Isolation, Culture, and Application of Marine Microalga *Dunaliella salina* (Volvocales, Chlorophyceae) as an Aqua Feed Additive

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Introduction

Microalgae are microscopic unicellular organisms capable to convert solar energy to chemical energy via photosynthesis. They contain numerous bioactive compounds that can be harnessed for commercial use. The potential of microalgal photosynthesis for the production of valuable compounds or for energetic use is widely recognized due to their more efficient utilization of sunlight energy as compared with higher plants. Microalgae can be used to produce a wide range of metabolites such as proteins, lipids, carbohydrates, carotenoids, or vitamins for health, food and feed additives, cosmetics, and energy production (Adams et al. 2009). However, microalgal biotechnology only really began to develop in the middle of the last century. Nowadays, there are numerous commercial applications of microalgae have been identified for example microalgae can be used to enhance the nutritional value of food and animal feed owing to their chemical composition; they play a crucial role in aquaculture. Moreover, they are cultivated as a source of highly valuable molecules. For example, polyunsaturated fatty acid oils are added to infant formulas and nutritional supplements, and pigments are important as aqua feed additive.

Microalgae have three fundamental attributes that can be converted into technical and commercial advantages. They are genetically a diverse group of organisms with a wide range of physiological and biochemical characteristics; thus they naturally produce many different and unusual fats, sugars, bioactive compounds, etc. In recent years, microalgae apart from being used as single-cell proteins, they are projected as living-cell factories for the production of bio-fuels and various beneficial biochemicals used in food, aquaculture, poultry and pharmaceutical industries due to presence of different useful compounds. Nowadays, they are consumed throughout the world for their nutritional value. Some of the most biotechnologically relevant

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microalgae are green algae (Chlorophycea) *Chlorella vulgaris, Haematococcus pluvialis, Dunaliella salina*, and the cyanobacteria *Spirulina maxima* which are widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives (Priyadarshani and Biswajit 2012).

Microalgae and Aqua Feed

Microalgal pigment has commercial uses as a natural food coloring and cosmetic ingredient. Some microalgae contain substantial amounts of carotene (besides beta-carotene). Other types of coloring appear in microalgae as well. Beta-carotene is used as a food coloring (with a major application in providing the yellow color to margarine) and as a food additive to enhance the color of the flesh of fish and the yolk of eggs and is used to improve the health and fertility of grain-fed cattle (Borowitzka and Borowitzka 1987). *Dunaliella salina* is grown for a source of the photosynthetic pigment, beta-carotene. Beta-carotene is used as an orange dye and as a vitamin C supplement. Algae are a nutritionally good fish food. Besides the high levels of protein, lipids, and carbohydrates, it contains appreciable amounts of valuable vitamins.

In India, the culture of Pacific white shrimp *Litopenaeus vannamei* which is an exotic species has been practiced intensively. Fishmeal is the most important ingredient of balanced feed formulas used in aquaculture. Fishmeal is dry brown colored flour obtained after cooking, pressing, drying, and milling whole fish trimmings. Fishmeal provides an excellent source of highly digestible protein, beneficial fatty acids, and essential vitamins and minerals. Fishmeal production has not risen recently because catch of wild fish has flattened. This has constrained the growth of aquaculture as an industry worldwide.

Fishmeal is considered as an essential ingredient in marine shrimp diets because of its balanced amount of essential amino acids and fatty acids, vitamins, minerals, and palatability (Suarez et al. 2009). Tacon and Metian (2008) reported that the aquaculture industry consumed 68.2% of global fishmeal production in 2006; however, fishmeal production has remained relatively constant since 1985 at about 7 million tons per year (IFFO 2006). The steady growth of aquaculture and consequent increase in demand for fishmeal has caused a significant increase in fishmeal prices in the last decade (Duarte et al. 2009; FAO 2009). It has led to an increase in feed prices reducing the use of this ingredient in diets for animals (Naylor et al. 2009). Hence, all over the world, aquaculturists, aqua feed formulators, and researchers are trying to replace the fishmeal with cost-effective and nutritionally appropriate alternative feed ingredients. Microalgal meal is considered as one of the promising alternative feed ingredients in aqua feed formulation. This paper has been focused on the methods involving collection, isolation, biochemical analysis, and application of marine microalga *Dunaliella salina* as an aqua feed additive in rearing of shrimp *Litopenaeus vannamei* post larvae.

Materials and Methods

Collection, Isolation, and Stock Culture of Dunaliella salina

Salt pan water samples of green, orange, and red colors were collected in sterile plastic vials from salt pans and screened for *Dunaliella* under compound microscope. The samples contained *Dunaliella* was transferred to De Walne's medium and kept at 24 °C in thermostatically controlled room, illuminated with cool fluorescent lamps at irradiance, under 12 h/12 h light/dark photo period. After 10 days the samples were serially diluted up to 10^{-4} and 0.1 mL spread on 2% De Walne's medium for further investigation. The cyanobacterial contaminants were eliminated by treating them with 3000 ppm of the antibiotic, streptomycin sulfate. The cultures were made axenic by triple antibiotic treatment as described by Droop (1967).

Stock Culture Maintenance

The stock culture was maintained in special air conditioning room. The stock cultures were kept in 1 and 2 litres culture flasks and 5 and 15 liters plastic containers. The seawater was filtered using filter bag (1 μm), the filtered seawater was sterilized by using automated autoclave, and after cooling water was transferred to the culture flasks. The culture flasks were plugged with cotton or covered by aluminum foil. All the vessels used for algal culture were sterilized properly and dried in an oven before use. Conway's medium was used for stock culture (Table 1). For 1 litre of filtered seawater the following nutrients were added:

- Solution A: 1 ml
- Solution B: 0.5 ml
- Solution C: 0.1 ml

About 10 ml of inoculum in the growing phase was transferred to the culture flasks, and culture was provided with 12:12 h light and dark cycle with 5000 lux by using two tube lights. After 10–15 days, the maximum exponential phase was obtained. The temperature and salinity was maintained in the range of 23–25 °C and 25–35 ppt., respectively, for entire culture period. The continuous aeration was provided for culture.

Estimation of Biochemical Composition

The biochemical constituents such as moisture, protein, carbohydrate, lipid, and ash were analyzed in microalga *D. salina* according to the standard procedure as follows:

Table 1 Composition of "Conway's medium" as follows

Composition	Gm/L
Solution – A	
Potassium nitrate	100 gm
Sodium orthophosphate	20 gm
EDTA (Na)	45 gm
Boric acid	33.4 gm
Ferric chloride	1.3 gm
Manganese chloride	0.36 gm
Distilled water	1 l
Solution – B	
Zinc chloride	4.2 gm
Cobalt chloride	4.0 gm
Copper sulfate	4.0 gm
Ammonium molybdate	1.8 gm
Distilled water	1 l
Solution – C	
Vitamin B1 (thiamin)	20 mg in 100 ml dist. water
Vitamin B12 (cyanocobalamine)	10 mg in 100 ml dist. water

Moisture Estimation

For moisture estimation, a known quantity of sample was taken, and the excess moisture was removed using a filter paper (Rajendran 1973). Then the sample was dried in a hot air oven at a constant temperature of 60 °C until the wet sample dried completely. The moisture was estimated by subtracting the dry weight of the sample from the wet weight of the sample. Percentage of moisture content was calculated as follows:

$$\text{Moisture}\,\% = \frac{\text{Wet weight of the sample- Dry weight of the sample}}{\text{Wet weight of the sample}} \times 100$$

Protein Estimation

Dunaliella salina weighing around 1 gm was taken. The algal sample was homogenized with double-distilled water, and the extract was centrifuged at 4000 rpm for 10 min. The extract was dissolved in 4 ml of 1 N NaOH solution. About 5 ml of freshly prepared alkaline copper solution containing 1 ml of 0.5% copper sulfate in 1% potassium tartrate and 50 ml of 20% sodium carbonate in 0.1 N sodium

hydroxide were added to the redissolved extract and allowed to stand for 20 min. 1 N NaOH solution and 0.1% bovine serum albumin served as blank and standard solution, respectively. The color developed was read at 650 nm with UV visible spectrophotometer. Standard was also run simultaneously and based on the OD value; the total protein concentration of the sample was calculated using the following formula:

$$\frac{\text{OD of the Unknown}}{\text{OD of the Known}} \times \text{Concentration of standard} = \text{Concentration of protein present in the Unknown sample}$$

Lipid Estimation

Lipid was estimated by using chloroform-methanol method as described by Folch et al. (1957). 10 mg of dried *D. salina* powder sample was taken in a test tube; 5 ml of chloroform methanol (2:1) mixture was added. The mixture was incubated at room temperature for 24 h after closing the mouth of the test tube with aluminum foil. After the incubation, the mixture was filtered using filter paper. The filter was collected in a 10 ml pre-weighed beaker, which was kept on the hot plate. The chloroform-methanol mixture was evaporated leaving a residue at the bottom of the beaker. The weight of empty beaker was subtracted from the weight of beaker with lipid to estimate the total amount of lipid present in algal sample using the following formula:

$$\text{Lipid}\,\% = \frac{\text{Amount of lipid in the sample}}{\text{Weight of sample taken}} \times 100$$

Carbohydrate Estimation

Carbohydrate was estimated according to the procedure of Dubois et al. (1956). Dried *D. salina* sample weighing 5 mg was homogenized with double-distilled water and centrifuged for 10 min at 3000 rpm. To the supernatant, 1 ml of 5% phenol solution and 5 ml of Con.H_2SO_4 were added, and it is allowed to stand for 30 min, and OD was measured at 490 nm. D-glucose was used as standard. The amount of carbohydrate present in the sample was estimated using the following formula:

$$\text{Carbohydrate}\,\% = \frac{\text{Standard value} \times \text{OD of the sample}}{\text{Weight of sample taken}} \times 100$$

Ash Estimation

Ash content in *D. salina* was estimated according to the method of AOAC (1995). Two gram of dried algal sample was added to a pre-weighed crucible, kept in furnace at 400 °C for 4 h, cooled in desiccators, and reweighed.

HPTLC Analysis of Samples for Amino Acids

Sample Digestion

The 100 mg of *D. salina* sample was weighed in an electronic balance and transferred into labeled glass test tubes. 1 ml of 6 M hydrochloric acid solution was added with sample in specified test tubes. These test tubes were sealed at the top under vacuum by high temperature gas flame, conducted triplicates of samples. All the sealed tubes were kept in a hot air oven at 110 °C for 48 h continuously.

Test Solution Preparation

After completion of digestion process, the top of the tubes were broken, available gident in the tubes was tranfered to glass beaker and the tubes contains digest was rinsed five times with distilled water. The acid in the digest was evaporated to core dry under vacuum using Roto-vac evaporator. The residual content was dissolved with distilled water and make up to 2.4 ml in a centrifuge tubes. This solution contains 41.6 µg raw sample in 1 µl distilled water and used as test solution for amino acid profile analysis by HPTLC technique.

HPTLC Analysis

Standard Amino Acid Solution Preparation

All the standard 20 amino acids were classified into 4 groups according to their R_f values to avoid merging of individual amino acids while eluted with mobile phase (Table 2).

Table 2 Amino acid standards

Group I	Group II	Group III	Group IV
Lysine	Proline	Histidine	Glycine
Asparagine	Serine	Arginine	Alanine
Glutamine	Cystine	Aspartic acid	Valine
Glutamic acid	Tyrosine	Threonine	Isoleucine
Methionine	Tryptophan	Leucine	Phenylalanine

Standard Amino Acid Concentration

Group I, Group II, Group III, and Group IV contain 10 mg of each five amino acids dissolved in 10 ml distilled water.

Sample and Standard Amino Acid Loading

1 µl of each test solutions was loaded as 5 mm band in pre-coated silica gel $60F_{254}$ TLC plate (10 cm × 10 cm) using 100 µl Hamilton syringe and CAMAG-LINOMAT 5 instrument. 1 µl of each Group I, II, III, and IV standards was loaded in the plate for analysis as separate track.

Spot Development

The sample loaded plate was kept in TLC twin trough developing chamber with respective mobile phase (amino acids), 20 min for chamber saturation. After chamber saturation the plate was developed in respective mobile phase up to 90 mm.

Photo Documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was documented using photo-documentation chamber (CAMAG-REPROSTAR 3) at visible light, UV 254 nm and UV 366 nm mode.

Derivatization

The plate was sprayed with respective spray reagent (amino acids) and dried at 100 °C in hot air oven. After derivatization, the plate was documented at visible light and UV 366 nm using CAMAG-REPROSTAR 3.

Scanning

Finally, the plate was fixed in scanner stage and scanned at 500 nm using CAMAG-TLC SCANNER 3. The Rf value and peak area of each track were observed for quantification study. The win CATS 1.3.4 version software was used. The concentration of amino acids present in algae was estimated as follows:

Calculations:	
Sample concentration	100 mg raw material in 2.4 ml of distilled water
Loaded volume of test solution	1 µl (41.6 *µg of raw material*)
Individual amino acid content in %	Conc. of amino acid in µg/41.6 µg × 100

GC-MS Analysis of Samples for Fatty Acids

Gas Chromatography

An Agilent 6890 gas chromatograph equipped with a straight deactivated 2 mm direct injector liner and a 15 m Alltech EC-5 column (250 µ I.D., 0.25 µ film thickness) was used to estimate the fatty acid concentration of microalga *D. salina*. A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35 °C, hold for 2 min, and then ramp at 20 °C per minute to 300 °C and hold for 5 min. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode).

Mass Spectrometry

A JEOL GCmate II benchtop double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000[1] software was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High-resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

Mass Spectrometry Library Search

Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software.

HPLC Analysis of Samples for Carotenoid

The HPLC instrument used consisted of a quaternary low-pressure mixing dual piston pump (Waters 600), a photodiode-array detector (Waters 996), and a fluorimeter (Waters 474) set at a detection wavelength of 430 nm for excitation and 670 nm for emission. The HPLC system was coupled with a computer equipped with the Millennium 2010 chromatograph manager software package (Waters-Millipore). The separating column was a 300 × 3.9 mm Nova-pack C18 with 4 μm particles. The carbon load was 7%; the pore size, 60 Å; and the pore volume was 0.30 ml g^{-1}. The injected sample volume was 100 μl, and the flow rate 0.6 ml min^{-1} for 35 min. The solvents and gradients were adapted from Kraay et al. (1992), but with a longer column, as recommended by Waters in case of high number of peaks. Identification and calibration of pigments was performed with, namely, chlorophylls *a* and *b* and β-carotene from Sigma; lutein, astaxanthin, phaeophytin, and neoxanthin from "The International Agency for 14C Determination," VKI, Denmark; and also lutein and zeaxanthin, which were kindly provided by Hoffman-La Roche laboratories. Standards of breakdown products of chlorophylls *a* and *b* were prepared following the procedures given by Brotas and Plante-Cuny (1998) and Plante-Cuny et al. (1993). The absorbance spectra of other algal pigments and their elution order were compared with literature data (Brotas and Plante-Cuny 1998; Jeffrey et al. 1997; Le Bris et al. 1998; Wright et al. 1991). From the total pigments identified, six are calibrated and their concentrations in the samples were estimated. Results were given in $\mu g/g^{-1}$ for dry algal cultures.

Formulation of Shrimp Feed with D. salina

Purchasing of Feed Ingredients

The branched feed ingredients such as soya bean, coconut oil cake, groundnut oil cake, tapioca flour, dry fishmeal, green gram, and egg were purchased from merchants; vitamin, mineral mix, and cod liver oil are obtained from medical shops. These ingredients were air dried and stored at desiccator in laboratory.

Pellet Feed Preparation (Bhavan et al. 2011)

The preparation of pellet feed was made according to the procedure of Bhavan et al. (2011) involving the following procedure:

Grinding

Dry fish, soya bean, coconut oil cake, groundnut oil cake, and green gram were ground well separately using micro pulverizer and sieved using commercial sieve.

Mixing

After grinding, the feed ingredients were weighed out as per the standard formula (proportion of feed shown in Table 1) and put into a mixer and homogenized the feed mix for 15 min, under room temperature.

Steam Cooking

The feed mixers were loaded in the thermostable trays, and trays are kept in streaming cooker. The feed mixers were cooked at 95–100 °C using stream cooker and kept for 5 min and allow the feed mixer to cool at room temperature.

Incorporation of Vitamin Mix, Cod Liver Oil, and Egg

The vitamin mix and cod liver oil was added to the stream cooked feed mix and thoroughly homogenized in a dough mixer. In this feed mix, 10% of distilled water and egg albumin were added and homogenized for another 10 min to bring the final mixture into a paste form.

Pelletization

The feed mix paste was loaded in the manual pelletizer fixed with 3 mm diameter disc, and pellets are collected in an aluminum tray.

Drying

The trays with moist feed was loaded into electrical trays at the temperature between 75 and 80 °C, and the feed was allowed to dry until the moisture content is less than 10%.

Checking the Quality of Feed

The dry feed pellets were physically examined for visual appearance such as uniformity, color, smooth surface, and fragment smell. The pellet feed was subjected to the analyses of proximate composition. The water stability of the pellets was also tested after 24 h of preparation.

Preparation of Experimental Feed

Combination of dry fish, groundnut oil cake, coconut oil cake, and soya bean was powdered in a grinder and was taken in different compositions as shown in Table 3. Along with this, the dried microalga *Dunaliella salina* powder was added in feed mix in known ratio as shown in Table 2. The feed without algae powder was considered as control feed. Tapioca flour and egg albumin were used as a binding agent. These feed ingredients are mixed well and brought into colloidal form. This feed mix paste was made into a pellet using pelletizer. Finally the pellets were dried in hot air oven at 27 °C for 48 h (Plates 1, 2, and 3).

Collection and Acclimatization of Experimental Animal

The PL 10 of *Litopenaeus vannamei* was obtained from the shrimp hatchery with proper aeration and then transported to the laboratory for further experiment.

Maintenance of Shrimp Larvae

During acclimatization, shrimp larvae were fed with control feed without *Dunaliella salina* powder. Culture water was routinely changed 7 days once to maintain a healthy environment for the shrimp apart from providing artificial aeration. This

Table 3 Composition of formulated experimental and control diets

S. No	Composition	G/100 g	
		Experimental feed with *Dunaliella salina*	Control diet without *Dunaliella salina*
1	Dry fishmeal	5	15
2	Groundnut oil cake	25	25
3	Coconut oil cake	10	10
4	Soya bean meal	25	25
5	Green gram	10	10
6	Wheat flour	5	5
7	Tapioca	3	3
8	Egg albumin	2	2
9	Cod liver oil	3	3
10	*Dunaliella salina*	10	–
11	Vitamin mix[a]	1	1
12	Table salt	1	1
	Total	100	100

[a]Becosules capsules
B-Complex forte with vitamin C for therapeutic use

Plate 1 Control feed ingredients

Plate 2 Experimental feed ingredients

Plate 3 Prepared formulated feed

Plate 4 Acclimatization of shrimp *Litopenaeus vannamei* larvae

ensures sufficient oxygen supply for the shrimp and an environment devoid of accumulated metabolic wastes (Plate 4).

Experimental Shrimp Rearing Condition and Feeding Regime

Salinity was maintained at 26‰. PL was stocked in 100 L tanks at 50 larvae/50 liter of filtered seawater and each diet is stocked in each tank. The shrimp larvae were fed twice a day for 21 days. The larvae were collected, measured, and weighed at weekly intervals for 3 weeks. PL was fed two times a day with formulated feed (control and experimental feed) at the rate of 10% of PL body weight. Survival, length, and weight measurements were made on weekly intervals. The mortality rate was assessed daily. The unconsumed feed, if any, was siphoned out 6 h after feeding. Likewise, fecal matter was also siphoned out prior to the next feeding.

Evaluation of Shrimp Larvae Growth Performance and Feed Efficiency Ratio

Growth performance of shrimp larvae was evaluated by individual weights which were taken at every 7 days interval throughout the experiment. The following response variables were determined from the experimental FRP tank:

$$\textbf{Weight gain}\left(\%\right) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Biochemical Analyses

The biochemical compositions, viz., moisture, protein, carbohydrate, lipid, amino acids, fatty acids, ash, and carotenoids, were estimated for both shrimp larvae and formulated feeds according to standard methods as explained previously.

Results

Growth Rate of Dunaliella salina

A maximum growth rate of 0.623 ± 0.035 OD in *D. salina* was obtained on 15th day (Fig. 1).

Pigment Extraction

Dunaliella salina showed maximum Chl "a" (0.273 µg/ml) on 16th day, and minimum value (0.019 µg/ml) was noticed on 1st day. Maximum value of Chl "b" (0.089 µg/ml) was noticed on 16th day, and minimum value (0.015 µg/ml) was noticed on 1st day. Total carotene was found maximum (0.196 µg/ml) on 10th day, and minimum value (0.049 µg/ml) was noticed on 4th day (Fig. 2).

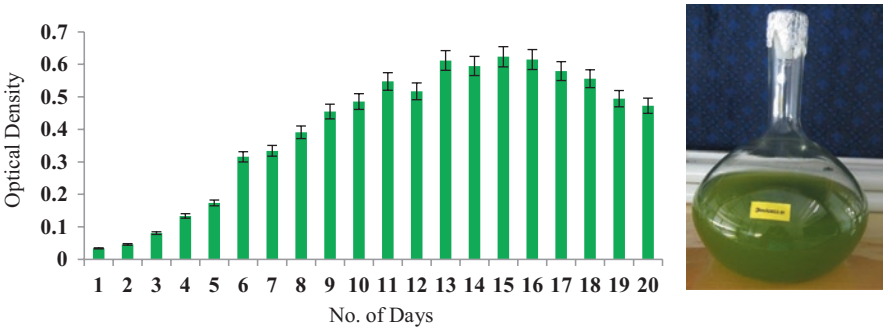

Fig. 1 Daily growth rate of *Dunaliella salina*

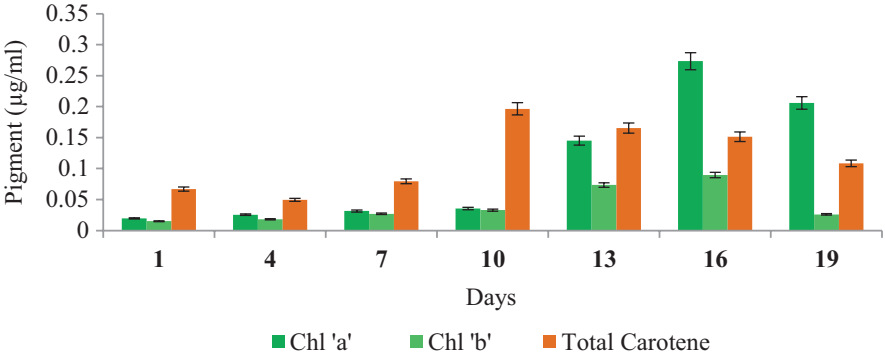

Fig. 2 Pigment extraction from marine microalga *Dunaliella salina*

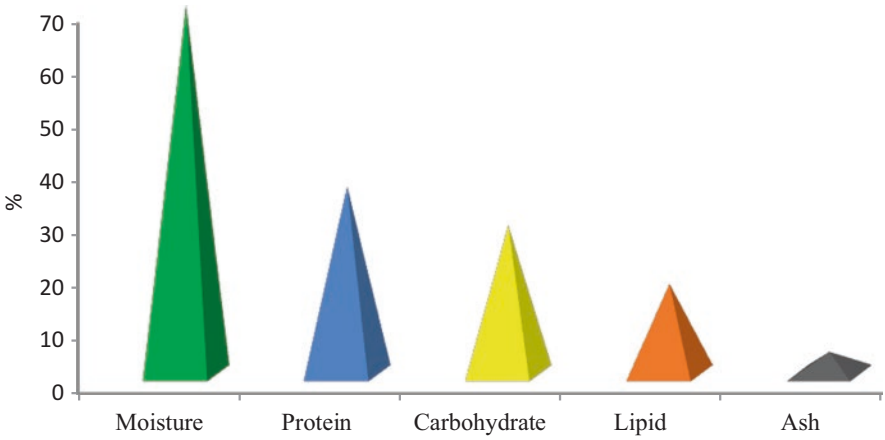

Fig. 3 Biochemical composition of *Dunaliella salina*

Biochemical Composition of D. salina

The biochemical composition showed high moisture content (69.44%). The recorded amount of total protein and carbohydrate in *Dunaliella salina* were 35.35% and 28.15%, respectively. The recorded concentration of lipid and ash were 17% and 4.07%, respectively (Fig. 3).

Amino Acid Profile

In the present study, the profiles of amino acids detected through HPTLC analyses from the marine microalga *Dunaliella salina* are presented in (Table 4). Totally 20 amino acids were detected; among these 9 are essential amino acids (histidine,

Table 4 Amino acid composition of marine microalga *Dunaliella salina*

S. No	Amino acids	*D. salina* %
	Essential amino acids	
1	Histidine	2.11
2	Isoleucine	1.27
3	Leucine	1.65
4	Lysine	2.30
5	Methionine	1.15
6	Phenyl alanine	1.51
7	Threonine	1.61
8	Tryptophan	3.72
9	Valine	1.20
	Nonessential amino acids	
10	Alanine	3.75
11	Arginine	3.75
12	Asparagine	–
13	Aspartic acid	2.11
14	Cystine	1.20
15	Glutamic acid	1.75
16	Glutamine	1.61
17	Glycine	1.75
18	Proline	6.63
19	Serine	–
20	Tyrosine	2.13

(–) denotes trace level

lysine, arginine, asparagine, threonine, isoleucine, tryptophan, leucine, and valine), and remaining 11 are nonessential amino acids (alanine, arginine, asparagines, aspartic acid, glycine, cystine, glutamic acid, glutamine, prolein, tyrosine, and serine). Among the essential amino acids noticed presently, tryptophan, lysine, and histidine were found dominant with 3.72%, 2.30%, and 2.11%, respectively. Similarly among the nonessential amino acids, prolein, alanine, and arginine were found predominant with 6.63%, 3.75%, and 3.75%, respectively.

Fatty Acid Profile

In the present study, the profiles of fatty acids detected through GC-MS analyses from the marine microalga *Dunaliella salina* are presented in Table 5 and Fig. 4. In this study, totally 12 fatty acids were detected, which include both unsaturated (essential) and saturated fatty acids (Table 5). There were ten saturated fatty acids

Table 5 Fatty acid composition of marine microalga *Dunaliella salina*

S.No	FAME formula	Common name	%
1	C11:0	Undecylic acid	0.85
2	C14:0	Myristic acid	1.87
3	C14:1	Myristoleic acid	7.75
4	C15:0	Pentadecylic acid	2.64
5	C15:1	Pentadecenoic acid	2.18
6	C16:0	Palmitic acid	4.29
7	C16:1	Palmitoleic acid	24.15
8	C16:2	Palmitic acid	32.92
9	C16:3	Phthalic acid	11.58
10	C17:0	Margaric acid	1.42
11	C20:0	Arachidic acid	5.97
12	C26:0	Cerotic acid	4.47

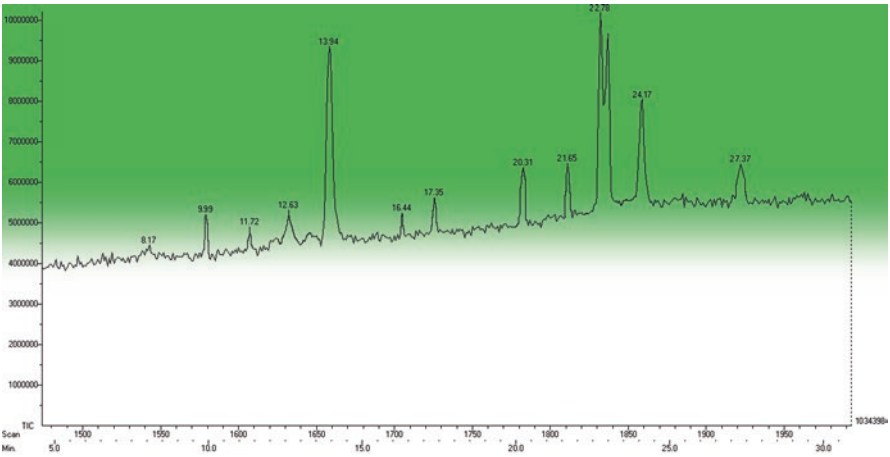

Fig. 4 Chromatogram of marine microalga *Dunaliella salina* showing fatty acid profile

(undecylic acid, myristic acid, pentadecylic acid, pentadecenoic acid, palmitic acid, phthalic acid and margaric acid, cerotic acid, arachidic acid); remaining two were unsaturated fatty acids (myristoleic acid and palmitoleic acid). In general, *Dunaliella salina* contains high level of saturated fatty acids such as C16:0 with 32.92% and C16:3 with 11.58% followed by unsaturated fatty acids C16:1 with 24.15% and C14:1 with 7.75%; other fatty acids are observed in the minimum percentage.

Table 6 Total carotenoids of marine microalga *Dunaliella salina*

Peak	Retention time	Pigments	References
1	3.159	Astaxanthin	Abd El-Baky et al. (2004)
2	5.681	Lutein	Abd El-Baky et al. (2004)
3	8.472	Chlorophyllide a	Wright et al. (1991)
4	12.143	Myxoxanthophyll	Garcia-Plazaola et al. (2012)
5	13.531	β-carotene	Abd El-Baky et al. (2004)
6	16.195	Chlorophyll "a"	Garcia-Plazaola et al. (2012)

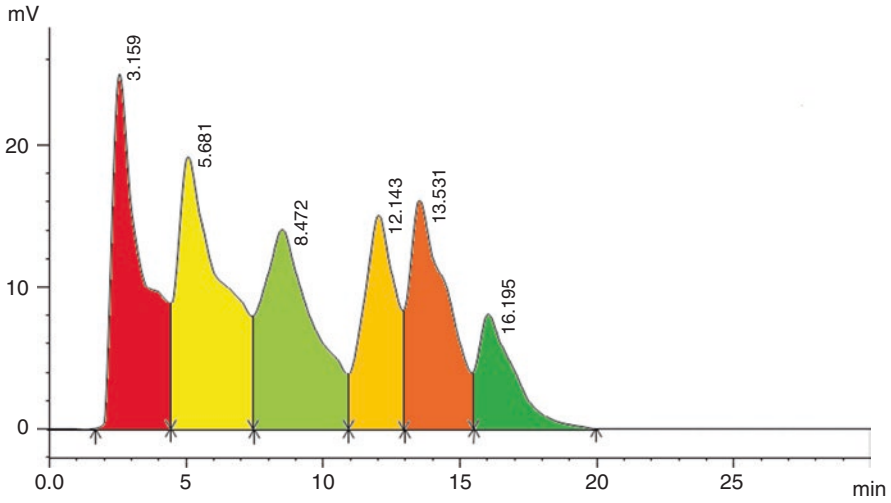

Fig. 5 HPLC profile of carotenoids of *Dunaliella salina*

Total Carotenoids Profile

A total of six pigments including astaxanthin, lutein, chlorophyllide "a," myxoxanthophyll, chlorophyll "a," and β-carotene were identified from the *Dunaliella salina* (Table 6; Fig. 5). The maximum pigment was determined to peak value of chlorophyll "a," β-carotene, and myxoxanthophyll and recorded minimum pigments were astaxanthin, lutein, and chlorophyllide "a."

Measurement of Length in Shrimp L. vannamei

The initial length of shrimp larvae was 1.34 cm. During the experiment trial, the length in control feed was 1.57 cm, 1.59 cm, and 1.6 cm, while in experimental feed, it was 1.58 cm, 1.73 cm, and 2.0 cm on 7th, 14th, and 21st day, respectively. Overall length was found to have increased gradually from 7th to 21st day in both the groups, whereas in experimental feed, higher growth rate was observed (Table 7).

Table 7 Measurement of length, weight, and survival in shrimp *L. vannamei* larvae

	Initial		7th day			14th day			21st day		
Feeding regimes	Length (cm)	Weight (gm)	Length (%)	Weight (gm)	Survival (%)	Length (cm)	Weight (gm)	Survival (%)	Length (cm)	Weight (gm)	Survival (%)
Control feed	1.34	0.001	1.57	0.003	97	1.59	0.013	90	1.6	0.027	88
Experimental feed	1.34	0.001	1.58	0.009	100	1.73	0.029	95	2	0.085	94

Measurement of Weight in Shrimp L. vannamei *Larvae*

The initial weight of shrimp larvae was 0.001 gm. During the experiment trial, the weight in control feed was 0.003 gm, 0.013 gm, and 0.027 gm, while in experimental feed, weight were 0.009 gm, 0.027 gm, and 0.085 gm on 7th, 14th, and 21st day, respectively. Overall weight was found to have increased gradually from 7th to 21st day in both the groups, whereas in experimental feed, maximum weight gain was observed compared to control feed fed animal (Table 7).

Biochemical Composition of Formulated Feeds

The maximum protein (40.01%) and lipid content (19.21%) were observed in experimental feed, while minimum moisture (80.51%), carbohydrate (22.54%), and ash (10.54%) were noticed in control feed (Fig. 6).

Total Carotenoids of Control and Experimental Feeds

The maximum total carotenoids (0.033 µg/gm) were noticed in experimental formulated feed, while the minimum total carotenoids of 0.023 µg/gm were noticed in control feed (Fig. 7).

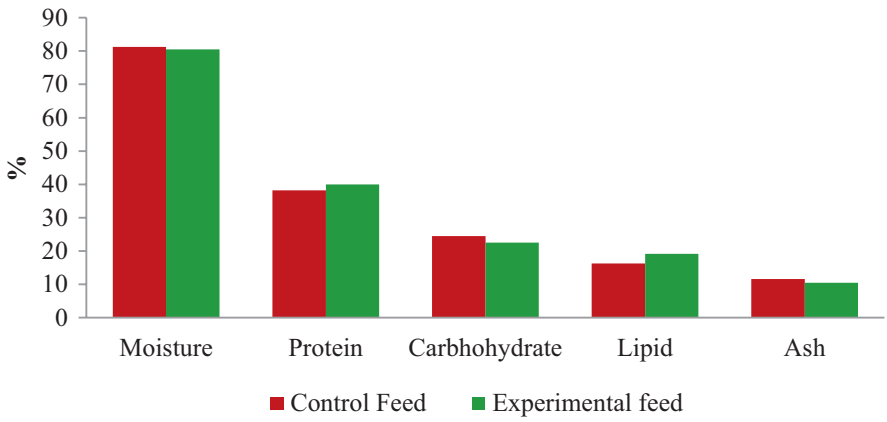

Fig. 6 Biochemical composition of formulated feeds

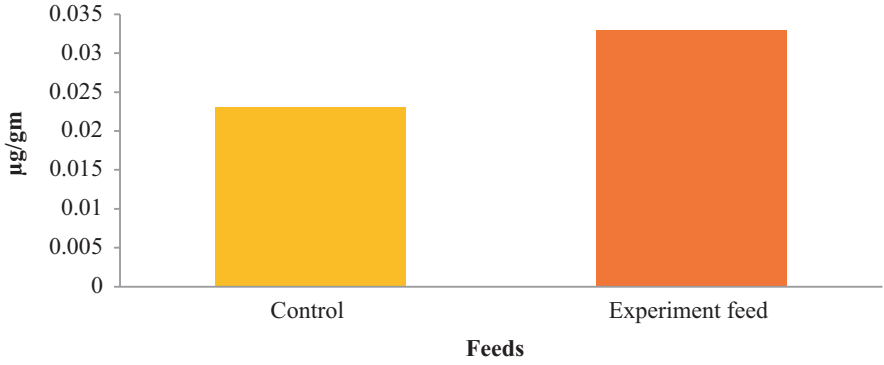

Fig. 7 Total carotenoids of control and experimental feeds

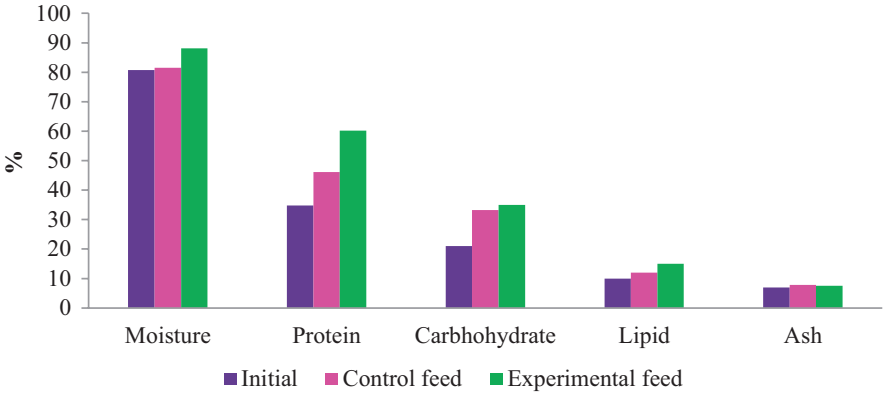

Fig. 8 Biochemical composition of shrimp larvae fed on different formulated feeds

Biochemical Composition of Shrimp Larvae Fed on Formulated Feeds

The recorded moisture, protein, carbohydrate, lipid, and ash contents in experimental feed fed shrimp larvae were 88.13%, 60.2%, 35%, 15%, and 7.5%, respectively, while the moisture, protein, carbohydrate, lipid, and ash contents in control feed fed shrimp larvae were 81.5%, 46.1%, 33.2%, 12%, and 7.8%, respectively (Fig. 8).

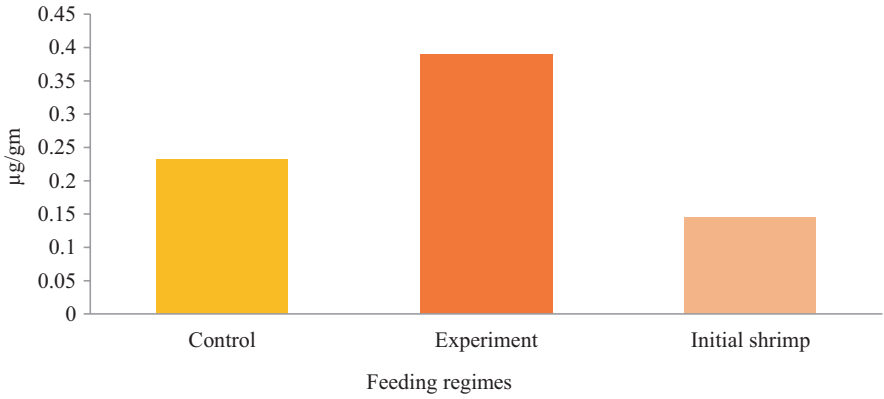

Fig. 9 Total carotenoids of shrimp larvae fed with control and experimental feeds

Total Carotenoid Contents of Shrimp Larvae Fed with Control and Experimental Feed

Total carotenoid contents of initial larvae, experimental feed fed shrimp larvae, and control feed fed shrimp larvae were 0.232, 0.39, and 0.145 µg/gm, respectively (Fig. 9).

HPTLC Amino Acid Analysis

In Feed

HPTLC analysis of various amino acids in formulated feeds is presented in (Table 8). The profile of amino acids detected through HPTLC in different formulated feeds is shown in Fig. 10. In the present study, however, the formulated feeds with alga showed almost equal proportion of various amino acids to that of control feed. The densitogram display of control and experimental feeds and control and experimental feed fed shrimp larva are shown in Fig. 11 HPTLC peak for standard amino acids and formulated feed amino acids are given in Fig. 12.

Table 8 Concentrations of amino acids detected through HPTLC in control and experimental feed and control and experimental feed fed shrimp larvae

Amino acids	Control feed (%)	Experimental feed (%)	Control feed fed shrimp larvae (%)	Experimental feed fed shrimp larvae (%)
Essential amino acids				
Histidine	13.9	12.1	15.9	16.9
Isoleucine	2.59	2.16	2.64	2.69
Leucine	3.22	2.59	3.34	3.10
Lysine	2.62	2.28	2.98	3.17
Methionine	2.35	1.97	2.37	2.42
Phenyl alanine	2.90	2.35	3.02	2.81
Threonine	4.27	3.84	4.13	4.66
Tryptophan	7.13	5.79	7.45	6.92
Valine	3.17	2.86	3.07	4.36
Nonessential amino acids				
Alanine	3.50	3.50	4.37	3.72
Arginine	2.28	1.99	2.69	2.93
Asparagine	–	–	–	–
Aspartic acid	6.11	6.17	7.96	6.56
Cystine	2.11	1.97	2.47	2.11
Glutamic acid	4.71	4.23	4.56	5.12
Glutamine	2.30	2.16	2.69	2.28
Glycine	12.5	10.9	14.9	16.1
Proline	7.45	6.49	8.79	9.51
Serine	–	–	–	–
Tyrosine	4.39	3.65	4.44	4.54

In Experimental Animals

Concentrations of amino acids detected through HPTLC in experimental shrimp are shown in (Table 8). The small difference of certain amino acid levels in the whole body among the dietary treatments may be due to the differences in the tissue levels of free amino acids; this can be in experimental feed fed shrimp larvae showing higher amino acid levels when compared to control fed shrimp larvae.

In the present study, the profiles of amino acids detected through HPTLC analyses from the muscle of the prawns are presented in Table 8. Totally 20 amino acids were detected; among these 9 are essential amino acids (histidine, lysine, arginine, asparagine, threonine, isoleucine, tyrosine, leucine, and valine); remaining 11 are nonessential amino acids (alanine, arginine, asparagines, aspartic acid, glycine, cystine, glutamic acid, glutamine, prolein, tyrosine, and serine). In control formulated feed amino acids such as histidine (13.9%), glycine (12.5%), tryptophan (7.13%), and prolein (7.45%) were found dominant.

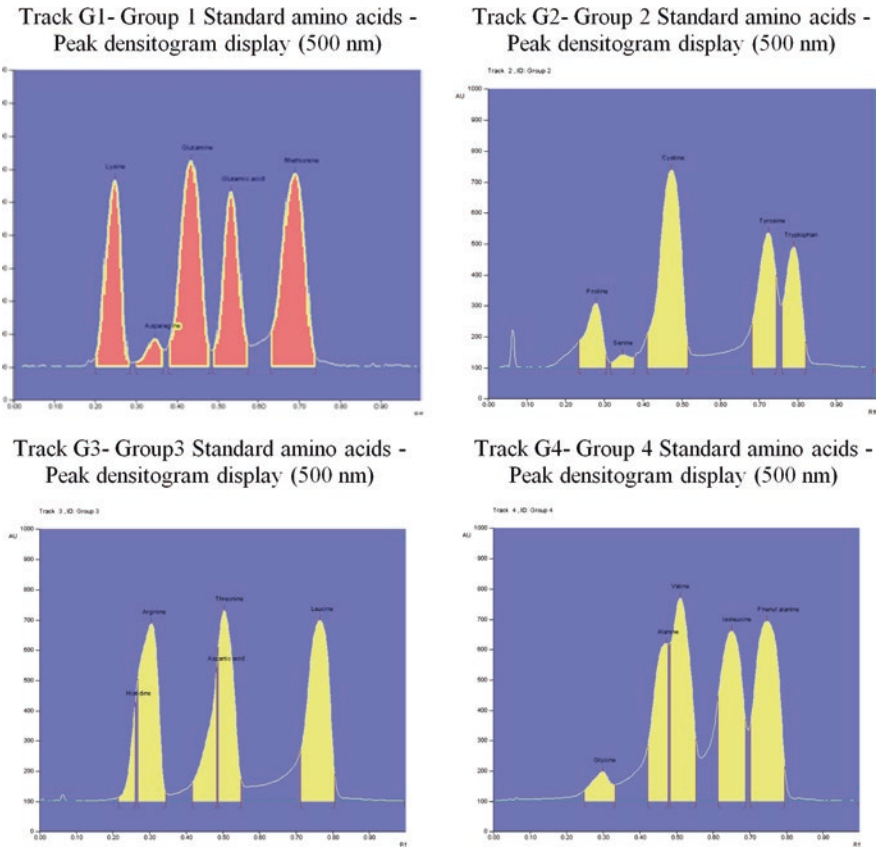

Fig. 10 HPTLC peak densitogram display for standard amino acids. Track G1 – Group 1 Standard amino acids – Peak densitogram display (500 nm). Track G2 – Group 2 Standard amino acids – Peak densitogram display (500 nm). Track G3 – Group3 Standard amino acids – Peak densitogram display (500 nm). Track G4 – Group 4 Standard amino acids – Peak densitogram display (500 nm)

The higher concentrations of amino acids were observed in experimental feed fed shrimp larvae which include histidine (16.9%), glycine (16.1%), and prolein (9.51%), while the lower concentration was noticed in control feed fed shrimp larvae including histidine (15.9%), glycine (14.9%), and prolein (8.79%). The experimental feed fed shrimp larvae showed maximum amino acids than control feed fed larvae.

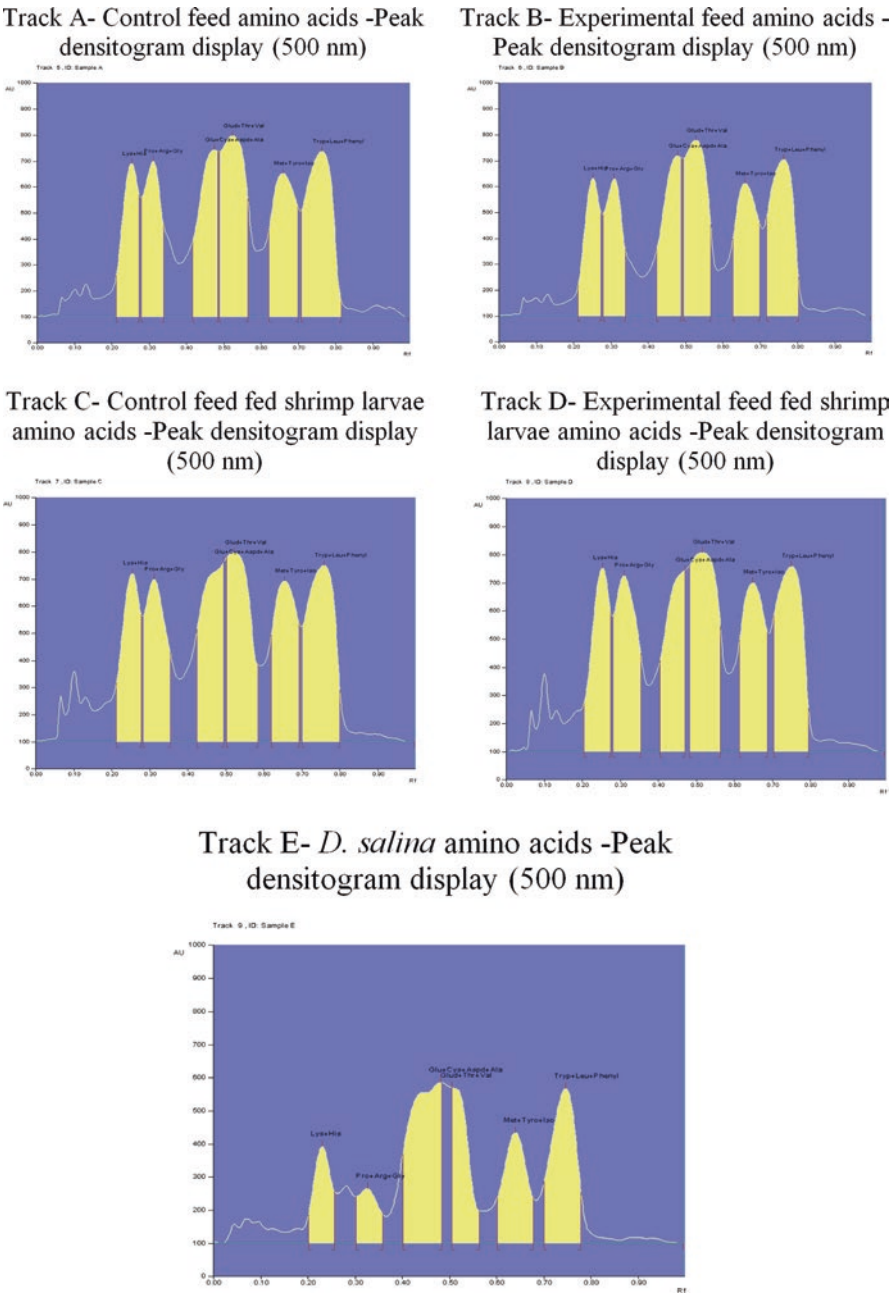

Fig. 11 HPTLC peak densitogram display for control and experimental feeds and control and experimental feed fed shrimp larvae. Track A – Control feed amino acids –Peak densitogram display (500 nm). Track B – Experimental feed amino acids – Peak densitogram display (500 nm). Track C – Control feed fed shrimp larvae amino acids – Peak densitogram display (500 nm). Track D – Experimental feed fed shrimp larvae amino acids – Peak densitogram display (500 nm). Track E – *D. salina* amino acids – Peak densitogram display (500 nm)

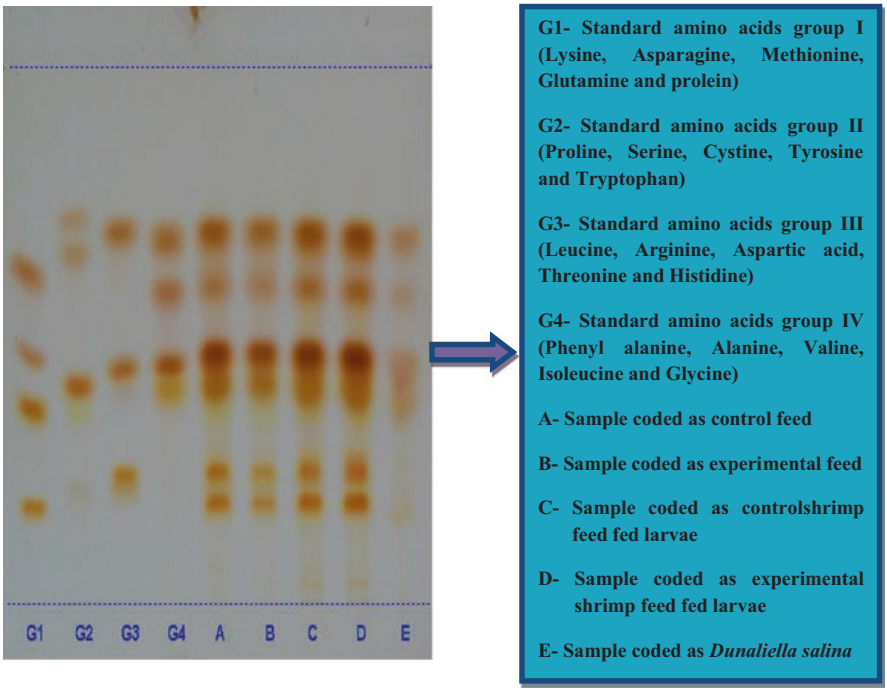

Fig. 12 HPTLC showing amino acids profiles of standard and experiment

Fatty Acid Profile

In the present study, the profiles of fatty acids detected through GC-MS analyses from the marine microalga *Dunaliella salina* are presented in Table 9 and Figs. 13 and 14. In this study, totally 17 fatty acids were detected, which include both unsaturated (essential) and saturated fatty acids (nonessential). There were 14 saturated fatty acids (myristic acid, pentadecylic acid, pentadecanoic acid, palmitic acid, margaric acid, and cerotic acid) which were recorded; remaining three were unsaturated fatty acids (palmitoleic acid, arachidic acid, and docosahexaenoic acid).

Control and experimental feed contains highest level of saturated fatty acids such as C18:0 with 31.69%, C16:1 with 3.19% and C26:0 with 2.41% and C18:0 with 24.75%, C26:0 with 17.89%, and C13:0 with 2.58%, respectively, followed by unsaturated fatty acids which was reported maximum in experimental feed that includes C16:2 with 42.37%. However, control feed showed the low level of unsaturated fatty acids, viz., C16:2 with 0.75% and C15: 0 with 2.12%. The other fatty acids are observed in the minimum percentage.

In the present investigation, experimental feed fed shrimp larvae contain highest level of saturated fatty acids such as C26:0 with 11.24%, C16:1 with 8.79%, and C15 with 8.59%. Similarly control feed fed shrimp larvae resulted the maximum of C26:0 with 7.26%, C14:0 with 6.54%, and C14:1 with 4.92%. The unsaturated fatty

Table 9 Fatty acids detected through HPTLC in control and experimental feed and control and experimental feed fed shrimp larvae

S.No	FAME	Common name	Control feed (%)	Experimental feed (%)	Control feed fed shrimp larvae (%)	Experimental feed fed shrimp larvae (%)
1	C12:0	Lauric acid	–	–	2.42	–
2	C13:0	Tridecylic acid	–	2.58	–	–
3	C14:0	Myristic acid	–	–	–	6.54
4	C14:1	Myristic acid	2.14	2.93	4.21	6.24
5	C14:2	Methyl myristate	2.31	–	–	–
6	C15:0	Pentadactyl acid	–	–	–	3.87
7	C15:1	Pentadecenoic acid	–	3.49	–	8.59
8	C16:0	Palmitic acid	2.12	–	–	–
9	C16:1	Palmitic acid	3.19	3.68	8.62	8.79
10	C16:2	Palmitoleic acid	0.75	42.37	7.59	–
11	C16:3	Methyl palmitoleate	–	–	3.64	–
12	C18:0	Stearic acid	31.69	24.75	–	–
13	C20:0	Arachidic acid	42.01	–	–	–
14	C22:0	Behenic acid	–	–	4.92	–
15	C22:6	Docosahexaenoic acid	–	2.14	61.28	48.62
16	C24:0	Lignoceric acid	–	–	–	5.92
17	C26:0	Cerotic acid	2.41	17.89	7.26	11.24

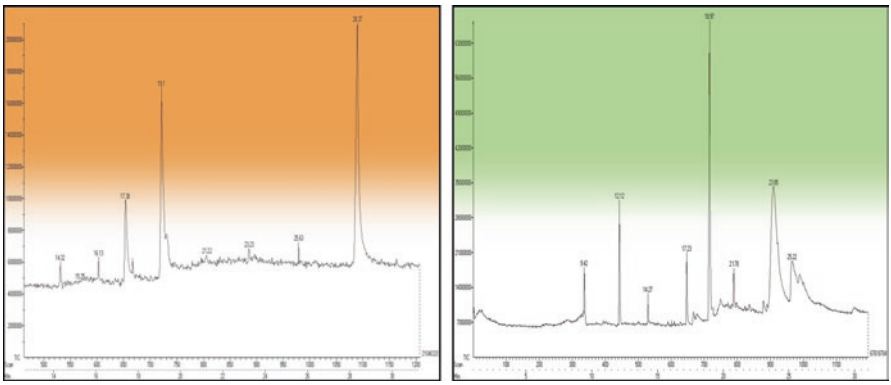

Fig. 13 Chromatogram of control and experimental feed showing fatty acid profile

acids were comparatively higher in control feed fed shrimp larvae (C22:6 with 61.28% DHA) than experimental feed fed shrimp larvae (C22:6 with 48.62% DHA). In general both the saturated and unsaturated fatty acids are comparatively higher in experimental feed and experimental feed fed shrimp larvae than control feed and control feed fed larvae.

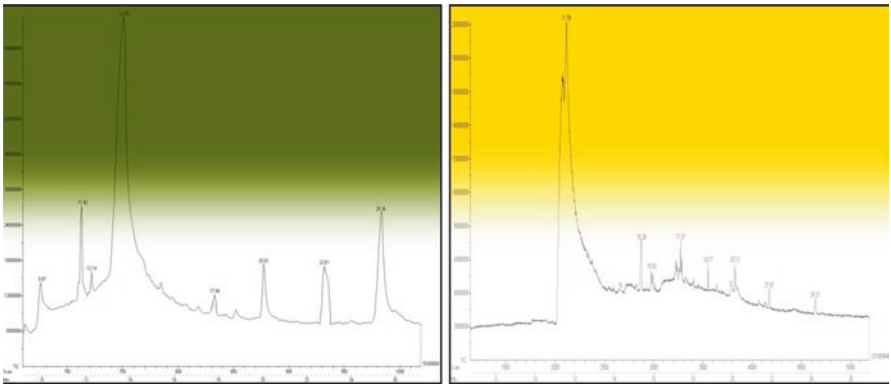

Fig. 14 Chromatogram of control and experimental feed fed shrimp larvae showing fatty acid profile

Table 10 HPLC profile of carotenoids of control and experimental feeds

Peak	Retention time			
	Control feed (%)	Experimental feed (%)	Pigments	References
1	8.428	8.596	Chlorophyllide "a"	Wright et al. (1991)
2	–	13.641	β-carotene	Abd El-Baky et al. (2004)
3	17.209	17.182	Pheophytin "a"	Brotas and Plante-Cuny (2003)

Total Carotenoid Profile

A total of three pigments were identified from the control and experimental feeds (Table 10) including chlorophyllide "a," β-carotene, and pheophytin "a." The maximum pigment was determined to peak value of experimental feed, and minimum pigments were observed in control feed in μg g^{-1} dry biomass (Fig. 15).

A total of five pigments were identified from the control and experimental feed fed shrimp larvae (Table 11) including chlorophyllide "a," lutein, neoxanthin, β-carotene, and pheophytin "a." The maximum pigment was determined to peak value of experimental feed fed shrimp larvae, and minimum pigment was observed in control feed fed shrimp larvae in μg g^{-1} dry biomass (Fig. 16).

Discussion

Algae with high nutritional value have remarkable potential as shrimp feed. *Dunaliella salina* has 90% of β-carotene and 10% of other carotenoids. Carotenoids are made up of α-carotene and xanthophylls like lutein, zeaxanthin, and cryptoxan. This is similar to those found in food and vegetables (Gouveia and Emphis 2003).

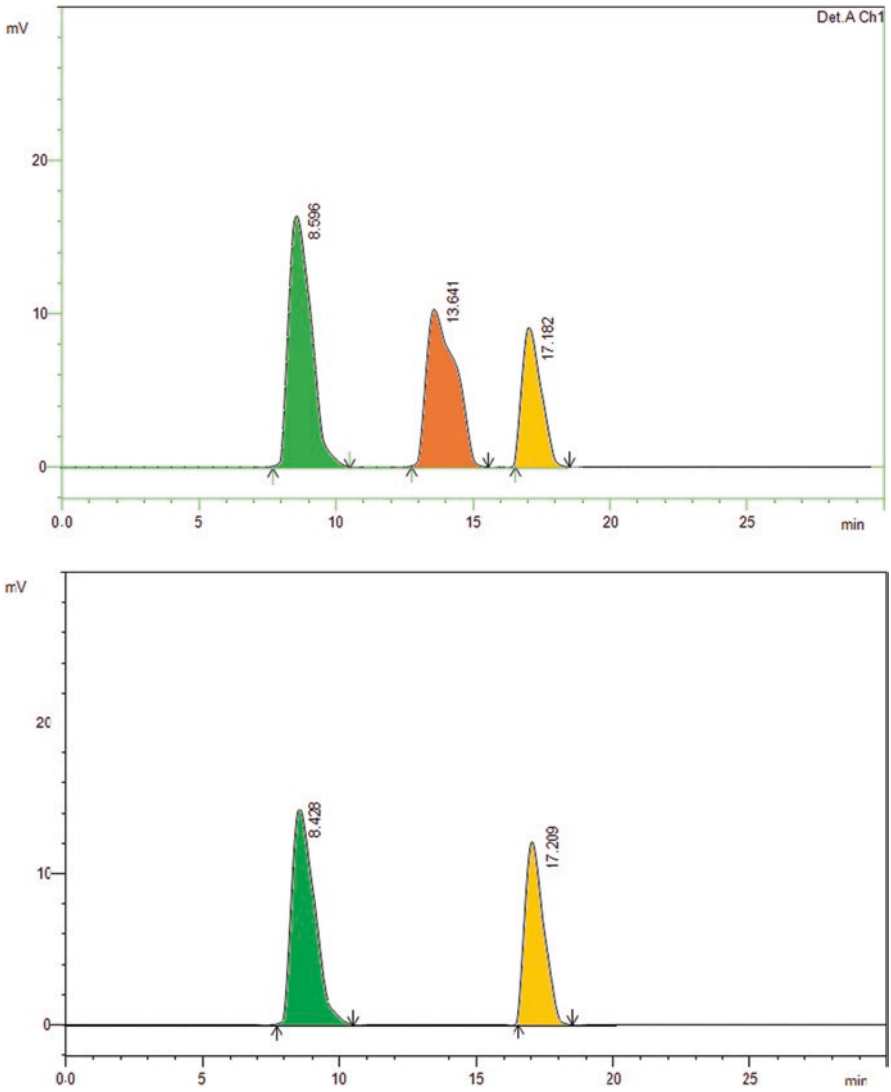

Fig. 15 HPLC profile of carotenoids of control and experimental feeds

Approximately 500 different carotenoids have been identified so far. *Dunaliella salina* which are widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives.

Fishmeal is an excellent source of protein and other essential nutrients to aquaculture species, but it is a limited natural resource. Fishmeal is considered as an essential ingredient in marine shrimp diets because of its balanced amount of essential amino acids, fatty acids, vitamins, minerals, and palatability (Suarez et al. 2009). Tacon and Metian (2008) reported that the aquaculture industry consumed

Table 11 HPLC profile of carotenoids of control and experimental feed fed shrimp larvae

Peak	Retention time		Pigments	References
	Control feed fed shrimp larvae (%)	Experimental feed fed shrimp larvae (%)		
1	5.154	5.225	Lutein	Abd El-Baky et al. (2004)
2	–	8.495	Chlorophyllide "a"	Wright et al. (1991)
3	11.529	11.657	Neoxanthin	Garcia-Plazaola et al. (2012)
4	–	13.562	β-carotene	Abd El-Baky et al. (2004)
5	17.089	17.148	Pheophytin "a"	Brotas and Plante-Cuny (2003)

68.2% of global fishmeal production in 2006. Fishmeal is a rich source of high quality protein, has relatively high-energy content, and is rich in important minerals such as phosphorus, B vitamins, and essential fatty acids. The steady growth of aquaculture and consequent increase in demand for fishmeal has caused a significant increase in fishmeal prices in the last decade (Duarte et al. 2009; FAO 2009). Hence in the present study, microalga *Dunaliella salina* was used as an alternative ingredient in formulated feed to replace the fishmeal partially.

Microalgae like *Spirulina*, *Chlorella*, *Scenedesmus*, *Dunaliella,* and *Nannochloropsis* are widely used as aquaculture feed for their high nutritional value (Venkataraman 1980; Avron and Ben-Amotz 1992; Lee 1997; Yamaguchi 1997). *Spirulina, Dunaliella,* and *Haematococcus* are also used as good sources of antioxidant pigments like carotenoids, lutein, astaxanthin, zeaxanthin, etc. in fish farming mainly for colored fishes (Chiu et al. 2001; Hanaa et al. 2003) for the intracellular protection of fish larvae against different diseases together with the bright coloration of fishes (Trinadha et al. 2003). *Dunaliella salina* food or food additive and a nutritional supplement mean that a high quality product is required. *Dunaliella salina* is also used as a source of natural pigments for the culture of prawns, salmonid fish, and ornamental fish. In the present study, the shrimp group fed with experimental diets showed a higher feed intake rate than that with the control feed during the experimental tenure. This might be due to the attractive color, flavor, and good nutrient composition of the experimental feeds.

The growth of the shrimp larvae depends on the quality of feed. The present study shows that the post larvae responded well to the experimental formulated diets which incorporated with microalga *Dunaliella salina*. The ingredients present in the formulated diets significantly influenced the performance of the juveniles, by resulting increase in final body length and weight. Survival rates obtained in this study with the experimental formulated diets seem to indicate that there were no overwhelming negative effects in utilization of nutrients by the microalga *Dunaliella salina.* Higher survival rate was reported in experimental shrimp larvae which could be due to the growth promoting substances occurred in the microalgae.

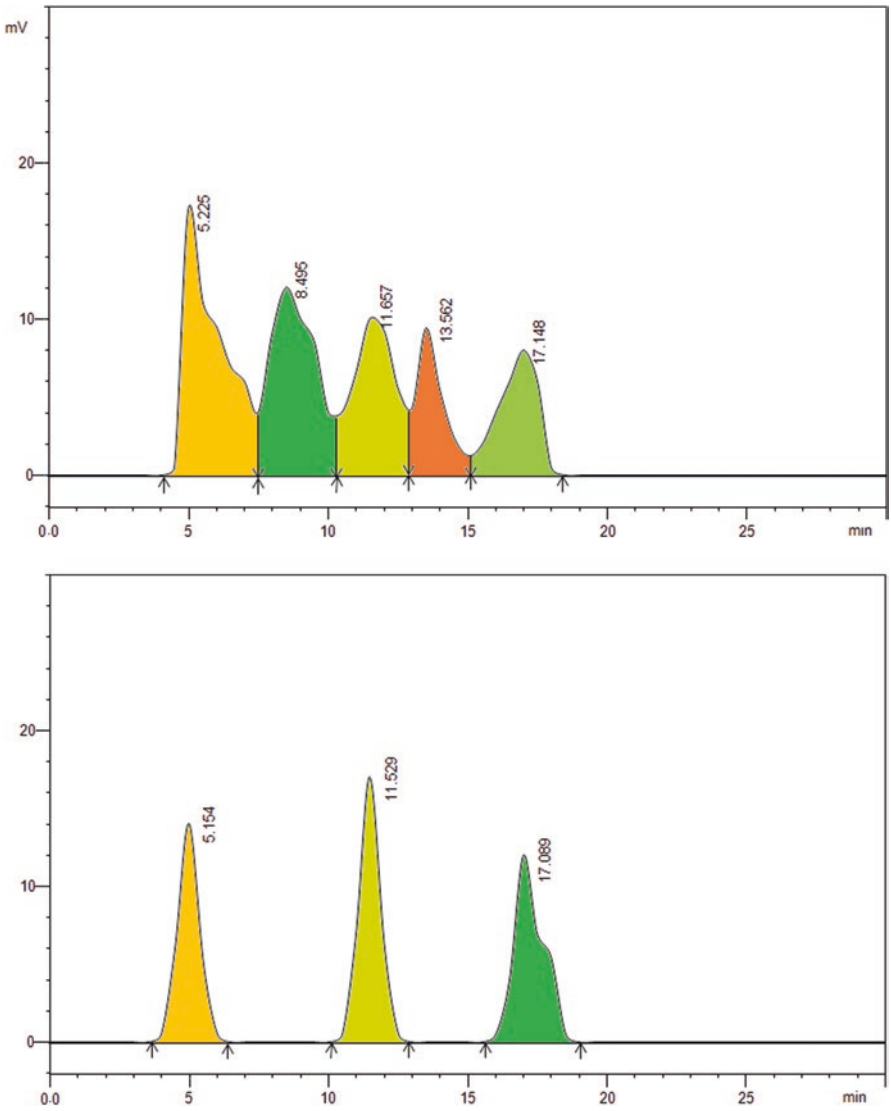

Fig. 16 HPLC profile of carotenoids of control and experimental feed fed shrimp larvae

The overall biochemical profile of the feed indicated the fact that the experimental feed had good nutritive value. As dietary protein is the most important factor affecting growth performance of shrimp (Kureshy and Davis 2002), most shrimp farmers prefer to use high protein feeds, especially in intensive culture systems. Amaya et al. (2007a) suggested that the successes of replacing animal protein in diets with alternative sources are due in part to the ability of shrimp to use natural productivity as a food supplement.

There is hardly any information on the use of microalgae as a dry feed component for shrimps, though there are ongoing efforts to replace fishmeal protein using terrestrial plant proteins. *L. vannamei* has been successfully grown on a predominantly plant protein diet containing solvent-extracted soybean meal, corn gluten meal, and corn fermented soluble, which together accounted for nearly 98% of the total dietary protein of 36% (Amaya et al. 2007a). The same research group has verified the concept of fishmeal-free shrimp feed in a pond trial (Amaya et al. 2007b). Furthermore, beneficial impact of algal inclusion on shrimp health has been reported recently; *L. vannamei* fed diets supplemented with marine algal meals rich in docosahexaenoic acid and arachidonic acid demonstrated significant improvement in immune responses (Nonwachai et al. 2010).

Various species of macroalgae and microalgae have been incorporated into fish feed formulations to assess their nutritional value, and many have been shown to be beneficial: *Chlorella* or *Scenedesmus* fed to Tilapia (Tartiel et al. 2008); *Chlorella* fed to Korean rockfish (Bai 2001); *Undaria* or *Ascophyllum* fed to sea bream (Yone et al. 1986); and *Ascophyllum, Porphyra, Spirulina*, or *Ulva* fed to sea bream (Mustafa and Nakagawa 1995). Often the algae chosen for fish feeding studies appear to have been selected largely for convenience, because they are low-cost and commercially available. For example, microalgae such as *Spirulina, Chlorella,* and *Dunaliella* can be produced by low-cost open pond technologies and are marketed as dry powders, and their nutritional profiles are well-documented.

Amino Acid Profiles

Proteins are made up of chains of amino acids which form the building blocks. They are utilized to form various cell structures and serve as a source of energy. An effective dietary protein source must satisfy an animal's requirement for both essential and nonessential amino acids (Guillaume 1997). Amino acids are required by all fish and shrimp species, and tryptophan is a precursor of the neurotransmitter serotonin (Savelieva, et al. 2008). Valine is involved in many metabolic pathways and is considered indispensable for protein synthesis and optimal growth (Wilson 2002). Histidine is also an indispensable amino acid involved in many metabolic functions including the production of histamines, which take part in allergic and inflammatory reactions. It plays a very important role in maintaining the osmoregulatory process and is related to energy production or is used in other metabolic pathways during certain emergencies/harsh conditions (Abe and Ohmama 1987).

From a diet formulation perspective, it should be noted that some responses to dietary protein source seem to be independent of their amino acid balance. Total protein content of a feed to a point where excessive amounts of many amino acids are included in an attempt to meet the requirement for one or more of the essential amino acids is shortest in supply. A diet should be formulated based on digestible amino acid values of feed ingredients and an ideal protein. *Spirulina* contains an unusually high amount of protein, up to 65% by dry weight, and is a complete

protein, containing all essential amino acids, along with good amounts of essential fatty acids, polysaccharide, phycobiliproteins, carotenoids, vitamins (especially B12), and minerals, making it a desired food source (Hu 2004).

The chemical composition of the experimental diets was equalled in protein and energy and at levels supposed to be optimal for Pacific white shrimp (Amaya et al. 2007a). All diets fed to shrimp in the present study had amino acid values similar to those reported by Suarez et al. (2009) for *L. vannamei*. In the present study, muscle composition of *L. vannamei* showed higher concentrations of histidine (16.9%), glycine (16.1%), and prolein (9.51%) in animal fed with diet supplemented with 10% of *Dunaliella salina*, whereas control feed fed group showed lower level of histidine (15.9%), glycine (14.9%), and prolein (8.79%). In the previous study, muscle tissue composition of *L. vannamei* resulted higher concentrations of arginine, lysine, and methionine in animals fed with diets supplemented with 10 and 40% of *Spirulina platensis*. However, it was found that the methionine content in animals fed with diet supplemented with 10% was lower when compared to the muscle demand values for this shrimp species fed with commercial diet. Whereas the imbalance, even if it is represented by a single essential amino acid, has an immediate effect on meeting the protein needs (Gadelha et al. 2013). As regards the energy balance (Fox et al. 2004), it was observed that the diet containing 40% *S. platensis* provided the best concentrations of amino acids for growth performance in animals.

The free amino acids have been shown to function in osmoregulation (Fang et al. 1992) and also have a major contribution to the flavor of seafoods (Thompson et al. 1980). Animals must consume dietary protein to obtain a continual supply of amino acids. After ingestion, it is digested or hydrolyzed to release free amino acids that are absorbed from the intestinal tract and then distributed to the various organs and tissues. The amino acid composition and concentration in the muscle of prawns may affect the quality of the prawn (Wang et al. 2004). Crustacean muscles contain high concentration of free amino acids, such as arginine, glycine, proline, glutamine, and alanine (Cobb et al. 1975). Amino acids are used by the tissues to synthesize new protein; thus animals do not necessarily require protein but do require the amino acids which comprise proteins. High protein diets are needed for good growth of most aquatic animals (NRC 1993). Hence, estimation of minimum requirement of essential amino acids (EAA) is indispensable to formulate cost-effective diets. The quantitative EAA requirements of fish and crustaceans are often determined by feeding experiments with diets containing graded levels of the particular amino acid to be examined (Wilson 1989). Deshimaru and Shigeno (1972) reported that the amino acid composition of the dietary protein should match of prawn tissue. These studies are all in agreement, indicating that arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are essential in the crustacean diet. Although not strictly required, tyrosine and cysteine should be considered semiessential as their presence in a diet reduces the requirement for phenylalanine and methionine, respectively (Guillaume 1997).

Arginine was proven to be crucial in energy metabolism by maintaining glycolysis under hypoxic conditions (Gade and Grieshaber 1986). Arginine plays an

important role in cell division, the healing of wounds, removing ammonia from the body, immune function, and the release of hormones (Tapiero et al. 2002; Stechmiller et al. 2005; Witte and Barbul 2003). Glutamic acid turned into glutamine, which is deaminated to produce NH_3 (Shen and Wang 1990). Aspartic acid can be synthesized from other amino acid and carbohydrate. The present study revealed that the presence of essential amino acids (EAA) like lysine, arginine, asparagine, threonine, isoleucine, tyrosine, leucine and valine provide the best growth and survival rates during commercial farming. However, the nonessential amino acids such as (NEAA) alanine, glycine, cystine, and serine were also identified.

According to Holme et al. (2009), diets containing amino acids in proportions similar to those in the shrimp muscles provide the best growth and survival rates during commercial farming, and the diet quality was not necessarily related to the total amount of proteins, but a well-balanced supplementation of amino acids can be found in *Dunaliella salina* present in its composition, with a complete protein containing all the essential and nonessential amino acids (Di Lifetec Co LTD 2009).

Fatty Acid Profiles

In this present study, totally 17 fatty acids were detected, which include both unsaturated (essential) and saturated fatty acids (Table 9). Among these 14 were saturated fatty acids, remaining 3 were unsaturated fatty acids. In the present study, the following fatty acids such as myristic acid, palmitic acid, stearic acid, oleic acid, linolenic acid, EPA, and DHA were found to be significantly higher in microalgal supplemented feed fed groups when compared to control group. The linolenic acid, arachidic acid, behenic acid, and lignoceric acid were found to be lower in microalgal supplemented feed fed groups when compared with control group. The total quantity of the fatty acids in experimental groups was found to be higher in *Dunaliella salina* incorporated feed groups compared to control group.

Feed is the largest production cost for commercial aquaculture plants, so improving feed efficiency in industrial systems has high priority. Moreover, fishmeal prices have risen in real terms in the past three decades and are likely to increase further with continuously growing demand. Therefore, microalgae are some of the most important feed sources in aquaculture (live feed for larvae of bivalves, crustaceans, and marine fish; food for rotifers and shrimps), due to their nutritional value and their ability to synthesize and accumulate great amounts of ω3 PUFA.

The polyunsaturated fatty acids (PUFA) of the linoleic (n-6) and linolenic (n-3) families have been recognized as important nutrients for growth and reproduction in fish (Izquierdo et al. 2001), crustaceans (Jeffs et al. 2002), and mollusks (Caers et al. 2000; Navarro and Villanueva 2000; Nelson et al. 2002). Cavalli et al. (1999) suggested that the higher levels of linoleic acid (18:2n-6) and n-3 highly unsaturated fatty acids (HUFA) increased fecundity, egg hatching efficiency, and larval quality of *M. rosenbergii*. In the present study, the fatty acids such as myristic acid, palmitic acid, stearic acid, oleic acid, linolenic acid, EPA, DHA, arachidic acid, behenic acid, and lignoceric acid were found in both experimental and control groups. The

total quantity of the fatty acids in experimental groups was found to be higher in algal supplemented feed fed groups when compared with control group.

The control and experimental feed fed shrimp larvae in the present study had amino acid values similar to those reported by Suarez et al. (2009) for *L. vannamei*. In the present investigation, the recorded improved growth performance in the shrimp fed with algae diet might be due to higher HUFA content available in algae. Digestibility of fatty acids is known to be influenced by a number of factors, including their chain length, degree of unsaturation, level of incorporation in dietary fat and by other constituent fatty acids, and their melting points (Lin et al. 2006). Moreover, the levels of polyunsaturated fatty acids (PUFA), such as linoleic, linolenic, eicosapentaenoic (EPA), docosahexaenoic (DHA), octadecatrienoic, and arachidonic acids, were found to be higher in experimental feed fed shrimp larvae when compared with control feed fed shrimp larvae.

Total Carotenoid Profiles

Algae with high nutritional value have remarkable potential as fish feed. Previous finding by Khatoon et al. (2010b) suggested that algal feed could be a better supplement for animal protein like *Tubifex*. The fish group fed with experimental diets showed a higher feed intake rate than that with the control feed during the experimental tenure. This might be due to the attractive color, racy flavor, and good nutrient composition of the experimental feeds. The high dietary carotenoid content might have contributed to elevated muscle carotenoid deposition. The occurrence of carotenoid in the experimental fishes might protect them against different diseases as suggested by Trinadha et al. (2003). In the present investigation, the overall carotenoid pigments in *Dunaliella salina* and experimental feed fed shrimp larvae were found highest than control feed and control feed fed group. The potential market for microalgae-derived food coloring is vast. *Dunaliella salina* is grown for a source of the photosynthetic pigment, beta-carotene. Beta-carotene is used as an orange dye and as a vitamin C supplement. Microalgae such as *Dunaliella salina*, *Haematococcus pluvialis*, and *Spirulina* are also used as a source of natural pigments for the culture of prawns, salmonid fish, and ornamental fish. Over the last four decades, several hundreds of microalgae species have been tested as food, but probably less than 20 have gained widespread use in aquaculture.

Previous studies with prawns, mostly *Penaeus japonicus*, have shown that dietary astaxanthin, β-carotene, or canthaxanthin led to the deposition of mainly astaxanthin esters in the carapace, as in the present study (Yamada et al. 1990; Chien and Jeng 1992; NeÁgre-Sadargues et al. 1993). However in the work of Yamada et al. (1990), astaxanthin was reported to be more effective for pigmentation than β-carotene or canthaxanthin. The present study simply showed that feeding of algal β-carotene was effective and efficient with the *P. japonicas* (Chien and Jeng 1992), a higher survival rate for animals fed with astaxanthin-supplemented diets than for ones fed a supplement of β-carotene or algal meal was reported. This is contrast with the present results obtained with the different species, *Penaeus monodon*.

Conclusion

The present study revealed that the optimization of parameters can increase the carotene level in *Dunaliella salina*. It is understood that *Dunaliella salina* can be capable of producing more amount of pigment especially the carotene which has multi commercial applications and uses. The present study concluded that the marine microalga *Dunaliella salina* can be considered as good candidate species for mass scale culture and further application as aqua feed additive to replace the high-cost fishmeal as partially or fully. Furthermore, the *Dunaliella salina* will be cultured in large-scale level in the coastal arid lands wherever possible and used as aqua feed additive in shrimp feed manufacturing for sustainable aquaculture practices.

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