Dietary supplementation of red alga *Pyropia* spheroplasts on growth, feed utilization and body composition of sea cucumber, *Apostichopus japonicus* (Selenka)

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Abstract

A feeding experiment was conducted in closed recirculating system to evaluate the effects of freeze dried *Pyropia* spheroplasts (*P*S) on growth and biochemical composition of sea cucumber *Apostichopus japonicus*. PS was prepared through enzymatic treatment to break down the complex mixture of polysaccharide cell wall. Sea cucumbers were fed formulated diets with 10 (Diet 1), 30 (Diet 2) and 50 g kg-1 (Diet 3) inclusion level of PS in closed recirculating systems. A diet without PS was used as a control (Diet 4). The experiment was conducted for 6 weeks maintaining water temperature 15±1°C, photoperiod 18:06 h (D:L). Feed were supplied ad-libitum at 16 h once in a day and excess feed and feces were removed in the next morning. Results showed that highest growth was observed in the 50 g kg^{-1} PS diet compared to other treatments. Total weight gain, mean weight gain, net yield, protein efficiency ratio (PER) and protein gain (%) was significantly higher in 50 g kg^{-1} PS diet (P<0.05). Significantly higher percentage of energy was allocated for growth in the diet 3. The highest specific growth rate, feed conversion efficiency (P<0.05) was observed in higher percentage of PS diet. Both the growth performance and biochemical analysis showed that

superior growth was observed with increasing the level of PS in the diet. This is comprehensible that PS can be used as a new, cheaper feed ingredient in the formulated diet of *A. japonicus* for commercial aquaculture.

Keywords: Red alga *Pyropia yezoensis*, spheroplasts, seaweed, growth, feed utilization, Sea cucumber *Apostichopus japonicus*

Introduction

Sea cucumber *Apostichopus japonicus* has long been exploited as a potential fishery resource in Japan, China, Republic of Korea and Russia Federation (Yuan, Yang, Zhou, Mao, Zhang, & Liu, 2006; Shahabuddin & Yoshimatsu, 2015). It has great potential as an aquaculture species in East Asia because of its nutritional and medicinal value (Zhang, Wang Y.G., & Rong, 2004; Gu, et al., 2010; Seo & Lee, 2011). Dried products are considered as ideal tonic food with higher protein and lower fat (Chen, 2003) and market demand has increased with increasing interest, leading to overfishing and causing a tremendous decline in the natural fishery resources (FAO, 2014; Conand, C., 2004). However, with great demand in the Asian markets makes it a high value species for aquaculture and therefore it is necessary to increase production through intensive aquaculture system.

Knowledge of dietary requirements is important for commercial aquaculture. Studies have examined the nutritional requirements (Okorie E. O., et al., 2008; Choi, Seo, & Lee, 2009; Okorie O. , Ko, Go, & Bae, 2011; Seo & Lee, 2011), feed ingredients (Yuan, Yang, Zhou, Mao, Zhang, & Liu, 2006; Liu, Dong, Tian, Wang , & Gao, 2009; Slater, Jeffs, & Carton, 2009; Seo, Shin, & Lee, 2011a; Seo, Shin, & Lee, 2011b; Dong, Wang, Gao, & Tian, 2013; Dong, Wang, Gao, & Tian, 2013), diet formation (Xia S. , et al., 2013), culture technology (Sui, 1989; Pei, Dong, Wang, Gao, & Tian, 2012), water quality (An, Dong, & Dong, 2007; Yuan, Yang, Wang, Zhou, & Gabr, 2010; Xie, Zhao, & Yang, 2013) of sea cucumber. Plant based ingredients specially macroalgae are considered as a part of the diet of *A. japonicus* and brown alga *Sargassum thunbergii* is to be the best choice (Xia S. , Yang, Li, Liu, Zhou, & Zhang, 2012). With the expansion of the culture system and rearing techniques, the alga was over exploited and became more expensive (Lobban & Harrison, 1994). Therefore, finding cheaper and optimal substitutes for brown algae is crucial. Nutritional studies conducted previously were mostly on larvae and juvenile, very few studies were found in sub-adult, especially for commercial aquaculture in closed recirculating system. Though the land based intensive recirculating system is a new promising culture method, however, it has not been drawn much attention yet (Chang, Yi, & Mu, 2003; Kang, Kwon, & Kim, 2003), due to the lack of commercial diet (Yuan, Yang, Zhou, Mao, Zhang, & Liu, 2006). Still little is known about the optimum nutrition requirements and economic diet of *A. japonicus*. The development of low cost and useful feed is expected as a substitute of brown algae for commercial aquaculture.

Seaweeds progressively considered as an ingredient of aquaculture feed (Mustafa & Nakagawa, 1995; Wassef, El-Masry, & Mikhail, 2001). The global annual aquaculture production of seaweeds is $20.3x10^6$ tonnes (FAO, 2014). Every year around 2% of the Nori produced worldwide are discarded (Ishihara, et al., 2008) and low quality dried laver with no commercial value has become a serious environmental problem. Studies confirmed that small amount of algae added to fish feed significantly improves growth, lipid composition and help in disease resistance (Nakagawa & Kasahara, 1986; Satoh, Nakagawa, & Kasahara, 1987; Xu, Yamasaki, & Hirata, 1993; Shahabuddin, et al., 2015). Among them Nori *Pyropia yezoensis* Ueda is one of the important candidates, that contains a higher percentage of protein as well as minerals and vitamins (Sugita, Yamamoto, & Yashimatsu, 2009; Shahabuddin, Khan, Arisman, Saha, Yoshimatsu, & Araki, 2015). Therefore, effective uses of the waste lever have been sought.

Anionic cell wall polysaccharides found in macroalgae limit the efficiency of protein digestion (Jordan & Vilter, 1991). Nori is composed of three kinds of polysaccharides, β-1,4-mannan, β-1,3-xylan and porphyrin; which limit the efficiency of its protein (Khan, Yoshimatsu, Kalla, Araki, & Sakamoto, 2008; Shahabuddin, Khan, Arisman, Saha, Yoshimatsu, & Araki, 2015). Spheroplasts isolation using polysaccharide degrading enzymes (Araki, Kayakawa, Tamaru, Yoshimatsu, & Morishita, 1994; Araki, Hayakawa, Lu, Karita, & Morishita, 1998), has shown promising result in using the single cell as a noble feed ingredient, that are easily digestible by aquatic organisms (Kalla, Yoshimatsu, Araki, Zhang, Yamamoto, & Sakamoto, 2008; Shahabuddin, Khan, Arisman, Saha, Yoshimatsu, & Araki, 2015; Shahabuddin, et al., 2015). *A. japonicas* has no specialized organs for physical grind and no gland for chemical digestion, therefore, the present experiment was designed to assess the dietary inclusion of single cell *Pyropia* spheroplasts (PS) as dietary ingredients for culturing the Japanese sea cucumber in closed recirculating system.

Materials and methods

Experimental diets

The experimental diets were made up of four isonitrogenous formulations with varying PS inclusion levels at 0, 10, 30, 50 g kg^{-1}. All the diets with average protein and crude lipid levels 35.0% and 3.1% respectively were formulated. Soybean meal was used as the primary protein source in all the diets and rice powder is used as the carbohydrate source. All the ingredients were ground and sieved to a particle size <100 μm and then mixed together completely. Table 1 shows the proximate analysis of ingredients and Table 2 shows the composition and proximate analysis results of the experimental diets. Feed ingredients were milled, mixed with other feed additives. The mixture was primed with distilled water to produce a suitable mash for extrusion and pelleted through 1.5 mm dye. Resulting extruded pellets were dried for 48 hours at 40^{o}C then stored in an airtight polythene bag and kept in frozen condition over the whole experimental period.

Table 1 Proximate compositions of ingredients used for the preparation of experimental diets of *A. japonicas* (% dry matter basis)

Items	Soybean meal	Rice powder	Rice bran	White fish meal	PS
Protein	47.2	10.4	24.4	65.8	31.1
Lipid	2.9	2.1	3.3	6.2	8.2
Carbohydrate	37.7	80.6	63.8	8.4	46.6
Ash	6.9	1	4.8	17.3	6.1
Moisture	5.3	5.7	3.7	2.3	8
Energy (KJ)	1531	1607	1602	1477	1610

Table 2 Formulation and chemical composition of experimental diets for *A. japonicus* cultured in closed recirculating system (% dry matter basis)

Items	Diet 1	Diet 2	Diet 3	Diet 4
Ingredients				
Soybean meal	50	50	50	50
Rice powder	15	15	15	15
Rice bran	15	15	15	15
White Fish meal	9	7	5	10
PS	10	30	50	0
Mineral premix	3	3	3	3
Vitamin premix	2	2	2	2
CMC	4	4	4	4
Chromic Oxide	1	1	1	1
Proximate composition				
Protein	35.7	35.4	34.3	34.7
Lipid	3.2	3.1	3.1	3.1
Ash	9.9	9.7	9.0	10.1
Carbohydrate	42.9	44.9	45.4	42.7
Energy (KJ)	1435.3	1455.3	1455.7	1416.7
EPA	1.65	2.20	3.43	1.53
DHA	3.16	2.15	1.79	4.28

Animal source and acclimatization

The experiment was conducted at the Laboratory of Shallow Sea Aquaculture of the Graduate School of Bioresources, Mie University, Japan in a closed recirculating system. Healthy sea cucumber was collected from the Shima peninsula of Mie prefecture and immediately transferred to the laboratory with maintaining the temperature. They were transferred to the fibreglass tanks in one close recirculating system. Prior to experiment, the animals were acclimatized to the laboratory conditions for two weeks. During the period sea cucumber were fed brown algae powder *Sargassum thunburgii* with sea mud every day at 16:00 h hours once a time.

Rearing condition

Sea cucumbers were housed in each of the twelve 10 L rectangular fibreglass tanks within a single water recirculation system. During the experiments, continuous aeration was provided and water flow was maintained 1 L min $^{-1}$ tank $^{-1}$. Water flow and aeration was maintained in such a way that feed on the bottom of the tank will not be disturbed. Water temperature was maintained at 15±0.5 ^{o}C and sea cucumbers were subjected to a photoperiod 18:6 h (D:L) using indoor fluorescent lights. Outflow water was passed through the protein skimmer to remove low molecular weight particles and then filtered by several layers of mechanical and biological filtration systems. The temperature was maintained by using digital thermal control system. UV was used to disinfect the inflow water. Artificial sea water was used during the experiment and salinity was maintained between 31 to 33 psu, dissolved oxygen was maintained above 5.0 mg L $^{-1}$. During the experiment 5% water of the recirculating system was exchanged each day and salt water ice was used to reduce the water temperature before adding to the system.

Experimental design and feeding trial

After acclimatization, a total of 24 healthy sea cucumbers was randomly selected and placed two individuals into 12 fibreglass (25x20x25 cm^3) to allocate one of the four dietary treatments in triplicate for 6 weeks in another closed recirculating system. The animals were fed ad-libitum once a day at about 16.00 h. The semi moist feed was placed at the bottom of the tank. During feeding water flow and aeration was stopped for about half an hour to settle down the feed at the bottom of the tank. Most of the feed was consumed and if remained, uneaten feed and feces were siphoned out from the tank at 14:00 h next day and gently separated from each other. The excess feed was dried at 60^{o}C to a constant weight to calculate the amount of feed consumed. Each animal was weighted individually every week and the feed was adjusted with increasing body weight. Feces were washed with distilled water several times to remove the salt, centrifuged and stored at -20 ^{o}C for analysis.

Preparation of Spheroplasts

Low-quality dried lavers made from *Pyropia yezoensis* were used to prepare PS for the present experiment. To break down the cell wall, three different kinds of cell wall degrading enzymes were used. Mixed enzymes includes 60 g sumizyme ACH, 200 units agarase and 100 units of β-1,3 Xylanase were added in the 30 L of water in a bucket and continuously stirred (1100 RPM min^{-1}) at 20 ^{o}C. Sumizyme ACH were used as it has β-mannanase activity. Four bundles of dried nori (4 bundle x10 pack in each bundle x10 sheet in each pack x 3g of each sheet=1200g) were immersed in the enzyme solution for 20 h. Spheroplasts were collected by centrifuging at 3000 RPM for 10 minutes, PS was collected as precipitate and immediately freeze-dried. These freeze-dried PS was grounded into powder form and used it in the experimental diets of sea cucumber.

Water quality analysis

Weekly water samples were collected for the analysis of total phosphorus (TP), ammonia nitrogen (NH_3-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N) using HACH portable data logging colorimeter DR-850.

Chemical analysis of diets, feces and animal samples

Proximate composition of the experimental diets, feces and animals were conducted by the method of AOAC (1990). The dry matter remaining after drying samples at 105^{o}C was combusted to ash at the 550°C for 4 h. Total nitrogen content was estimated by the Kjeldahl method. Crude protein was determined indirectly (N × 6.25). The crude fat was determined by soxhlet extraction method, The ash content was measured using a muffle furnace combustion at 550°C. The energy content of the diets, feces, and animal samples was measured using a calorimeter. Total lipids were extracted using a modified procedure (Folch, Lees, & Sloane-Stanley, 1957). Lipids were saponified and fatty acids were transmethylated using 5% hydrogen chloride methanol solution (HCL-MeOH) to obtain the fatty acid methyl esters (FAMEs). These FAMEs were analysed by gas chromatography (GC) (Simadzu, Japan). The GC was equipped with flame ionization detector (FID) and Omega wax fused silica capillary column (30 m × 0.32 mm i.d.). The detector, injector and column temperature were, and respectively with an analysis time of 60 min. The injection port and flame ionization detector were operated at 230°C and 240°C respectively. The column temperature of the omegawax was held at 200°C. Component peak was identified by comparison with standard mixtures (Japan oil chemist's society, 1996).

Data calculation

Daily weight gain (WG), mean weight gain (MW), specific growth rate (SGR %), feed conversion efficiency (FCE), protein gain *(*PG), Protein efficiency ration *(*PER) and protein productive value *(*PPV) were calculated as follows:

WG (g day^{-1})= W_t-W_0/T

MW (g)=W_t-W_0

SGR_w (% d^{-1})=($\ln W_t$-$\ln W_0$)/T×100

FCE (%)=(W_t-W_0)/C_w×100

PG (%)=P_t-P_0

PER (%)=(W_t-W_0)/P_w

PPV (%)=(P_t-P_0)/ P_w×100

Where W_t and W_0 are final and initial wet body weight (g) of sea cucumber respectively, T is the time of the experiment (days), C_w is the feed intake, P_t and P_0 are the final and initial body protein percentage respectively, P_w is the protein intake.

Energy budget was constructed according to the equation C=G+F+U+R (Carefoot, 1987).

Where C is the energy consumed, G is the energy for growth, F represents the energy loss to faeces produces, U is the energy loss as ammonia excretion and R stands for the energy loss in respiration.

The estimation of C, G, and F in the budget equation were calculated as follows:

$$C=C_w \times IF_e, \qquad G=W_t \times E_1-W_0 \times E_0, \qquad F=F_w \times FE_e$$

Where C_w and F_w are food intake and fecal production respectively; IF_e and FE_e are the energy contents of the feed and feces respectively; W_t and W_0 are the final and initial wet body weights of sea cucumber (g) respectively; E_t and E_0 are the final and initial energy contents of the sea cucumber respectively. Based on the nitrogen budget equation, U was determined as follows (Elliott, 1976):

$U= (C_N-G_N-F_N) \times 24.83$ (KJ g^{-1})

Where C_N is the nitrogen consumed from the feed, G_N is the nitrogen deposited in the sea cucumber tissue, F_N is the nitrogen lost in the feces, the constant 24.83 is the energy content in excreted ammonia (KJ g^{-1}). The value of R was calculated as the difference between energy consumption and the energy allocated to growth, feces and excretion using the equation R=C-G-F-U.

Statistical analysis

Means were calculated for each treatment (i.e. n=3). Mean values were analysed using SPSS 16.0 for Windows statistical package (SPSS, Chicago, USA). Values of growth performance, proximate analysis, water quality parameters were subjected to one-way analysis of variance (ANOVA) and significance differences ($P<0.05$) among treatments means were tested using least significant difference (LSD). The data are presented as mean ± SE of three replicate groups.

Results

Supplemental effects PS on growth performance of *A. japonicus*

The growth performance, nutrient utilization and survival of *A. japonicas* are shown in Table 3. The growth performance differed significantly among treatments fed diets with PS. *A. japonicas* grew significantly higher in PS diets compared to the control (P>0.05). Survival and evisceration were not affected by dietary PS level (P>0.05). Sea cucumber in all the treatments grew gradually with the time of the experiment. Growth variation observed among the treatments and the highest growth was observed in the diet 3. Daily weight gain (g), mean weight gain (g), net yield (kg m^{-3} $year^{-1}$), feed conversion efficiency (%) were significantly different among the treatments (P<0.05). Sea cucumber fed 50 g kg^{-1} PS diet had the highest SGR (%) (0.65±.02. The highest total weight gain was observed in the diet 3 and significantly higher than diet 1 and 4 (P<0.05, Figure 1). Mean weight gain of experimental animals also showed similar trend and significantly higher value was observed in the highest inclusion of PS compared to control. Using PS as an ingredient in the formulated diet in the closed recirculating system, net yield of *A. japonicas* showed that 45.27±4.03 kg could be produced per meter cube water per year and that is significantly higher than that of diet without using PS. Multiple comparisons showed that with the increasing PS level the weight gain was increased significantly (P<0.05).

Table 3 The growth performance and nutrient utilization of *A. japonicas* fed diets with different levels of PS in closed recirculating system for 42 days

Items	Diet 1	Diet 2	Diet 3	Diet 4
Daily weight gain (g day^{-1})	$0.34±0.08^{b}$	$0.51±0.12^{ab}$	$0.62±0.10^{a}$	$0.33±0.08^{b}$
Mean weight gain (g)	$14.08±3.28^{b}$	$21.03±5.32^{ab}$	$26.05±2.32^{a}$	$14.07±3.20^{b}$
FCE (%)	$49.43±10.81^{b}$	$73.07±15.46^{ab}$	$88.59±5.60^{a}$	$51.71±9.10^{b}$
SGR (%)	0.34±0.11	0.54±0.11	0.65±0.02	0.38±0.08
Net yield (kg m^{-3} $Year^{-1}$)	$24.46±5.70^{b}$	$37.01±9.25^{ab}$	$45.27±4.03^{a}$	$14.45±5.56^{b}$

Protein gain (g)	2.00 ± 0.25^{ab}	1.60 ± 0.25^{b}	2.57 ± 0.47^{a}	1.20 ± 0.12^{b}
Protein productive value (%)	20.18 ± 2.93^{ab}	17.85 ± 3.29^{ab}	28.95 ± 5.06^{a}	12.38 ± 2.62^{b}
Survival (%)	100	100	100	100

Values are means ± SE (n=3). Values with different superscript within the same row are significantly different (P<0.05).

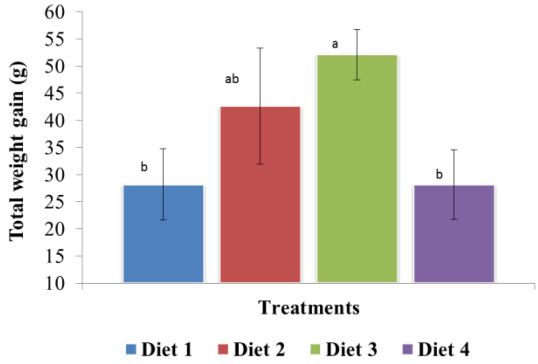

Figure 1 Total weight gain of *A. japonicas* during the experiment. Means (n=3) with different letters indicating significant difference and bar represent standard errors of the mean.

Effects of PS on feed utilization

PS diet showed significant difference in feed utilization. The FCR was lowest in the sea cucumbers feed the highest inclusion of PS in pellet diet (1.24±0.09) compared to the sea cucumber fed without PS diet (2.28±0.49). The feed conversion efficiency, increased as the level of PS was increased (P<0.05). The feed conversion efficiency was found highest in the 30 g kg^{-1} PS diet and significantly higher than the control (Table 3).

Effects of PS on biochemical composition of *A. japonicus*

Proximate composition of the ingredients and diets are shown in the Table 1 and 2 respectively. Protein percentage in PS was 31.1% with the comparatively higher percentage of energy. There were no significant difference between the protein, lipid, carbohydrate and ash content of four experimental diets. The average percentage of protein, lipid, carbohydrate and energy content of experimental diets was 35.03±0.32, 3.13±0.03, 43.98±0.69 and 1440.25±10.18 (KJ) respectively. The biochemical composition of *A. japonicus* in different treatments are presented in Table 4. Protein, lipid, carbohydrate and energy content in the body were significantly different among the treatments (*P*<0.05). Significantly higher protein and lipid percentage was observed in the higher inclusion of PS (diet 3) compared to control (*P*<0.05) and lower percentage of carbohydrate was found in diet 3 (*P*<0.05). The moisture contents of sea cucumber in all the treatments were higher than 91%, and were not different between the treatments (*P*>0.05). The energy contents was the highest in the diet 3 compare to other treatment and was significantly different between treatment (*P*<0.05).

Table 4 The biochemical compositions (%) of whole body in *A. japonicus* fed test diets in closed recirculating system

Items	Diet 1	Diet 2	Diet 3	Diet 4
Protein	3.5±40.3^{ab}	3.1±0.3^{ab}	4.1±0.5^{a}	2.60±0.2^{b}
Lipid	0.9±0.2^{b}	0.4±0.1^{b}	0.8±0.2^{a}	0.7±.0.2^{b}
Carbohydrate	0.7±0.2^{b}	0.7±0.2^{b}	0.4±0.1^{b}	1.5±0.3^{a}
Ash	2.9±0.3	2.9±0.7	2.9±0.9	3.0±0.1
Moisture	92.3±0.3	92.9±0.2	91.8±0.5	92.3±0.3
Energy (KJ)	96.3±4.9^{a}	79.0±0.0^{b}	104.7±4.3^{a}	93.3±0.7.6^{ab}

Values are means ± SE (n=3). Values with different superscript within the same row are significantly different (*P*<0.05).

Protein efficiency ratio, total protein gain (g) and protein productive value (PPV) of sea cucumber showed that, diet 3 was significantly higher than the diet 4 (control). The protein efficiency ratio was increased with increasing percentage of PS (Figure 1). The highest was observed in diet 3 and different than diet 1 and 4 (*P*<0.05). The highest PPV (%) value of 28.95±5.06 was found in the highest inclusion of PS and lowest (12.38±2.62) was observed in control. Indicating that formulated pellet diet with PS showed higher percentage of protein compared to non-PS diet. Fatty acid composition of whole body of *A. japonicas* are shown in Table 5. Total fatty acid analysis showed that a higher percentage of polyunsaturated fatty acids were found in the higher inclusion level of PS diet. The ratio of n-3 and n-6 was found higher in the diet 3 compare to other treatment and lower value of SFA/PUFA was observed in the same treatment.

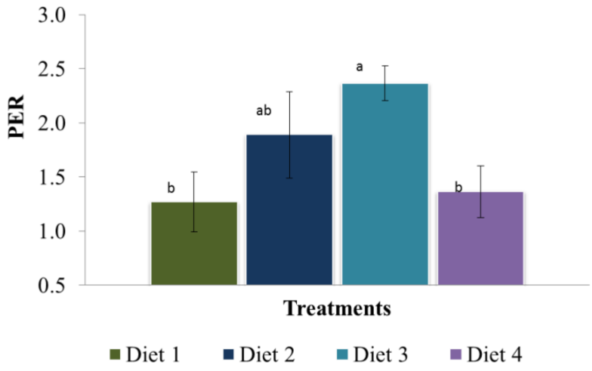

Figure 2 Protein efficiency ration (PER) of *A. japonicas* during the experiment. Means (n=3) with different letters indicating significant difference and bar represent standard errors of the mean.

Table 5 Major fatty acid composition of *A. japonicus* fed diets with different levels of PS in closed recirculating system for 42 days

Items	Diet 1	Diet 2	Diet 3	Diet 4
ΣSaturates	35.10	36.44	27.82	33.64
ΣMonoenes	44.18	37.46	33.39	38.10
ΣPolyenes	17.03	22.02	35.25	21.30
Others	3.69	4.08	3.55	6.96
SFA/PUFA	2.06	1.66	0.79	1.58
Σn-3/Σn-6	0.21	0.26	1.27	0.39

Effects of PS diets on water quality parameters

Weekly water quality parameters are presented in Table 6. The average dissolved oxygen and pH values in the recirculating system were 8.43±0.19 mg L^{-1} and 8.07±0.03 respectively. The NH_3-N was within the range and decreased with the time of the experiment, the average value was 0.45±0.13 mg L^{-1}. NO_2-N value was 0.12 (mg L^{-1}) was increased slowly at the begining and started to decrease hereafter. The NO_3-N level increased gradually with the time of the experiment, indicating that the biofilter system successfully denitrified the toxic water quality parameters to its neutralise form. Phosphorus level was increased might be due to the increasing amount of feed and feces during trials.

Table 6 Water quality parameters in the closed recirculating system during the experimental period of 42 days

Parameters	1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week	Average
DO (*P*pm)	9.20	8.70	8.00	8.00	8.20	8.5	8.43±0.19
pH	8.00	8.10	8.10	8.20	8.00	8.0	8.07±0.03
NH_3-N (mg L^{-1})	0.97	0.64	0.58	0.15	0.3	0.2	0.45±0.13
Phosphorus (mg L^{-1})	0.07	0.20	0.34	0.16	1.05	1.5	0.55±0.24
NO_2-N (mg L^{-1})	0.12	0.14	0.80	0.94	1.05	0.8	0.64±0.17
NO_3-N (mg L^{-1})	1.90	2.80	2.40	4.20	3.50	8.3	3.90±0.95

Effects of PS on the energy budget of *A. japonicus*

Energy allocations for the test *A. japonicus* are presented in Table 7. The energy budget of *A. japonicus* fed diets with PS showed significant difference among the treatments ($P<0.05$). The percentage energy lost in as by the animals was the highest (42.17±1.4 to 55.33±3.3%) followed by the energy lost in respiration (36.16±3.7 to 50.53±0.7%). The energy lost in feces and respiration accounted for the most proportions of the energy intake. The energy deposited as growth was only 2.2±1.3 to 8.2±0.6%. The significantly higher percentage of energy for growth was observed in diet 3 compared to all other treatments ($P<0.05$) and lowest was observed in the control.

Table 7 Allocation of energy of *A japonicus* fed diets with different levels of PS in closed recirculating system for 42 days

Items	Diet 1	Diet 2	Diet 3	Diet 4
F (% C^{-1})	50.97 ± 4.81^{ab}	55.33 ± 3.30^{b}	51.83 ± 0.78^{ab}	42.17 ± 1.41^{a}
G (% C-1)	3.60 ± 0.42^{b}	4.27 ± 1.58^{b}	8.20 ± 0.58^{a}	2.17 ± 1.34^{b}
U (% C-1)	$4.67\pm.013^{b}$	4.30 ± 0.62^{ab}	2.73 ± 0.57^{a}	5.00 ± 0.55^{b}
R (% C-1)	40.80 ± 4.46^{a}	36.17 ± 3.76^{a}	37.20 ± 1.10^{a}	50.63 ± 0.73^{b}

Values are means ± SE (n=3). Values with different superscript within the same row are significantly different ($P<0.05$).

Discussion

Growth performance

Systematic studies of the use of algae as an ingredient in commercial feeds for sea cucumber have been few. Different types of seaweeds have used to study the nutrition requirements of sea cucumber such as *Sargassum polycystum*, *Laminaria japonica*, *Sargassum thunbergii*, *Ulva lactuca*, *Zostera marina*, *Spirulina platensis* and *Undaria pinnatifida* (Liu, Dong, Tian, Wang , & Gao, 2009; Lui, Dong, Tian, Wang, & Gao, 2010; Seo & Lee, 2011; Slater, Jeffs, & Carton, 2009; Yuan, Yang, Zhou, Mao, Zhang, & Liu, 2006; Seo, Shin, & Lee, 2011b; Xia S. , Yang, Li, Liu, Zhou, & Zhang, 2012). Most of the researchers have used *S. thunbergii*, that have been considered to be optimum feed in land-based intensive culture system (Zhu, et al., 2007; Slater, Jeffs, & Carton, 2009) become expensive for aquaculture operation. To find out a cheaper source of ingredient to replace *S. thunbergii* is one of the main challenges. The results of this study show that PS diets showed significantly higher growth rate compare to the control and the inclusion of the PS in diets did not show any negative effect on growth performance and survival. Growth was increased with the increasing level PS in the diet. Using powder of brown seaweed *S. thunbergii* with sea mud in semi intensive farm showed the SGR (%) of 0.49±0.06 (Wu, et al., 2015) and 0.48±0.1 (Xia S. , et al., 2013). In the present experiment the SGR (%) was higher (0.65±0.02) than the brown seaweed. It was observed in another experiment that using algae powder with different ingredients including fish meal indicated that, extruded pellet diet showed higher SGR (%) and weight gain (%) compared to mesh (Xia S. , et al., 2013; Seo, Shin, & Lee, 2011a). We also used the extruded pellet diet as extrusion process improved the nutritional quality, palatability, pellet durability and pellet storage life (Barrows & Hardy, 2000). Sea cucumber fed the extruded pellet diet deposited much more energy for growth and lost less energy in feces and excretion compare to animal fed different form of diets. The animal fed mesh diet showed poorer energy deposition in growth (Xia S. , Yang, Li, Xu, & Rajkumar, 2013). It was also reported that processing method leads to the chemical and physic-chemical changes in the raw materials, which may impact the digestibility of nutrients (Xia S. , et al., 2013) Disruption of native protein structures by exposing enzyme-access sites, improves the protein digestibility (Camire, 2001; Barrows, Stone, & Hardy, 2007). Enzymatic treatments of *Pyropia* to prepare spheroplasts break down the complex polysaccharides in the simple form and made it suitable for absorption by the animal. Therefore the total weight gain, mean weight gain and SGR were found higher compared to the diet without PS. As an alternative of brown seaweed, fermented macro-algae powder (Yuan, Yang, Zhou, Mao, Zhang, & Liu, 2006) and distillers dried grain (Seo, Shin, & Lee, 2011b) was also suggested. During the fermentation process the microorganisms and their enzymes can improve nutritional quality of the substrates by degrading certain complex compounds into some digestible ones which can be effectively absorbed by animals (Jones, 1988; Wee, 1991). As *A. japonicus* has low digestive efficiency, that is why cell wall degraded single cell *Pyropia yezoensis* might be the best choice in the formulating diets.

Results of feeding polysaccharide to *A. japonicus* showed the improved growth and immunity against *Vibrio splendidus* (Bai, Zhang, Mai, Gu, & Xu, 2013). Moreover, the mannose binding lectin (MBL) that are known to activate the lectin pathway and which is thought to be important for host defence and were associated with phagocytosis in echinoderms (Bulgakov, Nazarenko, Petrova, Eliseikina, Vakhrusheva, & Zubkov, 2000) has already been isolated from *A. japonicus* (Bulgakov, et al., 2007).

Degradation of the cell wall of lavers improves the efficiency of using polysaccharide by the animal that might improve the growth rate compared to control. Low level of *Pyropia yezoensis* was reported to lead to improved growth performance of red sea bream (Mustafa, Wakamatsu, Takeda, Umino, & Nakagawa, 1995). The use of 50 g kg^{-1} PS in the diets of red sea bream *Pagrus major* did not show any negative effects (Kalla, Yoshimatsu, Araki, Zhang, Yamamoto, & Sakamoto, 2008) and 30 g kg^{-1} PS showed better performance in black sea bream *Acanthopagru schlegeli* (Khan, Yoshimatsu, Kalla, Araki, & Sakamoto, 2008). Better feed utilization was reported by using PS 50 g kg^{-1} for Nile tilapia *Oreochromis niloticus*

(Shahabuddin, et al., 2015) and 20 g kg^{-1} for short neck clam *Ruditapes philippinarum* (Shahabuddin, Khan, Arisman, Saha, Yoshimatsu, & Araki, 2015).

Feed utilization

In our experiment the FCE (%) was the highest (88.59±5.60) in the PS 50 g kg^{-1} whereas, lowest FCE value was observed in the 10 50 g kg^{-1}. FCE value increased with increasing level of PS. FCR value was recorded 1.24±0.09 which is the lowest among all other treatments. FCR of formulating a pellet diet with algae powder for the juvenile in the semi intensive culture system showed the value of 1.21±0.02 (Xia S. , et al., 2013) which is similar to the present experiment. Experiment with moist pellets of formulating the diet of fish meal and brown seaweed, showed the FCR value 1.20±0.07 (Seo, Shin, & Lee, 2011a). Higher FCR value was recorded using the brown seaweed with sea mud diet for juvenile sea cucumber (Xia S. , Yang, Li, Liu, Zhou, & Zhang, 2012; Wu, et al., 2015). The nutritional composition, taste and palatability of feed ingredients can influence feed intake and feed efficiency. The results of this study showed that PS mixed with soybean, rice powder, rice bran and white fish meal were useful ingredients to formulating sea cucumber diets.

Biochemical composition

Protein is the most expensive dietary ingredients, with a direct impact on growth performance. Nevertheless, information of possible dietary protein sources for *A. japonicus* is still limited. Although the land based culture system of the species has been established, however, proper diets have not been established due to the lack of information on protein (Seo & Lee, 2011). Optimum dietary protein level for maximum growth performances has been identified for aquaculture species (Lovell, 1989). In the present study protein content in the diet was maintained around 35% with lower lipid level (3.1%) as they require less amount of dietary fat. Study of the different protein sources in formulating diets on growth and body composition was maintained crude protein level 30% (Seo, Shin, & Lee, 2011a) and optimum dietary lipid levels 2% (Seo & Lee, 2011), suggested that a mixture of ingredients could be an effective diet for sea cucumber. In addition to this adequate level of carbohydrate in the diet is also important to reduce the catabolism of protein for energy. The dietary carbohydrate levels in the diets were 42.7 to 45.4 %. Previous research mentioned that formulated diet containing 43 to 60% carbohydrate is recommended for juvenile *A. japonicas* (Choi, Seo, & Lee, 2009). The EPA level was increased with the increasing percentage of PS and the highest was observed in 50 g kg^{-1} PS diet (Diet 3). Marine species generally require EPA for normal growth and development (Kim, Lee, Park, Bai, & Lee, 2002).

The observed Protein, lipid, ash and high moisture (91.8-92.8%) concur with the result of *A. japonicas* fed diet with brown seaweed (Seo, Shin, & Lee, 2011b; Seo, Choi, & Lee, 2010). No significant difference in body moisture and ash content was observed, however, significantly higher protein and lipid was observed in the 50 g kg^{-1} compared to control, might be due to the better utilization of feed, as the seaweed polysaccharide was broken down to single cell for easy absorption by the animal. Higher percentage of crude protein and crude lipid were also reported in the body muscle of red sea bream, black sea bream and Japanese short-neck clam fed diets with PS (Kalla, Yoshimatsu, Araki, Zhang, Yamamoto, & Sakamoto, 2008; Khan, Yoshimatsu, Kalla, Araki, & Sakamoto, 2008; Shahabuddin, Khan, Arisman, Saha, Yoshimatsu, & Araki, 2015).

Energy budget

Higher percentage of energy lost in feces would result in a lower percentage of energy deposition in the growth of *A. japonicus.* The energy budget in sea cucumbers fed diets 50 g kg^{-1} PS was 100C=51.9F +8.2G +2.7U+37.2R. The result is similar to the sea cucumbers fed diets with algae powder *Laminaria japonica*, *Sargassum thunbergii* and *Sargassum* sp. The energy budget of the algae powdered diet was 100C=67.1F+ 5.5G+2.7U+24.6R (Yuan, Yang, Zhou, Mao, Zhang, & Liu, 2006). Holothurians generally allocate lower energy in the growth and higher in the feces (Hu, 2004; Yuan, Yang, Wang, Zhou, & Gabr, 2010). Moreover, sea cucumber does not have any specialized organ for physical grind and no specialized gland for chemical digestion (Massin, 1982), in addition to the low enzyme activity in the digestive track allow them to lose more energy in feces (Cui, Dong, & Lu, 2000). In the present experiment the energy for growth was comparatively higher than the previous experiment indicating the better utilization of feed due to the breakdown of algal cell walls.

Conclusion

The results of the experiment suggest that dietary inclusion of enzyme treated *Pyropia* spheroplasts prepared from discarded Nori at a level of 50 g kg^{-1} could improve body weight and nutrient utilization of sea cucumber. These results suggest that PS can effectively be included in the diets for *A. japonicas* up to 50 g kg^{-1} without any negative effects as a low cost feed ingredient. Future studies should focus on the digestibility analysis of protein and lipid, as well as with higher inclusion of PS. The effects of PS supplementation should also be tested in different important warm and cold, fresh and seawater species commonly produced in aquaculture.

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