J Food Sci Technol
https://doi.org/10.1007/s13197-019-04144-3

ORIGINAL ARTICLE

Enhanced yield of *Scenedesmus obliquus* biomacromolecules through medium optimization and development of microalgae based functional chocolate

Su Aye Aye Hlaing[1] · Muhammad Bilal Sadiq[2] · Anil Kumar Anal[1]

Revised: 13 August 2019 / Accepted: 28 October 2019

Abstract The freshwater green microalga *Scenedesmus obliquus* was cultivated to enhance the contents of proteins, carbohydrates and lipids by using Box-Behnken experimental design. *S. obliquus* was cultured under phototrophic conditions by using Bold's Basal Medium with different cultivation parameters including pH (7, 8 and 9), salinity (10, 30 and 50 mM), and nitrogen source (0.125, 0.5 and 1 g/L). The highest biomass yield (64.9 ± 0.94 mg/L/day) was obtained by using optimized medium at a salinity concentration of 30 mM (w/v), and nitrogen sources of 0.125 g/L. The maximum content of protein, lipid and carbohydrates from *S. obliquus* optimized medium were 342.19 ± 0.28 mg/g, 241.41 ± 4.32 mg/g and 288.05 ± 1.12 mg/g of dry wt. respectively. The amino acid and fatty acid analysis of *S. obliquus* biomass indicated the presence of significant amount of essential amino acids and essential fatty acids. Furthermore, chocolate crispy bar was developed by fortification with encapsulated freeze-dried *S. obliquus* and evaluated for its oxidative stability and sensory analysis. The chocolate fortified with microalgae can be a potential source of essential fatty acids and amino acids in addition to other bioactive compounds.

Keywords Green microalgae · Biomass optimization · Encapsulated microalgae · Functional chocolate bar

Introduction

Algae contain a variety of bioactive substances, including proteins, lipids, carbohydrates, carotenoids, chlorophyll and vitamins. In recent years, algae gained considerable interest, as a rich source of bioactive compounds, such as phenolic compounds, fatty acids, amino acids, and carotenoids. These biomolecules from microalgae are often used as supplements and/or additives in food, nutraceuticals, cosmetics and feed products (Priyadarshani and Rath 2012). Algal oil was found to be very high in unsaturated fatty acids. Some of these unsaturated fatty acids are found in different species of algae, including arachidonic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), ϒ—linolenic acid, and linoleic acid. Many microalgae species have been evaluated for the production of single cell proteins, due to their ability to synthesize essential amino acids such as histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan and valine (Soletto et al. 2005). Because of its high overall digestibility, purified proteins have potential in applications in food, feed, and health products. Some microalgae contain more carbohydrates than lipid and protein, which can be further used to produce ethanol and other oligosaccharides.

Environmental factors affecting the growth and chemical composition of microalgae are influenced by the conditions of cultivation, incubation temperature, light intensity, photoperiod and pH (Chu et al. 2014; Ji et al. 2013). Among all the factors, nitrogen has a great effect on the lipids and fatty acids metabolism of various microalgae. The effects of the salinity, depleted nitrogen and

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13197-019-04144-3) contains supplementary material, which is available to authorized users.

✉ Anil Kumar Anal
anilkumar@ait.asia; anil.anal@gmail.com

[1] Department of Food, Agriculture and Bioresources, Asian Institute of Technology, PO Box 4, Klongluang, Pathum Thani 12120, Thailand

[2] School of Life Sciences, Forman Christian College (A Chartered University), Lahore 54600, Pakistan

Published online: 05 November 2019

phosphorus concentrations, pH and light intensity on the lipid productivity of *Chlorella vulgaris* were reported by Wu and Hsieh (2008). The growth rate of microalgae with different components are generally accelerated by the addition of nutrient stress (nitrogen, phosphorous and iron) and the increasing amount of oxygen (Aslan and Kapdan 2006). In order to improve the economic feasibility of using algal oil and protein as value-added products, algal biomass productivity and biomacromolecule content are the key parameters to be improved. Different cultivation strategies can improve the lipid content of microalgae, such as nitrogen depletion (Li et al. 2008), phosphate restriction (Reitan et al. 1994), high salinity (Rao et al. 2007), and high iron concentration (Li et al. 2008). *Scenedesmus spp.* is a kind of promising algae, because of high lipids content and strong environmental adaptability (Huo et al. 2015).

The benefits of polyunsaturated fatty acids (PUFA) in human health have been associated with the prevention of cardiovascular diseases (Astrup et al. 2011), hypertension and inflammation (Kris-Etherton et al. 2002). On the other hand, foods having higher concentration of PUFA are more prone to lipid oxidation. However, encapsulation of these types of cells in polymeric matrices can possibly protect from the undesired effects. The encapsulation of bioactive compounds and functional food ingredients to enhance the stability and fortification of the traditional foods is gaining interest (Shrestha et al. 2018). Nasrin and Anal (2015), encapsulated fish oil in resistant starch-soy protein isolate based microcapsule and fortified the bakery products by using these microcapsules.

The goal of this study was to optimize the production of *S. obliquus* under various nutrients depletion to enhance the biomass yield and bioactive compound including protein, lipids and carbohydrates using response surface method (RSM). This study also evaluated the development of functional crispy chocolate bar with *S. obliquus*, containing higher amount of PUFA. The encapsulation of microalgal cells protected from the oxidation and thus enhanced the shelf life as well.

Materials and methods

Scenedesmus obliquus (TISTR 8522), a freshwater microalga was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). The pure seed cultures were incubated with Bold Basal Medium (BBM, Sources Cannon 2007) at 25 ± 2 °C for 18 days. The strain was explored in terms of specific growth rate under the different concentrations of salinity and different nitrogen sources at a range of pH values under the light conditions of light: dark (14:10) h. Furthermore, all the chemicals used in this study were analytical grade and acquired from Sigma–Aldrich (St. Louis, USA).

Experimental design for the growth of algae

The green microalgae were grown by following the method of Arumugam et al. 2011, with some modifications. Bold Basal Medium (BBM) was selected as the standard medium (base case) to evaluate the influence of different medium components for the growth of *S. obliquus*. The concentration of NaCl and nutrient stress conditions like sodium nitrate as nitrogen sources were added to 500 mL Erlenmeyer flasks, containing freshly prepared 400 mL of BBM. The flasks were inoculated with 20 mL of *S. obliquus* (0.75 mg/mL) and temperature was maintained at 25 ± 2 °C. The cultures were aerated continuously with air containing 0.04% CO_2 that was bubbled through the glass tube fitted with stopper at a flow rate of 200 mL/min. The microalgal biomass suspension was measured at the wavelength of 680 nm using UV–Vis spectrophotometer (UNICAM UV/Vis Spectrophotometer, UK). Growth kinetics of microalgal cells was monitored every 24 h for 18 days at 25 ± 2 °C with light intensity of 24 ± 3 photon flux obtained from fluorescent lamps (Panasonic FL40SS—D/36) under the 14 h of light and 10 h of dark cycles. Microalgae cultivation experiments were performed in triplicates.

The Box-Behnken experimental design (Design Expert® software version 9.0.4.1) was formulated with the independent quadratic design and the factor variables were pH (X_1), NaCl (X_2) and $NaNO_3$ (X_3). The standard RSM design constituted a total of 24 sets of experiments, with factors pH (7, 8 and 9), NaCl (10, 30 and 50 mM) and $NaNO_3$ (0.125, 0.5 and 1 g/L) (Cam and Icyer 2015) as shown in Table 1. The "ANOVA for response surface quadratic model" was used to find the optimum conditions of four responses. The responses variables Y_1, Y_2, Y_3 and Y_4 were biomass productivity (mg/L/day), lipid (mg/g dry wt.), protein (mg/g dry wt.) and carbohydrate content (mg/g dry wt.) respectively. A second order polynomial model was used and presented in form of Eq. 1:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j=1}^{k} \beta_{ij} X_i X_j + \varepsilon_i \quad (1)$$

where, Y is the response variable, β_o is the intercept term, β_j is the linear effect, β_{jj} is the interaction effect, x_i and x_j are the factors independent variables and ε is the error.

Growth rate and harvesting of microalgal biomass

Samples (1 mL) of each *S. obliquus* culture (400 mL), were withdrawn every 24 h and analysed for microalgal growth using UV–Vis Spectrophotometric at the optical density of 680 nm. The algal biomass was further centrifuged (Kontron Centrikon T–324, Italy) at 5000 rpm for

Table 1 Experimental design for response surface method design in actual level of variable and the predicted and observed response functions for Box-Behnken design of optimization parameters for *Scenedesmus obliquus*

Run no.	Variables			Responses							
	pH	NaCl, (mM)	$NaNO_3$ (g/L)	Biomass productivity, (mg/L/day)		Lipid (mg/g DW)		Protein (mg/g DW)		Carbohydrate (mg/g DW)	
				Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed
1	8	10	0.125	55.67	49.78 ± 0.39	185.72	187.26 ± 0.13	233.60	178.71 ± 0.07	204.29	273.67 ± 1.39
2	9	30	0.125	48.21	45.09 ± 0.35	189.19	205.17 ± 0.17	301.75	285.23 ± 0.71	238.18	251.92 ± 2.34
3	8	50	0.5	45.34	41.97 ± 0.96	161.37	141.27 ± 1.36	221.19	149.73 ± 0.21	204.00	224.06 ± 1.73
4	7	30	1	46.16	41.68 ± 0.25	156.22	149.37 ± 1.14	311.36	262.89 ± 0.11	140.02	148.49 ± 2.19
5	8	10	0.5	47.79	44.92 ± 0.85	152.86	137.35 ± 2.83	216.82	209.87 ± 0.12	153.74	146.37 ± 0.43
6	9	30	0.5	49.70	48.85 ± 0.38	188.41	172.01 ± 1.78	305.30	297.08 ± 0.16	211.71	219.07 ± 1.08
7	8	30	0.125	45.10	48.49 ± 0.76	199.20	212.55 ± 1.28	263.92	327.48 ± 0.21	229.82	161.84 ± 1.65
8	7	50	1	59.59	62.87 ± 0.52	152.49	139.15 ± 1.52	263.66	309.37 ± 0.16	156.13	155.08 ± 0.40
9	7	30	0.5	53.46	55.07 ± 0.83	170.95	157.93 ± 2.99	329.94	313.35 ± 0.04	185.72	195.64 ± 4.26
10	8	50	0.125	39.48	37.41 ± 0.97	163.74	156.75 ± 2.29	193.47	237.01 ± 0.07	231.46	228.86 ± 0.47
11	7	10	0.125	64.51	62.96 ± 0.29	195.75	197.73 ± 3.62	321.38	335.22 ± 0.08	224.18	199.37 ± 0.78
12	9	10	0.125	61.80	61.58 ± 0.31	171.87	160.38 ± 4.87	242.26	218.88 ± 0.15	200.19	184.81 ± 0.69
13	7	10	0.5	54.13	55.23 ± 0.80	146.06	169.17 ± 2.21	306.52	342.19 ± 0.28	161.11	139.06 ± 1.48
14	8	30	1	40.13	45.93 ± 0.97	189.30	203.52 ± 3.40	248.25	287.25 ± 0.03	161.82	154.81 ± 1.17
15	8	50	1	50.54	55.56 ± 0.42	189.42	202.93 ± 3.71	229.72	216.92 ± 0.09	190.40	185.91 ± 0.53
16	8	10	1	46.64	44.42 ± 0.06	165.68	138.65 ± 4.93	170.18	136.56 ± 0.12	134.48	141.35 ± 0.46
17	7	50	0.5	57.73	48.31 ± 0.69	146.90	169.37 ± 3.09	252.57	248.58 ± 0.07	186.44	188.33 ± 1.12
18	9	10	1	46.64	50.68 ± 0.55	165.68	171.33 ± 4.20	170.18	163.78 ± 0.09	134.48	155.14 ± 1.65
19	9	50	0.5	47.93	54.13 ± 0.97	172.03	169.72 ± 3.68	286.26	282.07 ± 0.08	237.36	254.87 ± 2.72
20	7	30	0.125	56.97	64.90 ± 0.94	205.40	175.28 ± 0.39	322.55	299.16 ± 0.10	237.24	288.05 ± 1.12
21	9	10	0.5	56.43	64.04 ± 0.88	155.85	177.61 ± 4.36	223.56	299.29 ± 0.18	162.17	134.86 ± 0.49
22	7	50	0.125	54.38	55.90 ± 0.96	166.10	181.86 ± 1.94	222.93	220.16 ± 0.24	226.42	203.25 ± 2.13
23	9	50	1	56.45	55.29 ± 0.54	222.52	211.53 ± 2.16	292.23	298.19 ± 0.18	240.45	232.30 ± 1.29
24	9	30	1	49.08	38.80 ± 0.38	218.57	241.41 ± 4.32	281.60	292.22 ± 0.10	199.41	184.09 ± 0.83

The results were expressed as Mean ± SD of three replicates

10 min followed by washing twice with sterile saline water (0.9% w/v NaCl). The biomass slurries were further freeze-dried (Scanvac CoolSafe 55-4, Denmark) at − 55 °C for 24 h. The dried weight of the algal biomass was determined gravimetrically and stored in a sealed plastic bag at 4 °C for further analysis.

Determination of total lipid content and fatty acid composition

The dried sample (100 mg) was dispersed into the solvent mixture of chloroform: methanol (100 mL, 1:1 v/v) by following the method of Nielsen 2003, with slight modifications. The lipid content was extracted gravimetrically by using solvent extraction instrument (Soxtec model HT6, Tecator, Sweden). The extraction cup was dried in an oven at 105 °C for an hour, followed by cooling at room temperature (25 °C). The fatty acid methyl esters (FAMEs) of optimized algal biomass were analyzed by directly injecting algal extract (1 µL) into gas chromatography system (Agilent 6890 N Serial No. US10406046 ULYIPIU). The system was equipped with flame ionization detector (Wang et al. 2017) using a wax capillary column (100 m × 0.25 mm i.d., 0.2 µm, Supelco, USA). The column temperature was programmed from 140 °C (5 min hold) to 250 °C (17 min hold) at a rate of 3 °C/min. Helium was used as carrier gas at a flow rate of 1.1 mL/min. The FAMEs were identified by comparing their retention time with standard FAME mixture and fragmentation pattern with authentic standards (37-component FAME mixture, Supelco, USA) by using AOAC method, 996.06 (AOAC 2012).

Determination of protein content and analysis of amino acids

The protein content in microalgal biomass was measured using the Bradford assay (Bradford 1976). A pre-determined portion (100 mg) of each biomass sample was soaked in 10 mL of 1 N NaOH for 30 min. The extraction was carried out by ultra-sonication at 50 °C for 45 min. The samples were then subjected to centrifugation at 5000 rpm for 10 min and the supernatant was collected to analyze the protein contents (Sitthiya et al. 2018). The concentration of protein in each microalgae extract was determined using bovine serum albumin as the calibration standard.

The amino acid composition of optimized biomass was analyzed following the method described by Dhakal et al. (2018) with slight modifications. Freeze dried algal biomass (1 g) was added to 20 mL NaOH (4.2 M) for tryptophan quantification or 6 M HCl for the other amino acids. The sample was subsequently oven dried at 110 °C for 24 h. The hydrolyzed sample was further diluted to 100 mL of water and filtered through a membrane filter (pore size 0.45 µm) and evaporated to the dryness. The samples were injected into a Biochrom 30 + Amino Acid Analyzer (Biochrom, Cambridge, UK) for quantification of amino acids in the algal biomass.

Carbohydrate content

Carbohydrate content in microalgae was determined by using 3,5-dinitrosalicylic acid (DNS) method as described by Miao et al. 2004 with slight modifications. Freeze dried biomass (100 mg) was incubated in methanol (15 mL) for 2 h at 25 °C. The mixture sample (1.3 mL) was further incubated with 0.2 mL of 5 N NaOH for 10 min. The mixture was further incubated at 95 °C for 5 min to develop a red-brown color. Distilled water (8 mL) was added in the mixture after cooling in an ice water bath for a minute. The mixture solution was further vortexed for 2 min. Using DNS reagent, the optical density of the sample and the blank at 540 nm in a UV–Vis spectrophotometer was measured, and the carbohydrate content was estimated by using Eq. 2:

$$Carbohydratecontent(\%) = \frac{wt.ofcarbohydrate(fromstandardcurve) \times 100}{drycellmass(g)} \quad (2)$$

The carbohydrate content of algal biomass was determined by using glucose as standard.

Chlorophyll and carotenoid contents

Freeze dried algal biomass (0.5 g) was extracted with methanol (10 mL) in the test tube by stirring at 100 rpm and 25 ± 2 °C for 3 h. After homogenization, biomass was subjected to centrifugation (10,000 rpm, 10 min at 4 ± 1 °C). The supernatant (0.5 mL) was separated and mixed with methanol (4.5 mL) and further analyzed for total chlorophyll and carotenoid contents (Sumanta et al. 2014). The chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were estimated by using Eqs. 3, 4, 5 and 6 respectively.

$$\text{Chlorophylla}(\mu g/ml) = 16.72 \times OD_{665.2} - 9.16 \times OD_{652.4} \quad (3)$$

$$\text{Chlorophyllb}(\mu g/ml) = 34.09 \times OD_{652.4} - 15.28 \times OD_{665.2} \quad (4)$$

$$\text{Totalchlorophyll}(\mu g/ml) = \text{Chlorophylla} + \text{Chlorophyllb} \quad (5)$$

$$\text{Totalcarotenoids}(\mu g/ml) = \frac{(1000 \times OD_{470} - [1.63 \times \text{Chlorophylla} - 104.96 \times \text{Chlorophyllb}])}{221} \quad (6)$$

Encapsulation of freeze-dried biomass of *S. obliquus*

Alginate (2 g), Hi–maize resistant starch (2 g) were mixed together prior to disperse in sterile distilled water to obtain 4% (w/w) total solids under stirring at 100 rpm (Velp Scientifica, Europe). After homogenization, freeze-dried biomass was added into the mixture solution to obtain 5% (w/w) biomass in final mixture. The mixture (alginate, starch, freeze dry biomass) was emulsified with solution (200 mL) of canola oil with 0.1% lecithin to obtain the emulsion. The emulsification was carried out by mixing vigorously (400 rpm for 20 min) until fully emulsified. At emulsification, 100 mL of 1 M calcium chloride solution was added into the emulsified mixture and left for 60 min to sediment the microcapsules. After standing, upper oil layer was drained and the microcapsules were collected, washed with normal saline containing 5% (v/v) glycerol and stored at 4 °C for further use and analysis.

Preparation of chocolate crispy bar enriched with encapsulated microalgal cells

The encapsulated *S. obliquus* was used to formulate a chocolate crispy bar and evaluated for shelf life during storage period of 4 weeks. Samples were subjected to sensory and shelf life evaluation. The chocolate snack bar was made from chocolate (80 g) and rice crispy (15 g). The chocolate bar was cut into small pieces, then melted in the water bath (55 °C) following by stirring to a soft texture. The melted chocolate was cooled, mixed with rice crispy, and the mixture was added with 5% (w/w) microcapsule loaded with cell. Under aseptic conditions, the sample of chocolate snack bar was transferred to the aseptic polyethylene bags and stored at room temperature for 4 weeks. Samples of each treatment were performed at 1, 2, 3, 4 weeks for chemical analysis and sensory evaluation.

Free fat content and encapsulation efficiency

The free fat was estimated by following the method of Pisecky (1997), but petroleum ether was used instead of hexane. The sample (5 g) was dispersed into 30 mL of hexane, vortex for 2 min and filtered. The solvent free residue was dried in hot air oven (105 °C) and weighted. The amount of free fat in microcapsules was determined by following Eq. 7:

$$Freefat(\%) = \frac{Weightoffatextract}{Weightofsample} \times 100 \quad (7)$$

The total fat of the microcapsules was estimated according to IUPAC (1987).

Encapsulation efficiency (EE) was calculated by using Eqs. 8:

$$EE(\%) = \frac{Totalfat - Freefat}{Totalfat} \times 100 \quad (8)$$

Oxidative stability analysis

The oxidative stability of chocolate crispy bar containing microalgal cells was analyzed by measuring the peroxide value, following the method of Nasrin and Anal (2015). Peroxide value was measured by titration of liberated iodine with standard sodium thiosulphate solution according to the AOAC official method 965.33 (AOAC 1990). Formation of secondary oxidized products was measured by anisidine value, i.e. by the reaction of aldehydic compounds in oil and the anisidine and absorbance was measured at 350 nm (IUPAC 1987). The oxidative stability of chocolate crispy bar was determined for storage period of 30 days (0, 7, 14 and 30 days).

Sensory evaluation

The chocolate-rice crispy bar with and without microcapsules was evaluated for sensory attributes. The panelists were asked to compare samples for odor, texture, and flavor. The nine-point hedonic rank (1 = disliked extremely, 9 = liked extremely) was used. Samples were served in random order to the panelists. The results of the Hedonic scale test were analyzed as nonparametric test using SPSS.

Statistical analysis

The results were expressed as mean values with standard deviations. One-way analysis of variance (ANOVA) was carried out to determine significant differences ($P < 0.05$) between mean observations by using SPSS statistical software package (SPSS, version 17.0, Inc., Chicago, IL, USA).

Results and discussion

Experimental design for growth of algae

Nitrogen is an important source of nutrients that can be supplied in any form to promote the growth of algae. The phototrophic conditions under different pH, salinity, sodium nitrate, temperature control at 25 ± 2 °C and 14 h light and 10 h of dark cycles were used for the growth of *S. obliquus*.

The number of cells continuously increased until the end of the cultivation. Generally, all cultures showed exponential growth phase with the same duration, reaching the stationary phase on the first 7 days of cultivation. The condition (experiment 20); pH 7, 30 mM NaCl and

0.125 g/L $NaNO_3$, showed the highest growth rate, followed by the condition (experiment 21); pH 9, NaCl 10 mM and $NaNO_3$ 0.5 g/L. After 18 days of cultivation, there was a decrease in biomass, possibly due to depletion of nutrients. The weight of freeze-dried biomass for experiments 20 and 21 (Table 1) was slightly different (64.9 ± 0.94 and 64.04 ± 0.88 mg/L/day, respectively). After 18 days of cultivation, the average biomass production for non-optimized medium was 33.01 ± 0.35 mg/L/day at pH 7.

The influence of independent factors (pH, salinity, sodium nitrate) on response variables (biomass productivity, lipid, protein and carbohydrate) was predicted by using central composite design (CCD) and presented by quadratic model equations (equation S1-S5, supplementary material) and response surface and contour plots (Figure S1, supplementary material). The ANOVA of the regression model demonstrated that the model was significant. The combination of NaCl and $NaNO_3$ (X_2:X_3) or only adjusted pH (X_1) had a significant effect ($P < 0.05$) on biomass productivity. For lipid content, the combination of pH and $NaNO_3$ (X_1:X_3) or NaCl (X_2) had a significant effect ($P < 0.05$), whereas pH (X_1) or NaCl (X_2) were significant model terms for proteins. On the other hand, NaCl (X_2) or $NaNO_3$ (X_3) had a significant effect ($P < 0.05$) on carbohydrate content. P values were less than 0.05 for all responses, indicating that the model terms were significant for all cases. Moreover, the lack of fit value was not significant for all response parameters, indicating that the model was well fitted.

The biomass, lipid, protein and carbohydrate contents, predicted by quadratic model along with the observed experimental values are given in Table 1. The RSM experimental design revealed that the biomass productivity was ranged from 37.41 (mg/L/day) to 64.9 (mg/L/day), lipid content varied from 137.35 (mg/g) to 241.41 (mg/g), protein content was ranged from 136.56 (mg/g) to 335.22 (mg/g) and carbohydrate content was in the range of 141.35 (mg/g) to 288.05 (mg/g). The biomass productivity, lipids, proteins and carbohydrate contents for non-optimized medium were 33.01 ± 0.35 (mg/L/day), 151.41 ± 2.32 (mg/g), 237.65 ± 0.42 (mg/g) and 184.86 ± 0.49 (mg/g), respectively.

The highest concentration of biomass (64.9 ± 0.94 mg L^{-1} day^{-1}) was obtained in the medium at a saline concentration of 30 mM (w/v) after 18 days of cultivation at pH 7 while, the lowest biomass concentration (37.41 ± 0.97 mg L^{-1} day^{-1}) was observed in a medium with 50 mM (w/v) of salinity after 18 days of cultivation at pH 8. Kaewkannetra et al. (2012) reported maximum *S. obliquus* biomass yield (42 mg L^{-1} day^{-1}) at 50 mM NaCl after 15 days cultivation. Salama et al. 2013 found that the biomass yield of *C. mexicana* (40 mg L^{-1} day^{-1}) and *S. obliquus* (32.5 mg L^{-1} day^{-1}) was much higher for BBM added with 25 mM NaCl compared with BBM without NaCl. The growth rate of microalgae is substantially decreased due to insufficiency or excess of NaCl (Ruangsomboon 2012). Among the nutrients, nitrogen deficiency is one of the most important factors that affects the lipid metabolism of algae (Mujtaba et al. 2012). The increase in nitrogen content in nutrient medium and osmotic stress resulted in increased algal biomass concentration (Yilancioglu et al. 2014).

Fatty acid composition

The algal biomass obtained by optimized condition of RSM design was subjected to fatty acid composition analysis. *S. obliquus* biomass was found rich source of unsaturated fatty acids; α- linolenic acid (C18: 3n3), cis-9, 12-Linoleic acid (C 18:2 n6) and cis-9-Oleic acid (C18:1n9c) were the predominant fatty acids with a proportion of 50. 91, 12.03 and 10.71% (w/w) respectively, (Table 2). Among saturated fatty acids palmitic acid was predominant (21. 58%, w/w). The fatty acid profile of algal biomass depends on physiological stage of algae, salinity and type of medium (Darki et al. 2017). The unsaturated fatty acids reduce the incidence of cardiac diseases by lowering the cholesterol (Hiranrangsee et al. 2016). Salama et al. 2013, reported that among unsaturated fatty acids oleic acid (41%) and α-linolenic acid (20%) were the major fractions found in *S. obliquus* at 50 mM NaCl in nutrient medium. The lipid content in microalgae ranges from 1 to 40% and algal lipids are mainly important as they contain essential polyunsaturated fatty acids such as linoleic, EPA, and DHA (Chacón-Lee and González-Mariño 2010).

Table 2 Fatty acids (% w/w) composition of lipids produced by *Scenedesmus obliquus*

Fatty acid	% w/w
Myristic acid (C14:0)	0.33
Palmitic acid (C16:0)	21.58
Stearic acid (C 18:0)	1.15
Behenic acid (24:0)	0.49
Lignoceric acid (C 24:0)	0.33
Palmitoleic acid (C16:1 n7)	0.99
Cis-9-Oleic acid (C 18: 1n9c)	10.71
Nervonic acid (C24:1 n9)	0.99
Cis-9,12-Linoleic acid (C 18:2 n6)	12.03
Gamma-linoleic acid (C 18:3 n6)	0.49
Alpha-Linolenic acid (C18:3 n3)	50.91
Total	100.00

Amino acid composition

The use of microalgae protein in food industry is based on its nutritional quality (Becker 2007). Table 3 shows the amino acid contents of *S. obliquus* algal biomass. Among non-essential amino acids, glutamic acid (43.83 mg/g) and aspartic acid (31.58 mg/g) were predominant whereas, among the essential amino acids, leucine (31.92 mg/g), lysine (19.42 mg/g) and threonine (19.37 mg/g) were prominent. *S. obliquus* was found to be good source of essential and non-essential amino acids. Therefore, algal biomass rich in essential amino acids can be used to fortify the food with essential amino acids (Samarakoon and Jeon 2012). Microalgae can produce quality proteins and microalgae-based foods can be used to reduce the risk of cardiac diseases, as microalgae derived peptides have shown promising antioxidant and antihypertensive effects (Ejike et al. 2017).

Chlorophyll and carotenoid contents

The chlorophyll a and b contents in algal biomass obtained by optimized conditions were 3.040 ± 0.08 mg/g and 7.497 ± 0.234 mg/g of dry wt. biomass respectively. Whereas, the total chlorophyll and carotenoid contents were 10.54 ± 0.15 mg/g and 1.60 ± 0.07 mg/g of dry wt. algal biomass respectively. The chlorophyll and carotenoids are important pigments produced by algae due to their dietary values (Wells et al. 2017).

Table 3 Amino acids composition of *Scenedesmus obliquus* biomass

Amino acid profiles	Concentration (mg/g)
Alanine	30.37
Arginine	18.88
Aspartic acid	31.58
Cystine	2.05
Glutamic acid	43.83
Glycine	21.82
Histidine*	7.12
Isoleucine*	13.55
Leucine*	31.92
Lysine*	19.42
Methionine*	7.87
Phenylalanine	18.75
Proline	14.64
Serine	17.52
Threonine*	19.37
Tryptophan*	5.35
Tryosine	12.85
Valine*	16.12

*Essential amino acids

Encapsulation efficiency *S. obliquus* cells

The encapsulation efficiency of PUFA-producing microalgae cells was calculated by quantification of encapsulated oil and total oil. Encapsulation efficiency of microcapsules loaded with 5% microalgae cells was 70.25% whereas, it was 55.44% for microcapsules without cell loading. The actual percentage of oil efficiency for PUFA loaded cell was 14.81%. The microalgae and their functional ingredients such as astaxanthin, have been encapsulated to enhance the bioavailability and oxidative stability (Shrestha et al. 2018).

Oxidative stability analysis

The oxidative stabilities of chocolate crispy bar enriched with microcapsules of PUFA producing cell and a chocolate crispy bar without microcapsules, were evaluated during storage at room temperature for 4 weeks. During storage, peroxide and anisidine values were increased with time. The chocolate crispy bar enriched with *S. obliquus* microcapsules showed higher oxidation value than the chocolate crispy bar without microcapsules, however, the chocolate enriched with microalgal microcapsules showed less oxidation compared to the chocolate containing free microalgal cells (Table 4). Peroxide and anisidine values of chocolate crispy bar without microalgal cells (control) were also increased with time due to butter fat and other components contained within chocolate. The peroxide values of chocolate crispy bar without microalgae, with microalgae microcapsules and with free microalgae cells were 1.88, 2.18 and 2.72 meq/kg respectively, at 0 day whereas, at the end of storage period (30 days) the peroxide values were 7.19, 7.52 and 12.04 meq/kg respectively. A similar trend was observed for anisidine value, moreover, peroxide and anisidine values of chocolate crispy bar enriched with free cells of S. obliquus were increased rapidly compared with chocolate enriched with microalgae microcapsules and chocolate without microalgae. Finally, it was concluded that the emulsion encapsulation helped to slow down the process of oxidation by preventing cells which were rich in unsaturated fatty acids from oxygen and reaction with food matrix. The microalgae biomass can be incorporated in food products to fortify and enhanced the biological value of conventional foods (Chacón-Lee and González-Mariño 2010).

Table 4 Oxidative stability analysis of chocolate crispy bar enriched with mircoalgae

Days	Peroxide value (meq/kg)			Anisidine value (absorbance units/g)		
	Chocolate without microcapsules	Chocolate with microcapsules	Chocolate with free cell	Chocolate without microcapsules	Chocolate with microcapsules	Chocolate with free cell
0	1.88 ± 0.03^{a}	2.18 ± 0.06^{b}	2.72 ± 0.07^{c}	2.5 ± 0.003^{a}	2.57 ± 0.004^{a}	2.704 ± 0.006^{b}
7	2.96 ± 0.02^{a}	3.17 ± 0.04^{b}	4.21 ± 0.02^{c}	2.636 ± 0.003^{a}	2.728 ± 0.003^{b}	3.623 ± 0.003^{c}
14	4.29 ± 0.05^{a}	4.73 ± 0.04^{b}	7.8 ± 0.21^{c}	2.71 ± 0.003^{a}	2.866 ± 0.003^{b}	8.1 ± 0.13^{c}
30	7.19 ± 0.05^{a}	7.52 ± 0.03^{b}	12.04 ± 0.03^{c}	2.992 ± 0.004^{a}	3.009 ± 0.002^{a}	17.410 ± 0.003^{b}

Different superscript letters (a–c) indicate significant differences among mean observations within a row

Sensory evaluation

The sensory evaluation was done to find out the acceptability of the product by potential customers. The selected samples i.e. chocolate crispy bar with 5% encapsulated microalgae cells was compared with chocolate crispy bar without microcapsules (Figure S2, supplementary material). The odour, texture, flavour, and overall taste were evaluated, and output of the non-parametric Wilccoxon Signed Rank test is presented in Table 5. For the odour, 3 panellists detected

Table 5 Rank of the scores from the panelists for chocolate crispy bar with and without encapsulated PUFA-producing cell microcapsules

		N	Mean rank	Sum of ranks
Ranks				
Odor2–odor1	Negative ranks	3^{a}	6.00	18.00
	Positive ranks	9^{b}	6.67	60.00
	Ties	8^{c}		
	Total	20		
Texture2–texture1	Negative ranks	4^{d}	5.63	22.50
	Positive ranks	5^{e}	4.50	22.50
	Ties	11^{f}		
	Total	20		
Flavor2–flavor1	Negative ranks	7^{g}	5.50	38.00
	Positive ranks	9^{h}	10.83	97.50
	Ties	4^{i}		
	Total	20		
Total2–total1	Negative RANKS	5^{j}	6.50	32.50
	Positive ranks	8^{k}	7.31	58.50
	Ties	7^{l}		
	Total	20		

[a]Odor2 < odor1
[b]Odor2 > odor1
[c]Odor2 = odor1
[d]Texture2 < texture1
[e]Texture2 > texture1
[f]Texture2 = texture1
[g]Flavor2 < flavor1
[h]Flavor2 > flavor1
[i]Flavor2 = flavor1
[j]Total2 < total1
[k]Total2 > total1
[l]Total2 = total
1 = Chocolate sample with 5% PUFA-producing cell encapsulated microcapsules
2 = Chocolate sample without 5% PUFA-producing cell encapsulated microcapsule

fishy odour from sample which contained encapsulated microalgae. There was no significant difference in the texture attribute of both the samples as half of the panellists preferred both samples. The panellists preferred the flavour of sample without encapsulated microalgae. The microalgae (*Chlorella vulgaris and Spirulina maxima*) enriched pasta was found to have better firmness, flavour and sensory attributes (Fradique et al. 2010).

Microalgae enriched products are already available in market and gaining more interest due to health benefits. This urged the potential for the development of new microalgae-based products (Borowitzka 2013). Therefore, the development of microalgae enriched functional food can be a new era for novel food products with improved customer acceptance.

Conclusion

In this study, the growth of *S. obliquus* microalgae acquired from natural fresh water were investigated for 18 days periods of growth. The biomass productivity, lipid, protein and carbohydrate yield from *S. obliquus* were optimized using the response surface methodology. The biomacromolecules yield was dependent on pH, salinity and sodium nitrate and found to increase by 1.4 to 2 times as compared to non-optimized medium. The microalgae lipid and protein composition indicated the presence of various essential amino acids and essential fatty acids. High amount of PUFA (63.43% w/w) was found in *S. obliquus* lipids therefore, it can be considered as rich source of PUFA for nutraceutical, cosmetics, food and feed industries. The free cell *S. obliquus* sp. had oxidation process due to high amount of unsaturated fatty acids. Fortified chocolate enriched with encapsulated microalga showed lower peroxide and anisidine values indicating that encapsulated *S. obliquus* biomass can be used to fortify the food items.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

AOAC (1990) Official methods 965.33 of 15th edn. Association of Official Analytical Chemists, Washington

AOAC (2012) Official Method 996.06 of 19th edn, Fat (total saturated and unsaturated) in foods. Hydrolytic extraction gas chromatographic method, Gaithersburg, MD

Arumugam M, Agarwal A, Arya MC, Ahmed Z (2011) Influence of nitrogen sources on biomass productivity of microalgae *Scenedesmus bijugatus*. Bioresour Technol 131:246–249. https://doi.org/10.1016/j.biortech.2012.12.159

Aslan S, Kapdan IK (2006) Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecol Eng 28:64–70. https://doi.org/10.1016/j.ecoleng.2006.04.003

Astrup A, Dyerberg J, Elwood P, Hermansen K, Hu FB, Jakobsen MU et al (2011) The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: Where does the evidence stand in 2010? Am J Clin Nutr 93(4):684–688

Becker EW (2007) Microalgae as a source of protein. Biotechnol Adv 25:207–210

Borowitzka MA (2013) High-value products from microalgae—their development and commercialization. J Appl Phycol 25(3):743–756

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Cam M, Icyer NC (2015) Phenolics of pomegranate peels: extraction optimization by central composite design and alpha glucosidase inhibition potentials. J Food Sci Technol 52(3):1489–1497. https://doi.org/10.1007/s13197-013-1148-y

Cannon R (2007) Culturing of *Chlorella vulgaris*—Standard operating procedure. Daphnia Research group. http://www.biosci.rdg.ac.uk/Research/eb/daphnia.htm

Chacón-Lee TL, González-Mariño GE (2010) Microalgae for "healthy" foods—possibilities and challenges. Compr Rev Food Sci Food Saf 9(6):655–675

Chu FF, Chu PN, Shen XF, Lam PK, Zeng RJ (2014) Effect of phosphorus on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. Bioresour Technol 152:241–246. https://doi.org/10.1016/j.biortech.2013.11.013

Darki BZ, Seyfabadi J, Fayazi S (2017) Effect of nutrients on total lipid content and fatty acids profile of *Scenedesmus obliquus*. Braz Arch Biol Technol 60:e17160304. https://doi.org/10.1590/1678-4324-2017160304

Dhakal D, Koomsap P, Lamichhane A, Sadiq MB, Anal AK (2018) Optimization of collagen extraction from chicken feet by papain hydrolysis and synthesis of chicken feet collagen based biopolymeric fibres. Food biosci 23:23–30

Ejike CE, Collins SA, Balasuriya N, Swanson AK, Mason B, Udenigwe CC (2017) Prospects of microalgae proteins in producing peptide-based functional foods for promoting cardiovascular health. Trends Food Sci Technol 59:30–36

Fradique M, Batista AP, Nunes MC, Gouveia L, Bandarra NM, Raymundo A (2010) Incorporation of *Chlorella vulgaris* and *Spirulina maxima* biomass in pasta products. Part 1: preparation and evaluation. J Sci Food Agric 90(10):1656–1664

Hiranrangsee L, Kumaree KK, Sadiq MB, Anal AK (2016) Extraction of anthocyanins from pericarp and lipids from seeds of mangosteen (*Garcinia mangostana* L.) by Ultrasound-assisted extraction (UAE) and evaluation of pericarp extract enriched functional ice-cream. J Food Sci Technol 53(10):3806–3813

Huo SH, Wang ZM, Cui FJ, Zuo PX, Yuan ZH (2015) Enzyme-assisted extraction of oil from wet microalgae *Scenedesmus sp.* G4. Energies 8:8165–8174

IUPAC (1987) Standard methods for the analysis of oils, fats and derivatives, 7th edn. Blackwell Scientific Publications, Oxford

Ji F, Hao R, Liu Y, Li G, Zhou Y, Dong R (2013) Isolation of a novel microalgae strain *Desmodesmus sp.* and optimization of environmental factors for its biomass production. Bioresour Technol 148:249–254. https://doi.org/10.1016/j.biortech.2013.08.110

Kaewkannetra P, Enmak P, Chiu TY (2012) The effect of CO_2 and salinity on the cultivation of *Scenedesmus obliquus* for biodiesel production. Biotechnol Bioprocess Eng 17:591–597

Kris-Etherton PM, Harris WS, Appel LJ (2002) Fish consumption, fish oil, omega–3 fatty acids, and cardiovascular disease. Circulation 106(21):2747–2757

Li YQ, Horsman M, Wang B, Wu N, Lan CQ (2008) Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Appl Microbiol Biotechnol 81:629–636. https://doi.org/10.1007/s00253-008-1681-1

Miao X, Wu Q, Yang C (2004) Fast pyrolysis of microalgae to produce renewable fuels. J. Anal. Appl. Pyro 71:855–863

Mujtaba G, Choi W, Lee CG, Lee K (2012) Lipid production by *Chlorella vulgaris* after a shift from nutrient rich to nitrogen starvation conditions. Bioresour Technol 123:279–283

Nasrin TAK, Anal AK (2015) Enhanced oxidative stability of fish oil by encapsulating in culled banana resistant starch-soy protein isolate based microcapsules in functional bakery products. J Food Sci Technol 52(8):5120–5128. https://doi.org/10.1007/s13197-014-1606-1

Nielsen SS (2003) Determination of fat content. Chp 4, food analysis laboratory manual, 1st edn. Kluwer Academic, New York. https://doi.org/10.1007/978-1-4419-1478-1_6

Pisecky J (1997) Handbook of milk powder manufacture. Niro A/S, Copenhagen

Priyadarshani I, Rath B (2012) Commercial and industrial applications of micro algae—a review. J Algal Biomass Utln 3(4):89–100

Rao AR, Dayananda C, Sarada R, Shamala TR, Ravishankar GA (2007) Effect of salinity on growth of green alga and its constituents. Bioresour Technol 98:560–564. https://doi.org/10.1016/j.biortech.2006.02.007

Reitan KI, Rainuzzo JR, Olsen Y (1994) Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. J Phycol 30:972–979. https://doi.org/10.1111/j.0022-3646.1994.00972.x

Ruangsomboon S (2012) Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2. Bioresour Technol 109:261–265

Salama ES, Kim HC, Abou-Shanab RA, Ji MK, Oh YK, Kim SH, Jeon BH (2013) Biomass, lipid content, and fatty acid composition of freshwater *Chlamydomonas mexicana* and *Scenedesmus obliquus* grown under salt stress. Bioprocess Biosyst Eng 36(6):827–833

Samarakoon K, Jeon YJ (2012) Bio-functionalities of proteins derived from marine algae—a review. Food Res Int 48(2):948–960

Shrestha S, Sadiq MB, Anal AK (2018) Culled banana resistant starch-soy protein isolate conjugate based emulsion enriched with astaxanthin to enhance its stability. Int J Biol Macromol 120:449–459. https://doi.org/10.1016/j.ijbiomac.2018.08.066

Sitthiya K, Devkota L, Sadiq MB, Anal AK (2018) Extraction and characterization of proteins from banana (*Musa Sapientum* L.) flower and evaluation of antimicrobial activities. J Food Sci Technol 55(2):658–666

Soletto D, Binaghi L, Lodi A, Carvalho JCM, Converti A (2005) Batch and fed-batch cultivations of Spirulina platensis using ammonium sulphate and urea as nitrogen sources. Aquaculture 243:217–224. https://doi.org/10.1016/j.aquaculture.2004.10.005

Sumanta N, Haque CI, Nishika J, Suprakash R (2014) Spectrophotometric analysis of chlorophylls and carotenoids from commonly grown fern species by using various extracting solvents. Res J Chem Sci 4(9):63–69

Wang P, Sun M, Ren J, Djuric Z, Fisher GJ, Wang X, Li Y (2017) Gas chromatography—mass spectrometry analysis of effects of dietary fish oil on total fatty acid composition in mouse skin. Sci Rep 7:42641. https://doi.org/10.1038/srep42641

Wells ML, Potin P, Craigie JS, Raven JA, Merchant SS, Helliwell KE, Smith AG, Camire ME, Brawley SH (2017) Algae as nutritional and functional food sources: revisiting our understanding. J Appl Phycol 29(2):949–982

Wu WT, Hsieh CH (2008) Cultivation of microalgae for optimal oil production. J Biotechnol 136:521–1521. https://doi.org/10.1016/j.biortech.2009.03.019

Yilancioglu K, Cokol M, Pastirmaci I, Erman B, Cetiner S (2014) Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. PLoS ONE 9(3):e91957. https://doi.org/10.1371/journal.pone.0091957