from biofloc sample according to Folch et al. (1957). Fatty acid methyl esters were prepared based on the procedure given in AOAC (1995). Briefly, the lipid extract was boiled in a condenser with 2 mL methanolic NaOH for first 5 min followed by 2 mL BF_3-methanol for the next 2 min. To recover the fatty acid methyl ester (FAME) in organic phase, 5 mL heptane was added and heated for 8 min, and washed with saturated NaCl solution into a separating funnel. The upper FAME layer was stored in glass vials at 0 °C for further analysis in GC–MS.

The methylated fatty acids were separated using GC–MS (QP2010, Shimadzu, USA) equipped with DB Wax (30 m × 0.25 mm internal diameter × 0.25 μm film thickness) capillary column (Cromlab SA, Spain). Helium was used as carrier gas. Injector and detector temperatures were set at 250 °C. Injection was performed in split mode (1:15) with an injection volume of 1 μl FAME. The initial column temperature was maintained at 50 °C for 2 min. The temperature was set to increase at the rate of 10 °C per minute until the final temperature reached 230 °C and kept for 35 min. Fatty acid methyl esters were separated at a constant pressure of 82.5 KPa. The peaks were identified by comparing the mass spectra with the mass spectral data base.

2.8. Determination of water quality parameters

The water quality parameters like salinity, temperature and pH were measured using an ATAGO hand refractometer, thermometer and pH meter respectively. Total alkalinity, turbidity, dissolved oxygen, total ammonia-N (TAN), nitrite-N (NO_2N) and nitrate-N (NO_3N) were analyzed immediately after sample collection following the procedures described in APHA (1998). Biofloc volume in production tanks were determined by sampling 1000 mL water into a series of Imhoff cones. The volume of the floc plug accumulated on the bottom of the cone was determined (Avnimelech and Kochba, 2009).

2.9. 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;

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

$$\text{SGR}\ (\%) = (\text{Log}_e\ \text{final weight–Log}_e\ \text{initial weight/Experimental days}) \times 100$$

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

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

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

2.10. Analysis of digestive enzymes

After completion of the feeding experiment, 18 inter-molt shrimp from each treatment groups (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 activity of digestive enzymes. The hepatopancreas and gut of the shrimp were dissected out, weighed and homogenized with

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

Nutrients	B0	B4	B8	B12	Level of significance
Organic matter	82.12 ± 0.10[c]	81.75 ± 0.14[bc]	81.51 ± 0.10[ab]	81.17 ± 0.06[a]	**
Moisture	8.10 ± 0.42[a]	7.98 ± 0.17[a]	7.88 ± 0.04[a]	7.91 ± 0.15[a]	NS
Crude protein	37.88 ± 0.03[a]	37.92 ± 0.33[a]	37.77 ± 0.14[a]	38.05 ± 0.29[a]	NS
Crude lipid (EE)	7.67 ± 0.26[a]	7.91 ± 0.10[a]	8.10 ± 0.15[a]	8.02 ± 0.05[a]	NS
Ash	17.88 ± 0.10[a]	18.25 ± 0.14[ab]	18.49 ± 0.10[bc]	18.84 ± 0.06[c]	**
Crude fiber	3.16 ± 0.08[a]	3.37 ± 0.18[a]	3.16 ± 0.13[a]	3.35 ± 0.01[a]	NS
NFE[b]	25.32 ± 0.73[a]	24.58 ± 0.93[a]	24.62 ± 0.01[a]	23.85 ± 0.17[a]	NS
Gross energy	401.23 ± 0.71[a]	401.57 ± 0.25[a]	401.79 ± 1.13[a]	400.23 ± 0.52[a]	NS

Different superscript in the same row indicate significant difference amongst experimental diets (Tukey multiple range test ** = 0.05).
[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.

Table 5
Fatty acid composition of biofloc (% of fatty acids by peak area).

Sl. no.	Fatty acid	Percentage
1	Caproic acid, C 6:0	0.12
2	Caprylic acid, C 8:0	0.15
3	Capric acid, C 10:0	0.74
4	Lauric acid, C 12:0	0.09
5	Tridecanoic acid, C 13:0	3.06
6	Pentadecanoic acid, C 15:0	1.67
7	Palmitic acid, C 16:0	46.54
8	cis-7-hexadecenoic acid, C 16:1 (n−9)	5.25
9	Palmitoleic acid, C 16:1 (n−7)	0.21
10	Heptadecanoic acid, C 17:0	0.85
11	Stearic acid, C 18:0	1.96
12	Oleic acid, C 18:1 (n−9)	9.19
13	cis-vaccenic acid, C 18:1 (n−7)	15.37
14	Linoleic acid, C 18:2 (n−6)	10.67
15	α-linolenic acid, C 18:3 (n−3)	0.1
16	Nonadecanoic acid, C 19:0	0.4
17	Arachidic acid, C 20:0	0.4
18	Eicosapentaenoic acid, C 20:5 (n−3)	ND
19	Behenic acid, C 22:0	0.49
20	Docosahexaenoic acid, C 22: 6 (n−3)	ND
21	Lignoceric acid, C 24:0	0.42
22	Nervonic acid, C 24:1 (n−9)	2.33

ND; Not detected.

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. The reaction was stopped by adding 2 mL DNS reagent, and kept in boiling water bath for 5 min. 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 mg^{-1} protein min^{-1} 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. The reaction was stopped by adding 3 mL of DNS reagent and the mixture was kept in boiling water bath for 10 min. 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 of 2.5 mL of 1% (w/v) casein prepared in 0.01 N NAOH, 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. 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 mg^{-1} protein min^{-1} at 37 °C.

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 with enzyme source is 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.

2.11. Statistical analysis

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 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. Microbial composition of biofloc

Total heterotrophic, *Vibrio*, *Bacillus*, *Lactobacillus* and fungal counts (cfu mL^{-1}) during the biofloc production cycle are given in Table 2. Total heterotrophic count increased from 0.23 ± 0.06 to $245.67 \pm 61.32 \times 10^8$ cfu mL^{-1} during the biofloc production cycle. The major microbial genera noticed in the harvested biofloc were *Vibrio*, *Lactobacillus*, *Bacillus* and fungi. The harvested biofloc contained $240.83 \pm 47.00 \times 10^4$ cfu mL^{-1} *Vibrio*, $345.00 \pm 37.75 \times 10^4$ cfu mL^{-1} *Bacillus* and $6.67 \pm 1.67 \times 10^1$ cfu mL^{-1} *Lactobacillus*. On an average, $526.67 \pm 44.10 \times 10^2$ cfu mL^{-1} of fungus were noticed in the harvested biofloc.

3.2. Proximate composition of biofloc and experimental diets

Total yield of biofloc per production cycle was 178 g and, 4.03 ± 0.36 kg wheat flour was used to produce 1 kg of bioflocs. Proximate

Table 6
Physicochemical parameters of water during biofloc production cycle.

Parameters	Initial	2nd day	3rd day	5th day	Level of significance
Temperature (°C)	28.33 ± 0.17^a	28.27 ± 0.13^a	27.47 ± 0.23^b	27.23 ± 0.15^b	**
pH	7.43 ± 0.07^a	7.70 ± 0.15^a	7.83 ± 0.15^a	7.80 ± 0.12^a	NS
Salinity (ppt)	13.67 ± 0.17^a	14.08 ± 0.08^{ab}	14.42 ± 0.08^b	14.29 ± 0.11^b	*
Alkalinity (mg $CaCO_3$ L^{-1})	94.67 ± 8.74^a	96.33 ± 2.03^a	96.00 ± 9.23^a	100.33 ± 6.89^a	NS
Total ammonia-N (mg L^{-1})	5.16 ± 0.28^b	4.09 ± 0.27^{ab}	3.56 ± 0.35^b	0.68 ± 0.11^c	**
Dissolved oxygen (ppm)	9.33 ± 0.17^a	5.42 ± 0.23^b	5.06 ± 0.22^b	5.25 ± 0.30^b	**
Turbidity (NTU)	9.00 ± 1.53^a	93.33 ± 8.82^a	266.67 ± 10.93^b	530.00 ± 56.86^c	**
Biofloc volume (ml L^{-1})	0.25 ± 0.05^a	2.47 ± 0.09^b	4.83 ± 0.60^c	6.93 ± 0.09^d	**

The means (mean ± SE) with no superscript letter in common per factor indicate significant difference.
If the effects were significant, ANOVA was followed by Tukey test. *$P < 0.05$; **$P < 0.01$; NS, not significant.

Table 7
Physicochemical parameters in the experimental tanks during the study period (mean ± SE).

Parameters	B0	B4	B8	B12	Level of significance
Temperature (°C)	30.68 ± 0.40[a] (29.00–32.50)	30.79 ± 0.46[a] (28.80–32.40)	30.64 ± 0.37[a] (29.20–32.50)	30.96 ± 0.30[a] (29.00–32.20)	NS
pH	7.83 ± 0.13[a] (7.46–8.40)	7.96 ± 0.13[a] (7.43–8.50)	7.90 ± 0.12[a] (7.48–8.50)	7.93 ± 0.11[a] (7.50–8.50)	NS
Salinity (ppt)	14.30 ± 0.26[a] (13.20–15.20)	14.26 ± 0.28[a] (13.11–15.30)	14.26 ± 0.27[a] (13.10–15.20)	14.58 ± 0.17[a] (14.10–15.40)	NS
Alkalinity (mg $CaCO_3$ L^{-1})	127.78 ± 2.41[a] (116.0–138.5)	129.31 ± 1.60[a] (120.0–136.2)	125.00 ± 2.60[a] (112.0–140.0)	128.33 ± 2.67[a] (120.0–143.0)	NS
Nitrite-N (μg L^{-1})	38.52 ± 5.10[a] (19.96–56.11)	35.42 ± 4.70[a] (22.50–58.70)	39.33 ± 5.36[a] (20.45–60.50)	43.32 ± 5.62[a] (22.12–64.00)	NS
Nitrate-N (μg L^{-1})	106.57 ± 12.76[a] (55.50–142.00)	95.99 ± 11.36[a] (50.40–135.00)	107.36 ± 13.46[a] (52.50–140.00)	110.54 ± 11.95[a] (59.10–146.00)	NS
Total ammonia-N (μg L^{-1})	107.31 ± 9.83[a] (65.00–137.80)	104.78 ± 10.19[a] (60.10–134.50)	108.69 ± 10.06[a] (67.70–142.00)	112.29 ± 9.26[a] (58.90–140.50)	NS
Dissolved oxygen (ppm)	5.91 ± 0.17[a] (5.10–6.50)	5.92 ± 0.20[a] (5.10–6.80)	5.79 ± 0.18[a] (5.10–6.60)	5.87 ± 0.20[a] (5.15–6.77)	NS

The means with no superscript letter in common per factor indicate significant difference. Ranges are in parenthesis.
If the effects were significant, ANOVA was followed by Tukey test. NS, not significant.

composition (%) of biofloc and experimental diets are presented in Tables 3 and 4. The dried biofloc contained 24.30 ± 0.28% crude protein, 3.53 ± 0.35% crude lipid and 29.24 ± 0.64% nitrogen free extract (NFE). The mean ash content and acid insoluble ash content was 31.98 ± 1.01 and 10.75 ± 1.06% of dried biofloc 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 B8 and B12 compared to control (B0) and B4. The crude protein content ranged from 37.77 ± 0.14 to 38.05 ± 0.29% in the experimental diets.

3.3. Fatty acid profile of biofloc

The fatty acid profile of biofloc by GC–MS analysis is given in Table 5. Out of twenty fatty acids identified in the biofloc, palmitic acid (46.54%), cis-Vaccenic acid (15.37%), linoleic acid (10.67%) and oleic acid (9.19%) were the abundant fatty acids. The most poly-unsaturated fatty acids (PUFA) were linoleic acid (10.67%) and α-linolenic acid (0.1%). However, omega 3 poly unsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were not detected in biofloc.

3.4. Physicochemical parameters

The water quality parameters during biofloc production cycles are presented in Table 6. The dissolved oxygen level reduced from 9.33 ppm on the initial to 5.25 ppm on the 5th day. During the production cycles, total ammonia-N (TAN) level reduced from 5.16 to 0.68 mg L^{-1} on the day of biofloc harvesting. Rate of TAN reduction was 13.78% per hour. The mean turbidity and biofloc volume increased from 9.00 to 530.00 NTU and 0.25 to 6.93 mL L^{-1} respectively, from the initial to the 5th day of production cycle. No significant difference ($p > 0.05$) among the treatments was observed in water quality parameters during the experimental period (Table 7).

3.5. Growth performance

Growth performance of *P. monodon* juveniles over the time period are presented in Table 8. Final body weight among treatments differed significantly with 16.9 and 13.9% significantly higher ($p < 0.01$) final body weight noticed in B8 and B4 respectively, compared with control. However, the body weight of the group, B12 (5.65 ± 0.22 g) did not differ significantly with control, B0 (5.80 ± 0.12 g). Feed conversion ratio (FCR), protein efficiency ratio (PER) and survival showed a highly significant difference among the treatments ($p < 0.01$). A better FCR, 1.84 ± 0.09 was observed in B4 which was significantly lower from control (2.81 ± 0.19) and B12 (3.58 ± 0.14). Similarly, significantly higher PER, 3.48 ± 0.17 was registered in B4 compared to 2.80 ± 0.13 and 2.78 ± 0.18 in control and B12 respectively. However, no significant difference in FCR and PER was observed between B4 and B8.

3.6. Digestive enzymes

Specific activity of amylase in hepatopancreas was 57.6% significantly higher ($p < 0.01$) in B4 compared with control (Fig. 1) while no significant difference was noticed among B8, B12 and control, B0. However, gut amylase activity did not differ significantly ($p > 0.05$) among the treatments. Cellulase activity in hepatopancreas differed significantly ($p < 0.01$) with the highest specific activity in B4 which was 45.5 and 112.5% significantly higher compared to B0 and B12 respectively (Fig. 2). As for gut, the highest cellulase ($p < 0.05$) activity recorded in B8 (0.05 ± 0.01 U mg^{-1} protein) and the lowest activity in control (0.02 ± 0.001 U mg^{-1} protein).

Table 8
Growth performance of *Penaeus monodon* juveniles fed with graded level of biofloc supplemented diets.

Growth parameters	B0	B4	B8	B12	Level of significance
Initial wt. (g)	2.90 ± 0.06[a]	2.83 ± 0.06[a]	2.92 ± 0.11[a]	2.83 ± 0.04[a]	NS
Final wt. (g)	5.80 ± 0.12[a]	6.61 ± 0.13[b]	6.79 ± 0.21[b]	5.65 ± 0.22[a]	**
Weight gain (%)	100.03 ± 2.57[a]	133.71 ± 5.68[b]	132.96 ± 5.99[b]	100.27 ± 10.39[a]	**
Weight gain/week (g)	0.34 ± 0.01[a]	0.44 ± 0.02[b]	0.45 ± 0.02[b]	0.33 ± 0.03[a]	**
FCR[1]	2.29 ± 0.11[ab]	1.84 ± 0.09[a]	1.95 ± 0.04[ab]	2.31 ± 0.14[b]	*
SGR[2]	1.16 ± 0.02[a]	1.41 ± 0.04[b]	1.41 ± 0.04[b]	1.15 ± 0.09[a]	**
PER[3]	2.80 ± 0.13[a]	3.48 ± 0.17[b]	3.27 ± 0.06[ab]	2.78 ± 0.18[a]	*
Survival (%)	87.30 ± 4.20 [a]	95.24 ± 2.75[a]	87.30 ± 1.59[a]	88.89 ± 4.20[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 test. *P < 0.05; **P < 0.01; NS, not significant.
FCR[1] = Feed conversion ratio; SGR[2] = Specific growth rate PER[3] = Protein efficiency ratio.
Values are presented as mean ± SE.

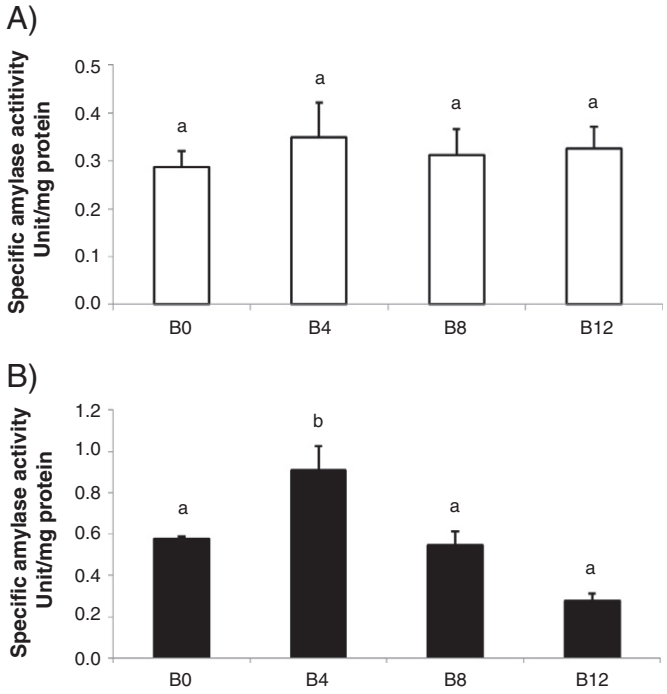

Fig. 1. Specific amylase activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of biofloc in A) Gut and B) Hepatopancreas, Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p < 0.05$). The level of biofloc supplementation was 0 (B0), 4 (B4), 8 (B8), and 12% (B12).

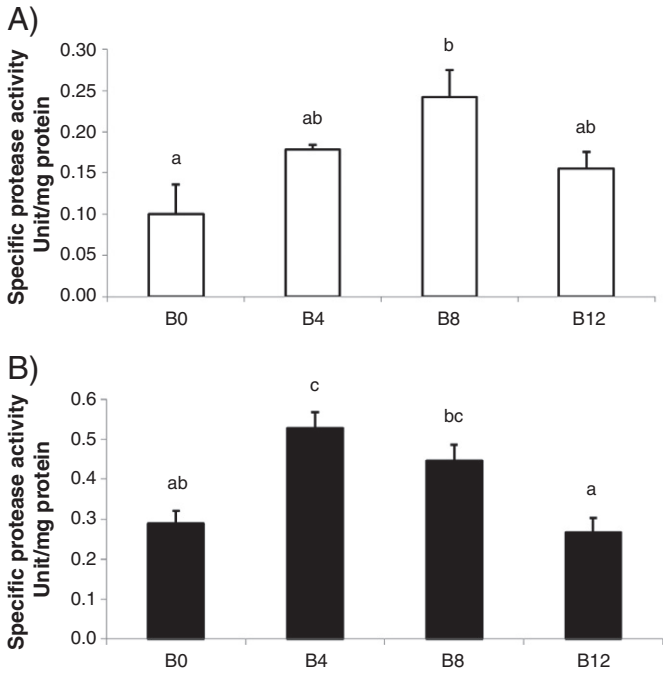

Fig. 3. Specific protease activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of biofloc in A) Gut and B) Hepatopancreas, Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p < 0.05$). The level of biofloc supplementation was 0 (B0), 4 (B4), 8 (B8), and 12% (B12).

Gut protease activity (Fig. 3) differed significantly ($p < 0.05$) with 141.6, 78.6 and 54.9% increases in B8, B4 and B12 respectively compared with B0. As for hepatopancreas, 82.6 and 54.4% increase in protease activity was observed in B4 and B8 respectively compared with control. Similarly, hepatopancreas lipase activity showed 61.0 and 28.1% increases in B4 and B8 respectively compared with control (Fig. 4).

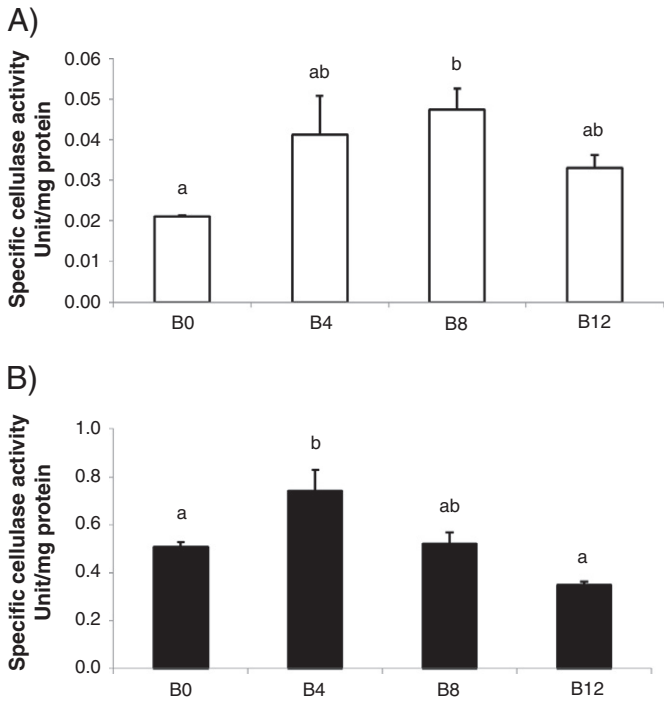

Fig. 2. Specific cellulase activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of biofloc in A) Gut and B) Hepatopancreas, Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p < 0.05$). The level of biofloc supplementation was 0 (B0), 4 (B4), 8 (B8), and 12% (B12).

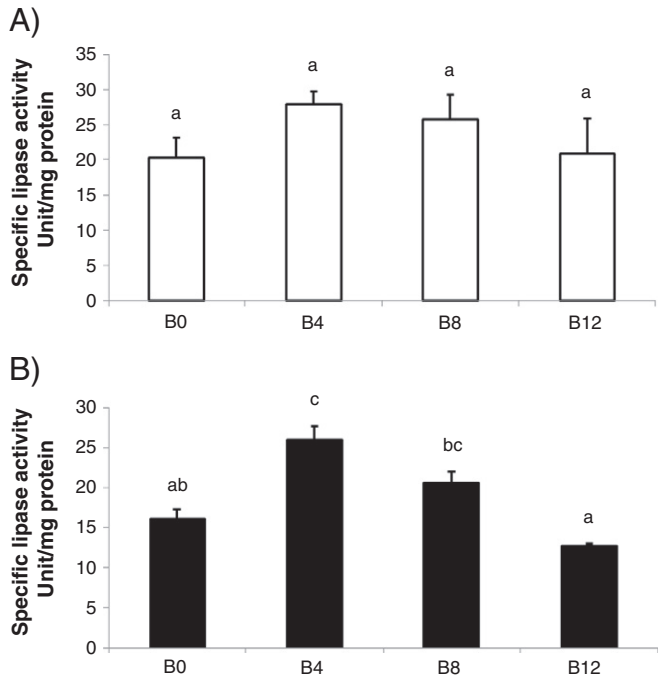

Fig. 4. Specific lipase activity in *Penaeus monodon* juveniles fed with experimental diets supplemented with graded level of biofloc in A) Gut and B) Hepatopancreas, Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p < 0.05$). The level of biofloc supplementation was 0 (B0), 4 (B4), 8 (B8), and 12% (B12).

4. Discussion

Beneficial role of *in situ* based biofloc system in penaeid shrimp culture are well documented (Hari et al., 2006; Xu and Pan, 2012). Recently, it has been reported that use of biofloc as a dietary ingredient in shrimp diet enhances the growth rate of *L. vannamei* (Kuhn et al., 2009, 2010). The present study illustrates the role of biofloc as a dietary supplement on growth performance and digestive enzyme activity in *P. monodon*.

In the present study, C:N ratio was maintained at 10:1 to efficiently convert the inorganic nitrogen into microbial protein (Avnimelech, 1999). Wheat flour was used as carbohydrate source for its easy availability and production of good quality floc (Azim and Little, 2008; Ballester et al., 2010). In the present experiment, 4.03 ± 0.36 kg wheat flour was used to produce 1 kg of microbial flocs. Earlier, Kuhn et al. (2009) reported 1 kg microbial flocs production from 1.5 kg of sucrose in a bioreactor. This may be because being a disaccharide; sucrose is readily available for microbial utilization, while wheat flour has complex long chain polysaccharides. Even though, bioreactors have better conversion efficiency, the present production system is simple and advantageous as it used cheap and readily available ammonium sulphate as nitrogen source and wheat flour as carbon source.

Total microbial community in biofloc production system reached a density of 10^8 cfu mL^{-1} and matches with the earlier findings (Burford et al., 2003). The major microbial community developed in the biofloc belonged to Vibrionaceae, *Bacillus* sp., and *Lactobacillus* sp. with majority as gram negative bacteria. Aggregates of particulate organic matter, zooplankton communities like ciliates, rotifers and a small amount of autotrophic microalgae were also noticed. Similarly, Ballester et al. (2010) reported that biofloc is composed of attached heterotrophic bacteria, filamentous cyanobacteria, dinoflagellates, ciliates, flagellates and rotifers. Various factors like salinity, light and type of culture system affects the microbial composition of floc. For example, Ju et al. (2008a) reported the dominance of algal communities over bacterial biomass in flocs collected from outdoor shrimp culture units. Lower dominance of autotrophic community noticed in the present study might be due to lack of direct sunlight in the biofloc indoor production facility. The presence of diverse group of bacteria especially, *Bacillus* sp. and *Lactobacillus* sp. indicate that biofloc can be considered as a source of potential probiotics.

Proximate analysis of the biofloc in the present study is in agreement with the findings of Ballester et al. (2010) who reported 30.4% crude protein (CP) with wheat flour and molasses as carbohydrate sources. Nutritional composition of biofloc varies with type of carbohydrate source, microbial community structure, culture condition etc. For instance, Crab et al. (2010) observed that biofloc developed from glycerol inoculated with *Bacillus* contained higher protein (58% CP) than biofloc developed from glycerol, acetate (42–43% CP) and glucose (28% CP). The lower crude protein level noticed in the harvested biofloc compared to earlier findings (Crab et al., 2010; Ekasari et al., 2010) may be due to differences in bacteria taking part in floc formation (Rittmann and McCarty, 2001). For example, substrates like acetate and glycerol used in the previous studies (Crab et al., 2010) might have promoted the bacteria involved in cellular growth and increased the protein content in biofloc whereas wheat flour might have promoted bacteria that produce large amounts of exopolysaccharides. Ju et al. (2008a) reported that chlorophyll-dominated biofloc contained higher crude protein content (42%) than flocs dominated by diatoms (26–34%) and bacteria (38%). This further suggests that the microbiota that constitutes the biofloc is likely to affect the protein content of the bioflocs.

In the microbial food web, microalgae and zooplankton are the richer source of lipids compared to bacteria (Zhukova and Kharlamenko, 1999). Out of 20 fatty acids detected in biofloc, palmitic acid, cis-vaccenic acid, linoleic acid and oleic acid were the dominant fatty acids, and omega 3 fatty acids detected in trace level. This is in consonance with the findings of Crab et al. (2010) who reported that biofloc contain palmitic acid, palmitoleic acid and linoleic acid in the highest and omega 3 fatty acids in trace levels. The lower level of PUFA in biofloc may be due to the dominance of heterotrophic bacteria compared to PUFA rich microalgal community (Meyers and Latscha, 1997).

Inclusion of biofloc as a dietary ingredient in shrimp diet found to improve the growth performance of *L. vannamei* (Ju et al., 2008b; Kuhn et al., 2009, 2010). In the present study, dietary supplementation of biofloc at 4 and 8% levels significantly enhanced the growth, PER and reduced the FCR in tiger shrimp. It has been documented that bioflocs are the rich source of many bioactive compounds such as carotenoids, chlorophylls, phytosterols, bromophenols, amino sugars (Ju et al., 2008a) and anti-bacterial compounds (Crab et al., 2010). This suggests that microbial components, unknown growth factors or probiotic microorganisms like *Bacillus*, *Lactobacillus* present in the biofloc might have resulted in significantly higher growth rate and better FCR in shrimp fed with biofloc incorporated diet.

Biochemical composition of the diet plays an important role in digestive enzyme profile in shrimp (Gamboa-Delgado et al., 2003). In the present study, dietary supplementation of biofloc significantly improved ($p < 0.05$) the specific activity of digestive enzymes like amylase, cellulase, protease and lipase in the treatment groups compared with control. The increased digestive enzyme activities in B4 and B8 might have enhanced the digestion and nutrient absorption which might have contributed significantly higher ($p < 0.05$) growth rate in these treatment groups. A previous study by Xu and Pan (2012) in biofloc based system, reported significantly higher digestive enzyme activities and better growth performance in *L. vannamei*. Similarly, enhanced level of digestive enzymes activity has been reported in fish and shrimp fed with probiotics, microalgae and periphyton supplemented diets (Anand et al., 2013b; Lara-Flores et al., 2003; Ziaei-Nejad et al., 2006). The presence of microbial components in the biofloc supplemented diet might have stimulated the production of endogenous enzymes by the shrimp hepatopancreas compared with control.

In the present study, the biofloc supplementation at the 12% level (B12) did not result in proportionate increase in growth rate or improvement of FCR compared with control. Further, the digestive enzyme activities in B12 were not significantly higher than other treatment groups. Earlier, Kuhn et al. (2010) replaced the fish meal by biofloc in *L. vannamei* diet and recorded significantly higher growth rate at 10 and 15%, and non-significant difference at 21 and 30% dietary inclusion level of biofloc. The present findings agree in general with those of Wang (2007) and Anand et al.(2013b) who reported that increase in dietary supplementation of probiotic or periphytic algae in shrimp diet do not increase proportionately the digestive enzyme activities and growth of shrimp. Moreover, reduction in growth rate of fishes was recorded at higher level of microbial supplementation (Ajiboye et al., 2012; Kiessling and Askbrandit, 1993) as microbial products at higher level tend to reduce the feed palatability and digestibility (Kiessling and Askbrandit, 1993). The higher ash level recorded in the B12 diet compared to all other groups also might have influenced the digestibility and growth performance of shrimp. However, the growth performance of B12 was comparable to control suggesting that dietary supplementation of higher level of biofloc do not have growth retardation effects in shrimp juveniles. Seeing the cost-effectiveness, the inclusion of biofloc at 4% level is beneficial in improving growth performance and digestive enzyme activities in shrimp.

5. Conclusion

The study demonstrates that dietary supplementation of biofloc at 4–8% level had beneficial effects on growth performance and digestive enzyme activities in *P. monodon*. Current method of biofloc production using ammonium sulphate as nitrogen and wheat flour as carbon source is cheaper and easier compared to bioreactors. These findings may encourage feed manufacturers to consider biofloc as a viable alternative dietary supplement. Future studies are required to determine the amino

acid profile, mineral profile and non-protein content of the biofloc with respect to nutritional requirement of shrimp.

Acknowledgment

The authors are grateful to Dr. A.G. Ponniah, Director, Central Institute of Brackishwater Aquaculture, Chennai and Dr. W. S. Lakra, Director, Central Institute of Fisheries education, Mumbai for providing the required facilities to conduct this study.

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