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Enhancing the omega-3 fatty acid synthesis of marine microalgae using indigenous red seaweed resources of Gulf of mannar region as liquid fertilizers

Krishna Moorthy Abarna (abarnakrish1996@gmail.com)
Fisheries College and Research Institute, Tamil Nadu Dr.J.Jayalalithaa Fisheries University, India

Rani V
Fisheries College and Research Institute, Tamil Nadu Dr.J.Jayalalithaa Fisheries University, India

Padmavathy P
Fisheries College and Research Institute, Tamil Nadu Dr.J.Jayalalithaa Fisheries University, India

Jawahar P
Tamil Nadu Dr. J. Jayalalitha Fisheries University

Uma A
Tamil Nadu Dr. J. Jayalalitha Fisheries University

Kalidas C
ICAR-Tuticorin Regional Station of CMFRI

Manimozhi N
Fisheries College and Research Institute, Tamil Nadu Dr.J.Jayalalithaa Fisheries University, India

Santhanam P
Marine Planktonology and Aquaculture Laboratory, Department of Marine Science, Bharathidasan University, Tiruchirappalli-24

Gowthami A
Marine Planktonology and Aquaculture Laboratory, Department of Marine Science, Bharathidasan University, Tiruchirappalli-24

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Abstract

Fatty acids of microalgae particularly EPA and DHA has great attention worldwide in recent times due its major role in human neurological development and microalgae is considered to be a best source. Hence, the present study was aimed to utilize indigenous seaweeds as a bio-stimulant in enhancing the fatty acid synthesis of microalgae. Three different seaweed liquid fertilizers (SLFs) prepared from indigenous seaweed species viz., *Gracilaria edulis* (SLF-1), *Gelidiella pannosa* (SLF-2) and *Sargassum wightii* (SLF-3) contain enormous amount of essential nutrients required for microalgal growth. Nitrogen, potassium and iron levels were higher in SLF-1 whereas phosphorous, calcium and zinc levels were higher in SLF-2. The growth performance of both alga on three different SLFs revealed that the SLFs prepared from the red seaweed was highly efficient than the brown seaweed. Both the red SLFs significantly enhanced the growth performance, physiological and biochemical response of microalgae. In addition, SLFs had enhanced the EPA and palmitoleic acid concentration in *N. oculata* and *D. salina* respectively. SLFs 1 and 2 enhanced the EPA content of *N. oculata* by 19.85% and 6.35% whereas in *D. salina* the palmitoleic acid concentration was increased by 18.07% and 21.43% respectively. DHA was detected only in the microalgae cultured using SLFS but at trace levels of 0.01-0.02%. These study results suggest the potential application of seaweeds as cost effective culture media for microalga with an added advantage of enhancing its nutritional properties.

Highlights

- Seaweed liquid fertilizers (SLFs) were prepared from red and brown seaweeds by enzymatic hydrolysis process
- SLFs prepared from red seaweeds were found to be highly efficient than the brown seaweed SLF.
- SLFs boosted the growth performance, physiological and biochemical response of microalgae
- Fatty acids such as EPA and palmitoleic acid concentrations were enhanced by SLFs by 19% and 21% in the cultured microalgae
- DHA was detected only in the SLF cultured microalgae but at trace levels

Introduction

Marine microalgae, the primary producers were found to be a rich source of omega 3 fatty acids and considered to be a promising sustainable source of EPA. These fatty acids have drawn much attention at present due to its enormous health benefits on reducing human diseases such as stroke, arrhythmia, asthma, depression etc,. Microalgal lipids have also heeded as a source for biofuel production, because of their high lipid yielding capacity than many other energy crops used so far (Chisti, 2007; Griffiths and Harrison, 2009). In addition to it, microalgae have appended role in $C0_2$ mitigation as well as bioremediation (Oancea et al. 2013). Growth and lipid synthesis of microalgae is mainly influenced by the method of cultivation, energy source and nutrients available in the growth media (Chen et al. 2011). Among which the nitrogen source and concentration in the media used for cultivation plays a major

factor in deciding the microalgal biomass and lipid productivity. Therefore, selection of the media and the nutrient composition is crucial to achieve the best biomass and lipid yield (Hsieh and Wu, 2009; Yeh and Chang, 2011). Microalgae can be cultivated using freshwater, brackish water, sea water and also using the waste water but the major factor that decides the growth is the amount of essential nutrients present in the culture media (Krzywonos et al. 2014; Krzemińska et al. 2014; Zabochnicka-Świątek, 2017).

At present, the more focussed part in microalgae cultivation is to improve the method of cultivation that will increase the biomass production and decrease the production cost (Becker, 1994; Gouveia and Oliveira, 2009; Nayak et al. 2013). Commercial media of inorganic type are generally used for the biomass production which results in high cultivation cost (Soeder, 1980). In aquaculture sector, microalgae have been used as feed for crustaceans, mollusc and some fish species; in industrial sectors for biofuel production and for several pharmaceutical applications, for which the microalgal cultivation requires high-cost commercial media which still remains a hindrance in large scale production sector (De Pauw et al. 1983). To overcome this problem, production of a low cost, practically viable and nutritionally non-compromising media from natural or sustainable source should be standardized.

Seaweeds have been used as fertilizers for agricultural crops since many years ago. Fertilizers prepared from seaweed are organic and they are sustainable to be obtained from natural resources. The extract of seaweed contains bio-stimulant which has the potential to increase the microalgal growth. They also contain essential nutrients, antioxidants, bioactive molecules (Akila and Jeyadoss, 2010) and phytohormones (Sridhar and Rengasamy, 2010) that may enhance the growth rate (Ramarajan, et al. 2013; Pacholczak et al. 2016).

Utilization of resources such as seaweeds that are under exploited for media production is an effective way for satisfying the high quantity nutrient requirement for mass scale microalgal biomass production which will lead to industrial developments (Lam and Lee, 2012). In order to explore the efficiency of seaweed resources for utilization as growth media and bio-enhancer of nutritional properties of microalga, this study was conducted using three different seaweeds: two red species such as *Gracilaria edulis*, *Gelidiella pannosa* and a brown seaweed species *Sargassum wightii* which were collected from the Gulf of mannar region, Thoothukudi coast, India. Similarly, in consideration with the practical viability and economic view point, selection of the suitable algal species with high lipid yielding potential must be chosen (Del Río et al. 2015). Hence, two green marine microalgae with high lipid yielding potential and predominantly available species along the coastal region of Gulf of mannar region such as *Nannochloropsis oculata* and *Dunaliella salina* were chosen.

Materials and methods

Marine microalga cultivation condition

Two green marine microalgae *Nannochloropsis oculata* belonging to the chlorophyte group and a halophilic dinoflagellate *Dunaliella salina* were bought from the Central Marine Fisheries Research

Institute (ICAR-CMFRI) marine microalgal laboratory, Thoothukudi, Tamilnadu, India. The procured algal species were maintained by sub-culturing in the F/2 medium. The algal species were maintained under the optimum environmental conditions (Temperature: 18 ± 2 C, Photoperiod: light:dark = 18:6 h, light intensity: 3000 lux). The cultures were agitated daily for two times to make the algal cells in suspension.

Preparation of SLFs

Seaweeds were collected from Hare Island of Gulf of mannar region, South east coast of India. Collected seaweeds were identified as *Gracilaria edulis*, *Gelidiella pannosa* (red seaweeds) and *Sargassum wightii* (brown seaweed) using standard morphological key characters (Rao and Mantri, 2006; Mantri et al. 2020). The identified seaweeds were separated and washed thoroughly to remove the salts, dirt and attached organisms. After thorough cleaning, it was dried under shade for 24 hours and then oven dried for 4 hours at 60 C. The dried seaweeds were powdered using mincer. One kg of dried powder of the three seaweeds were taken and added in 3% NaCl in the ratio of 1: 9. 10g of cellulase enzyme (Enzyme Bioscience Pvt. Ltd.) was added to this mixture to enhance the fermentation process (Uchida and Murata, 2002). After 15 days of fermentation the mixture was filtered and then centrifuged using refrigerated centrifuge (Remi, C-24 plus) at 5000 rpm for 20 minutes. The supernatant was collected and used as liquid fertilizer for the cultivation of marine microalga.

Nutrient analysis

Nessler's method (Crosby, 1968) and Amino Naphthol Sulphonic method (Allen, 1940) was adopted to estimate the ammoniacal nitrogen and phosphorous content of the SLFs respectively. Macro elements such as sodium and potassium content were analysed using the Flame photometer (Hald, 1947). Micro elements viz., molybdenum, zinc, iron, magnesium, manganese, cobalt, calcium and copper were estimated using Atomic Absorption Spectroscopy (AAS). The nutrient compositions of the SLFs analysed are presented in Table:1

Experimental design

The experiment was conducted using different concentrations of SLFs viz., 1, 2, 4, 6, 8 and 10% as microalgal culture media and the control was carried out with F/2 medium. Algal inoculum of 5% was added to all the treatments and control. All the experiments were accomplished in triplicates.

Determination of cell density and growth rate

The microalgal growth analysis was carried out for 14 days by counting the number of algal cells present in one mL of culture using Haemocytometer (Improved Neubauer chamber) under the light microscope (Nikon Eclipse TS 100). From this observation, the microalgal specific growth rate (μ) was estimated by the formula given by Levasseur et al. (1993):

$$\mu = \ln (x_2) - \ln (x_1) / t_2 - t_1$$

Where μ represents the growth rate per unit amount of cell concentration, x_1 and x_2 are cell concentration at time I (t_I) and time 2 (t_2), respectively.

The doubling time (day) of microalgal cells can be determined from the growth rate using the following equation given by Fogg and Thake (1987):

td = ln 2/ μ

Determination of biomass dry weight

Dry weight of biomass was estimated by filtering 5 mL of culture to obtain the wet biomass, the salts adhered with algal cells were removed by adding ammonium bicarbonate (10 mL) which is then filtered by a filtrate containing an acetate membrane (pore size: 0.7 μm). The obtained biomass was kept in hot air oven at 80^{o}C for 12 h and kept in desiccator. The weight of the dried filter paper containing biomass was measured to calculate the dry algal biomass weight (Rizwan et al. 2017).

Pigment analysis

From all the treatments, 5 ml of sample was taken and centrifuged using Remi C-24 plus refrigerated (4^{o}C) centrifuge at 5000 rpm for 10 minutes. After the centrifugation, the algal pellet was collected, to which N, N, dimethylformamide (5 mL) was added and kept as such for 24 hours. Then the same centrifugation process was carried out again and the OD value was measured at 461, 647 and 664 nm using the spectrophotometer (PerkinElmer, Lambda 25).

The pigments: chlorophyll-*a* (Chamovitz, 1993); chlorophyll-*b* (Chamovitz, 1993) and carotenoid (Moran, 1982) present in the microalgae were calculated as follows:

Chlorophyll − *a* (μg/mL) = OD664 × 11.92

Chlorophyll − *b* (μg/mL) = − 5.6 × OD664 + 23.26 × OD647

Carotenoid (μg/mL) = [OD461 − (0.046 × OD664)] × 4

Protein analysis

Protein estimation was carried out following the standard procedure of Lowry et al. (1951). Reagent I was prepared by mixing three different reagents A (2% Na_2CO_3 in 0.1 N NaOH), B (1% NaK tartrate in H_2O) and C (0.5% $CuSO_4.5H_2O$ in H_2O) in the ratio of 48:1:1 whereas reagent II was prepared by mixing Folin phenol with water in the ratio of 1:1. Protein content was estimated by mixing 1 ml of algal samples with 4.5 ml of reagent I and 0.5 ml of reagent II and left for 30 minutes. The absorbance was estimated using the spectrophotometer (PerkinElmer, Lambda 25) at 660 nm.

Lipid analysis

Lipid analysis was carried out by centrifuging 40 mL of microalgal culture from all the treatments for 5 minutes at 3000 rpm. After centrifugation, chloroform: methanol: water was added to the pellet in the ratio of 1:2:0.8 (v/v/v) and a final of 7.6 mL was achieved which was subjected to sonication process (Labman Scientific Instruments) for 1 minute at 20 kHz and 100 W. It was made to remain at room temperature to cool and then vortexed (30 seconds) and centrifuged at 3000 rpm (5 minutes). This resulted in three different layers, the upper methanol and bottom chloroform with lipid layer. The upper layer was collected separately and the same process of extraction was carried out twice to recover if any lipids present. Then both the chloroform layers were combined and kept in oven for drying at 80^{o}C for 24 h to evaporate the chloroform. The resultant lipid was weighed and expressed as lipid content per ml of microalgal culture (Rizwan et al. 2017).

Fatty acid analysis

One gram of freeze dried microalgal sample was added with 0.5 mg of C13:0 (internal standard) mixed in 1 mL of methanol, 0.7 mL of 10 N potassium hydroxide and 5.3 mL of methanol. The mixture was kept in water bath for 1.5 hours at 55°C. It was for every 20 minutes to completely dissolve the sample, then allowed to cool and 0.58 mL of 24 N H_2SO_4 was added to it. It was again kept in water bath to synthesize fatty acids which was cooled and 3 mL of hexane was added followed by centrifugation. The hexane layer containing FAME was collected separately and stored at − 20°C until GC analysis (Fallon et al. 2007).

Statistical analysis

Standard deviation and mean of the experiment values conducted in triplicates were calculated by SPSS 22.0 (SPSS Inc. Illinois). The difference between the treatments and control was found out using the one-way ANOVA. The mean difference at the significance level of 0.05 was carried out by Duncan's Multiple Range Test.

Results

Nutrient composition of SLFs

Seaweed liquid fertilizers (SLF-1, SLF-2, SLF-3) prepared by fermentation process using three different seaweeds contains enormous level of nutrients that are listed in Table 1. Three SLFs had high level of macro nutrients such as nitrogen, phosphorous, potassium, calcium, magnesium and sodium. In which, SLF-1 had high amount of nitrogen (1117 mg/L), potassium (6613 mg/L) and sodium (9369 mg/L) than the other two SLFs. Other macronutrients such as phosphorous (176 mg/L), calcium (4412 mg/L) and magnesium (453 mg/L) levels were significantly higher in SLF-2. In case of trace nutrients, iron (132 mg/L) and manganese (15 mg/L) levels were higher in SLF-1 whereas zinc (181 mg/L) and copper (3.8 mg/L) level was higher in SLF-2. Comparatively lower level of nutrients was present in SLF-3 (Table.1). A strong fermented smell was observed from the 10th day of fermentation process. The colour of the three

media viz., SLF-1, SLF-2 and SLF-3 were moderately dark, slight yellow to transparent and dark brown respectively.

Table.1 Nutrient composition of SLFs

Nutrient composition of SLFs			
Macro elements (mg/L)	**SLF-1**	**SLF-2**	**SLF-3**
N	**1117**	555	283
P	118	176	154
K	6613	2957	5287
Ca	1286	4412	3365
Mg	314	453	353
Na	9369	9009	9441
Trace elements (mg/L)	**SLF-1**	**SLF-2**	**SLF-3**
Fe	132	105	53
Mn	15	11	5
Cu	3.4	3.8	3.5
Zn	123	181	152
Co	NT	NT	NT
Mo	NT	NT	NT

Effect of SLFs on growth of N. oculata

In SLF-1 grown microalga, the maximum cell density of 21.6, 29.74 and 24.8 *10^6 cells/mL were found at 4, 6 and 8% concentration with a biomass production of 0.3065, 0.4219 and 0.3519 g/L on the 12th day of culture respectively whereas in control (F/2) the maximum cell density was 26.8 *10^6 cells (0.3961 g/L) (Fig. 1). The result suggests that 6% concentration of SLF-1 as the best media concentration for obtaining maximum cell growth which is even higher than the control. The maximum microalgal cell density observed in SLF-2 were 26, 32.06 and 28.32 *10^6 cells/mL with a biomass production of 0.3689,0.4549 and 0.4018 g/L at 6, 8 and 10% concentration respectively on the 14th day whereas in control 27.16 *10^6 cells/mL (0.3995 g/L) were found. This concludes that 8% concentration as the optimum concentration for enhanced biomass production (Fig. 2).

The maximum cell density of 14.8, 18.98 and 13.44 *10^6 cells/mL was observed in the SLF-3 at 1, 2 and 4% concentration with a biomass production of 0.2100, 0.2693 and 0.2049 g/L on the 14th day whereas

in control 26.47 *10^6 cells/mL (0.3711 g/L) were found (Fig. 3), which suggest that 2% as the best concentration but the production was significantly lower than the commercial media grown microalgae (Table 2).

Table.2 Growth parameters of *N. oculata* in three different SLFs

SLF-1	*N. oculata*			
	SGR (/d)	Doubling time (hours)	Biomass concentration (g/L)	Biomass productivity (g/L/d)
Control	0.1544 ± 0.0018	4.4900	0.3961 ± 0.0379	0.0283 ± 0.0084
1%	0.0835 ± 0.0023	8.3035	0.1816 ± 0.0248	0.0130 ± 0.0037
2%	0.1233 ± 0.0037	5.6208	0.2815 ± 0.0938	0.0201 ± 0.0043
4%	0.1310 ± 0.0046	5.2894	0.3065 ± 0.0165	0.0219 ± 0.0073
6%	0.1601 ± 0.0173	4.3290	0.4219 ± 0.0736	0.0301 ± 0.0028
8%	0.1436 ± 0.0183	4.8268	0.3519 ± 0.0378	0.0251 ± 0.0019
10%	0.1082 ± 0.0078	6.4063	0.2384 ± 0.0745	0.0170 ± 0.0024
SLF-2	**SGR (/d)**	**Doubling time (hours)**	**Biomass concentration (g/L)**	**Biomass productivity (g/L/d)**
Control	0.1399 ± 0.0072	4.7537	0.3995 ± 0.0289	0.0285 ± 0.0024
1%	0.0717 ± 0.0221	5.9514	0.1646 ± 0.0363	0.0118 ± 0.0047
2%	0.0872 ± 0.0017	5.2624	0.2015 ± 0.0471	0.0144 ± 0.0056
4%	0.1169 ± 0.0034	4.9695	0.2965 ± 0.0726	0.0212 ± 0.0035
6%	0.1337 ± 0.0183	4.5926	0.3689 ± 0.0895	0.0263 ± 0.0028
8%	0.1499 ± 0.0072	4.4938	0.4549 ± 0.0365	0.0325 ± 0.0034
10%	0.1403 ± 0.0248	4.3273	0.4018 ± 0.0456	0.0287 ± 0.0017
SLF-3	**SGR (/d)**	**Doubling time (hours)**	**Biomass concentration (g/L)**	**Biomass productivity (g/L/d)**

SLF-1	*N. oculata*			
	SGR (/d)	Doubling time (hours)	Biomass concentration (g/L)	Biomass productivity (g/L/d)
Control	0.1241 ± 0.0035	4.3625	0.3711 ± 0.0546	0.0265 ± 0.0045
1%	0.0803 ± 0.0072	6.5068	0.2100 ± 0.0471	0.0150 ± 0.0069
2%	0.0994 ± 0.0254	8.5738	0.2693 ± 0.0515	0.0192 ± 0.0172
4%	0.0784 ± 0.0185	8.7271	0.2049 ± 0.0152	0.0146 ± 0.0025
6%	0.0517 ± 0.0145	10.3443	0.1447 ± 0.0475	0.0103 ± 0.0034
8%	0.0427 ± 0.0085	12.8922	0.1288 ± 0.0258	0.0092 ± 0.0075
10%	0.0238 ± 0.0045	14.5510	0.1007 ± 0.0856	0.0072 ± 0.0035

Effect of SLFs on growth of D. salina

D. salina grown under various concentration of SLF-1 suggest that 6% concentration as the best concentration because it resulted in maximum cell growth of 24.05 *10^6 cells/mL with a biomass production of 0.6310 g/L followed by 4 and 8% organic medium with cell density of 20.83 and 18.32 *10^6 cells/mL achieved on the 12th day of culture period. The control (F/2 media) grown microalgae resulted in maximum growth of 19.11 *10^6 cells/mL (0.4739 g/L) on the 14th day (Fig. 4). In SLF-2 grown microalga, the maximum cell density of 22.95 *10^6 cells/mL was achieved with a biomass production of 0.5954 g/L on 10th day grown at 10% concentration which is significantly higher than the control ie., 16.2 *10^6 cells/mL (0.4739 g/L) (Table 3). Other treatments with concentration of 8 and 6% showed a maximum cell density of 19.93 and 17.58*10^6 cells/mL with the biomass production of 0.4998 g/L and 0.4601 g/L whereas in control 17.83 *10^6 cells/mL was achieved on the 12th day respectively (Fig. 5). SLF prepared from the brown seaweed resulted in the cell density of 9.55 and 12.45*10^6 cells/mL at 2 and 4% concentration on the 14th day with a biomass production of 0.2499 and 0.3258 g/L which was significantly lower than the control 19.11*10^6 cells/mL (0.5001 g/L) (Fig. 6). Above 4% concentration exposure, there was a high reduction in the microalgal cell density.

Table.3 Growth parameters of *D. salina* in three different SLFs

SLF-1	*D. salina*			
	SGR (/d)	**Doubling time (hours)**	**Biomass concentration (g/L)**	**Biomass productivity (g/L/d)**
Control	0.1505 ± 0.0043	4.6057	0.4739 ± 0.0766	0.0339 ± 0.0044
1%	0.1341 ± 0.0077	5.1696	0.3829 ± 0.0339	0.0273 ± 0.0038
2%	0.1454 ± 0.0243	4.7685	0.4433 ± 0.0456	0.0317 ± 0.0073
4%	0.1613 ± 0.0138	4.2983	0.5451 ± 0.0685	0.0389 ± 0.0028
6%	0.1725 ± 0.0223	4.0180	0.6310 ± 0.0953	0.0451 ± 0.0096
8%	0.1504 ± 0.0085	4.6096	0.4732 ± 0.0131	0.0338 ± 0.0087
10%	0.1509 ± 0.0013	4.5928	0.4766 ± 0.0482	0.0340 ± 0.0053
SLF-2	**D. salina**			
	SGR (/d)	**Doubling time (hours)**	**Biomass concentration (g/L)**	**Biomass productivity (g/L/d)**
Control	0.1343 ± 0.0394	5.1612	0.4739 ± 0.0222	0.0339 ± 0.0058
1%	0.1178 ± 0.0043	5.8825	0.3826 ± 0.0687	0.0273 ± 0.0039
2%	0.1263 ± 0.0136	5.4863	0.4274 ± 0.0174	0.0305 ± 0.0018
4%	0.1294 ± 0.0469	5.3552	0.4449 ± 0.0837	0.0318 ± 0.0026
6%	0.1320 ± 0.0036	5.2505	0.4601 ± 0.0316	0.0329 ± 0.0055
8%	0.1384 ± 0.0098	5.0085	0.4998 ± 0.0687	0.0357 ± 0.0068
10%	0.1518 ± 0.0043	4.5648	0.5954 ± 0.0445	0.0425 ± 0.0075
SLF-3	**D. salina**			

SLF-1	*D. salina*			
	SGR (/d)	Doubling time (hours)	Biomass concentration (g/L)	Biomass productivity (g/L/d)
	SGR (/d)	Doubling time (hours)	Biomass concentration (g/L)	Biomass productivity (g/L/d)
Control	0.1546 ± 0.0038	4.4826	0.5001 ± 0.0348	0.0357 ± 0.0042
1%	0.0871 ± 0.0086	7.9608	0.2078 ± 0.0347	0.0148 ± 0.0035
2%	0.1013 ± 0.0356	6.8444	0.2499 ± 0.0156	0.0179 ± 0.0085
4%	0.1217 ± 0.0048	5.6969	0.3258 ± 0.0548	0.0233 ± 0.0033
6%	0.0786 ± 0.0174	8.8213	0.1861 ± 0.0378	0.0133 ± 0.0024
8%	0.0774 ± 0.0136	8.9580	0.1832 ± 0.0878	0.0131 ± 0.0017
10%	0.0566 ± 0.0733	12.2561	0.1397 ± 0.0287	0.0100 ± 0.0089

Effect of SLFs on protein content

The result of protein analysis suggests that *N. oculata* grown under SLF-1 at 6% concentration had maximum protein content of 1.0831 mg/mL followed by 4 and 8% concentration with the value of 0.8426 and 0.7963 mg/mL. The microalgae grown in SLF-1 had significantly higher protein content than the control (0.8439 mg/mL). In SLF-2 culture treatment, the maximum microalgal protein content obtained was 1.09, 1.21 and 1 mg/mL at 6, 8 and 10% respectively which were significantly higher than the control (0.7439 mg/mL). The results positively correlate with the biomass production ie., the treatments with higher biomass production had higher protein content. SLF-3 grown microalgae exhibited significantly lower protein content in comparison with the commercial F/2 medium grown culture. The maximum protein concentration achieved was 0.7739 mg/mL at 2% concentration which was very lower than the control (0.8117 mg/mL) (Fig. 7).

Higher protein content was observed in *D. salina* grown under 6% SLF-1with 1.0915 mg/mL protein value followed by 0.82 and 0.8 mg/mL protein content in 8 and 10% medium concentration. The result suggests that SLF-1 at 6% concentration as the best media that resulted in protein value which was significantly higher than control (0.937 mg/mL). The protein value was observed to be high in *D. salina* cultured in 10% SLF-2 resulting in protein value of 1.0843 mg/mL followed by 1.0023 and 1.0423 mg/mL

in 6 and 8% respectively. These values were significantly higher than the protein content of microalgae grown in F/2 medium. In SLF-3 grown microalgae, the maximum protein content was found in the culture grown under 4% concentration with 0.636 mg/mL followed by 0.3942 and 0.5173 mg/mL in 1 and 2% concentration which were significantly lower than the control (1.005 mg/mL) (Fig. 8).

Effect of SLFs on pigment synthesis

Pigment contents such as chlorophyll a, b and carotenoid of microalgae cultured in SLFs were analysed. *N. oculata* grown in SLF-1 and SLF-2 resulted in higher chl-*a* production ie., 5.0163 and 5.10873 µg/mL at 6 and 10% concentration respectively which were found to be significantly higher than the control (4.1766 µg/mL) whereas the microalgae grown in SLF-3 resulted in lower chl-*a* pigment synthesis ie., 3.2987 µg/mL which was significantly lower than the control (Fig. 9). The carotenoid production was also found to be high in the microalgae cultured in red seaweed medium ie., SLF-1 and SLF-2 with the production value of 1.9646 and 2.2147 µg/mL which were significantly higher than the control (1.8543 µg/mL) which denotes that the red seaweed medium has enhanced the carotenoid production. SLF-3 medium has reduced the carotenoid production with the production value of 1.5692 µg/mL which was lower than the control (Fig. 10).

The maximum chl-*a* pigment synthesis of *D. salina* was noted at 6% SLF-1, 10% SLF-2 and 4% SLF-3 with the value of 5.9917, 5.2687 and 2.9052 µg/mL respectively. The chl-*a* production in both red seaweed organic medium was noted to be significantly higher than the control (3.0014 µg/mL) (Fig. 11). Similarly, significantly higher chl-*b* and carotenoid production values than the control (2.7329 and 2.1366 µg/mL) was found in 6% SLF-1 and 10% SLF-2 with the content of 3.8826,3.9732 µg/mL and 3.6587, 4.2768 µg/mL respectively whereas in microalgae cultured in brown seaweed medium the chl-*b* and carotenoid production values were 2.0279 and 1.8622 µg/mL respectively which were significantly lower than the control (Fig. 12,13).

Effect of SLFs on lipid synthesis

In SLF-1, SLF-2 and SLF-3 treated *N. oculata*, the maximum lipid production of 0.2107, 0.1886 and 0.1348 mg/mL was found at 6, 8 and 2% concentration respectively. Red seaweed medium resulted in an increase of lipid production of microalgae which was significantly higher than the control (0.176 mg/mL) whereas in brown seaweed medium there was a significant reduction of lipid synthesis (Fig. 14). *D. salina* grown using the SLFs resulted in enhanced lipid production. Lipid production of *D. salina* grown in SLF-1 and SLF-2 were 0.2675 and 0.3 mg/mL at 6 and 10% respectively which were noted to be significantly higher than the control (0.1807 mg/mL) (Fig. 15).

Effect of SLFs on fatty acid synthesis

Twenty-two different types of fatty acids were detected in *N. oculata* cultured using commercial media (F/2) whereas in SLFs grown microalgae the fatty acid chain length was increased to 32. In control,

among the 22 fatty acids the dominant fatty acid was found to be Methyl palmitoleate and Methyl cis 5,8,11,14,17-eicosapentaenoate (EPA) at the rate of 42.99% and 25.11% respectively. In SLF-1 grown microalgae, the dominant fatty acid was found to be EPA at the concentration of 44.96% which was 19.85% higher than the control whereas in SLF-2 grown microalgae, the EPA was found to be 31.46% which was 6.35% higher than the control. Followed by EPA, the dominant fatty acid in both SLF- 1 and 2 grown microalgae was methyl palmitoleate at the rate of 37.53% and 20.95% respectively. In control, DHA was found to be nil whereas in microalgae grown using SLF-1 and 2, DHA was detected at trace levels at the rate of 0.009 and 0.018% respectively. These results shows that both the SLFs had significantly enhanced the EPA content of the microalgae (Table.4).

Table.4 Fatty acid composition of *N. oculata* in different culture media (% of total FAME)

Fatty acids	Concentration (%)		
	Control	SLF-1	SLF-2
C4:0	0.080 ± 0.014	0.009 ± 0.003	0.064 ± 0.009
C6:0	0.078 ± 0.027	0.012 ± 0.007	0.014 ± 0.005
C8:0	0.293 ± 0.086	0.183 ± 0.017	0.122 ± 0.054
C10:0	0.236 ± 0.094	0.116 ± 0.023	0.138 ± 0.007
C11:0	ND	0.011 ± 0.002	0.019 ± 0.006
C12:0	0.654 ± 0.025	0.345 ± 0.105	0.771 ± 0.276
C13:0	ND	0.005 ± 0.001	0.099 ± 0.016
C14:0	4.476 ± 0.573	3.327 ± 1.001	9.352 ± 0.954
C14:1[cis-9]	ND	0.005 ± 0.002	0.101 ± 0.038
C15:0	0.798 ± 0.005	0.731 ± 0.024	2.239 ± 0.144
C15:1 [cis-10]	ND	0.004 ± 0.002	0.019 ± 0.006
C16:0	12.677 ± 3.068	13.251 ± 3.008	5.093 ± 1.007
C16:1[cis-9]	42.995 ± 5.16	37.530 ± 6.418	20.955 ± 2.567
C17:0	0.633 ± 0.005	0.336 ± 0.007	1.207 ± 0.53
C18:0	1.595 ± 0.128	1.252 ± 0.027	1.902 ± 0.21
C18:1 [trans-9]	ND	0.173 ± 0.019	0.510 ± 0.002
C18:1 [cis-9]	6.305 ± 0.049	6.020 ± 0.183	14.522 ± 4.297
C18:2 [cis-9,12]	2.190 ± 0.084	3.079 ± 0.482	8.244 ± 0.627
C20:0	0.043 ± 0.039	0.070 ± 0.009	0.170 ± 0.025
C18:3 [cis-6,9,12]	0.186 ± 0.093	0.742 ± 0.016	1.453 ± 0.004
C20:1 [cis-11]	0.371 ± 0.065	0.185 ± 0.046	0.669 ± 0.016
C18:3 [cis-9,12,15]	ND	0.247 ± 0.03	1.320 ± 0.843
C20:2 [cis-11,14]	ND	0.021 ± 0.007	0.602 ± 0.102
C22:0	ND	0.007 ± 0.001	0.233 ± 0.024
C20:3 [cis-8,11,14]	0.037 ± 0.016	0.329 ± 0.025	1.005 ± 0.02
C22:1 [cis-13]	ND	ND	0.015 ± 0.004

*ND-Not Detected

Fatty acids	Concentration (%)		
	Control	SLF-1	SLF-2
C20:4 [cis-5,8,11,14]	0.685 ± 0.004	0.516 ± 0.025	0.636 ± 0.011
C22:2 [cis-13,16]	ND	ND	0.008 ± 0.002
C24:0	ND	0.002 ± 0.005	0.049 ± 0.001
EPA	25.118 ± 2.037	44.967 ± 4.515	31.466 ± 5.011
C24:1 [cis-15]	ND	ND	0.003 ± 0.002
DHA	ND	0.009 ± 0.002	0.018 ± 0.006
*ND-Not Detected			

D. salina cultured using F/2 media had high level of EPA with the concentration of 42.43%. Followed by EPA, methyl palmitoleate content was found to be 27.03%. In the treatments SLF- 1 and 2 grown microalgae, EPA concentration was found to be 44.98% and 42.97% respectively which was significantly lower in comparison with the control. In case of methyl palmitoleate, the concentration in SLF-1 and 2 treatment was 45.10% and 48.46% respectively which was found to be 18.1% and 21.46% higher in comparison to the control respectively. Similar to *N. oculata*, DHA was found to not detected in control whereas in *D. salina* grown using SLF-1 and 2, DHA was detected at trace levels at the rate of 0.025 and 0.021% respectively. This study strongly reveals the efficiency of SLFs in increasing the fatty acid content of the cultured microalgae (Table.5)

Table.5 Fatty acid composition of *D. salina* in different culture media (% of total FAME)

Fatty acids	Concentration (%)		
	Control	SLF-1	SLF-2
C4:0	0.029 ± 0.017	0.104 ± 0.052	0.006 ± 0.027
C6:0	0.023 ± 0.011	0.004 ± 0.002	0.003 ± 0.001
C8:0	0.243 ± 0.135	0.200 ± 0.018	0.049 ± 0.014
C10:0	0.128 ± 0.056	0.144 ± 0.027	0.041 ± 0.016
C12:0	0.291 ± 0.093	0.406 ± 0.056	0.161 ± 0.087
C13:0	0.079 ± 0.052	0.005 ± 0.001	0.015 ± 0.006
C14:0	3.006 ± 0.293	3.435 ± 1.286	2.355 ± 0.128
C14:1[cis-9]	0.117 ± 0.045	0.006 ± 0.004	0.055 ± 0.006
C15:0	0.869 ± 0.067	0.680 ± 0.139	0.466 ± 0.046
C16:0	10.082 ± 1.517	16.136 ± 4.522	13.702 ± 2.483
C16:1[cis-9]	27.033 ± 4.304	45.107 ± 7.291	48.466 ± 5.271
C17:0	0.476 ± 0.013	0.414 ± 0.016	0.355 ± 0.002
C18:0	0.913 ± 0.044	1.878 ± 0.044	1.652 ± 0.013
C18:1 [cis-9]	5.412 ± 0.372	9.997 ± 0.736	15.445 ± 0.863
C18:2 [cis-9,12]	7.124 ± 0.883	4.020 ± 0.548	2.872 ± 0.193
C20:0	0.062 ± 0.004	0.160 ± 0.028	0.171 ± 0.069
C18:3 [cis-6,9,12]	0.149 ± 0.028	0.687 ± 0.384	0.347 ± 0.109
C20:1 [cis-11]	0.092 ± 0.041	0.151 ± 0.028	0.067 ± 0.009
C18:3 [cis-9,12,15]	0.382 ± 0.065	0.340 ± 0.039	0.208 ± 0.075
C21:0	0.138 ± 0.018	0.188 ± 0.045	0.005 ± 0.001
C20:2 [cis-11,14]	0.179 ± 0.084	0.413 ± 0.102	0.248 ± 0.106
C22:0	0.014 ± 0.002	0.060 ± 0.003	0.033 ± 0.002
C20:3 [cis-8,11,14]	0.389 ± 0.065	0.465 ± 0.127	0.296 ± 0.073
C22:1 [cis-13]	0.023 ± 0.007	0.021 ± 0.005	0.007 ± 0.002
C20:3 [cis-11,14,17]	0.019 ± 0.003	0.007 ± 0.002	0.017 ± 0.002
C24:0	0.028 ± 0.001	0.004 ± 0.001	0.006 ± 0.005

*ND-Not Detected

Fatty acids	Concentration (%)		
	Control	SLF-1	SLF-2
EPA (Methyl cis 5,8,11,14,17)	42.436 ± 6.051	44.987 ± 4.05	42.973 ± 2.129
DHA (cis-4,7,10,13,16,19)	ND	0.025 ± 0.005	0.021 ± 0.004
*ND-Not Detected			

Discussion

Three SLFs had abundant nutrients that are required for microalgal growth. Macro and trace nutrients were found to be high in both red seaweed media (SLF-1, SLF-2) in comparison with the brown seaweed media (SLF-3). The essential macronutrients N and K were high in SLF-1 whereas P content was high in SLF-2. An important trace nutrient that plays a major role in microalgal growth is iron which was found to be more in SLF-1 followed by SLF-2 whereas low in SLF-3. Among the macro nutrients, sodium and potassium was most abundant followed by calcium and nitrogen. In the study conducted by Zheng et al. (2016), the kelp waste extract contained enormous amount of nitrogen (5723 mg/L), phosphorous (5529 mg/L) and potassium (60.54 mg/L) whereas iron was present at not detectable levels. The results of the nutrient analysis suggest that the SLFs prepared from the seaweeds were loaded with essential nutrients for microalgal growth. This media can act as a biostimulant that can satisfy the nutrient requirement of microalgae for growth as well as lipid production. Villares et al. (2007) has reported that seaweeds contain plenty of macro, micro and trace nutrient elements. Biomass and lipid productivity of the microalgae depends on the nutrients available in the culture media. Nutrient elements present in seaweed extract were found to be water soluble in nature hence get easily absorbed by plants and helps in reducing the nutrient deficiency problems (Mohanty et al. 2013). The results of the present study suggest that high levels of nutrients were present in the SLFs prepared from red seaweed in comparison with brown seaweed media. These results are in agreement with the report of Raja et al. (2015) that the extract of the red seaweed *K. alvarezii* had higher amount of nutrients than the brown seaweed *T. cornoides* extract. Thus, it can be concluded that, the red seaweed could be effectively utilized as an organic source for the media preparation for the growth and maintenance of the marine microalgae.

SLFs had a strong stimulation on the growth of both microalgae. The results of the growth study suggests that SLF-2 as the best culture medium followed by SLF-1 which enhances the biomass production of *N. oculata* to a greater level in comparison with SLF-3 as well as the control. The best media dose for enhancing the biomass production using SLF-1, SLF-2 and SLF-3 were 6, 8 and 2% respectively. Two red seaweed SLFs resulted in higher biomass production in comparison with the commercial media suggesting the efficiency of the seaweed as an effective microalgal culture media. Similarly, enhancement in the growth of microalgae using Kelp waste extract was reported by Zheng et al. (2016) that *Chlorella* sp., *C. sorokiniana*, *S. maxima* and *P. tricornutum* exhibited a higher growth in KWE than the control (commercial media). Studies on seaweed extract added with commercial media also

enhanced the biomass production. A study on the effect of red seaweed extracts of *Gracilaria corticata* and *Grateloupia lithophila* added as supplement with the commercial BBM media resulted in an increase of cell density of *Chlorella vulgaris* than the BBM exclusively grown microalgae (Lakshmi and Sheeja, 2021).

The halophilic dinoflagellate *D. salina* grown under three different SLFs suggest that the best medium to obtain maximum biomass production was SLF-1 followed by SLF-2. Both the red seaweed media resulted in higher biomass production than the commercial medium grown microalgae. SLF-3 showed a significantly lower cell density than the control. The result of the present study suggests the best concentrations of SLF-1, SLF-2 and SLF-3 for achieving maximum biomass production as 6, 10 and 4% respectively. SLF-1 resulted in higher biomass value even at low medium concentration (6%) than the SLF-2 (10%). These results well corelate with the findings of Rohani-Ghadikolaei et al. (2012) that the seaweed extracts (*U. lactuca*, *E. intestinalis, G. corticate*) used as supplement along with the commercial F/2 medium resulted in enhancement of *Isochrysis galbana* growth in comparison with those achieved by commercial media exclusively. Another report by Cho et al. (1998) also confirms that there is enhancement in growth of microalga *Isochrysis galbana* when grown in the extract of seaweed *Monostroma nitidum*. Crouch and Van Staden (1993) stated the reason behind the enhancement of microalgal growth by seaweed extract (SWEs), that SWEs are not only abundant with supplementary nutrients but also with the growth enhancing substances such as auxins and cytokinins, which results in the enhancement of the growth of microalgae.

In the present study, both the microalgae showed significantly lower growth in SLF-3 treatment. The two main reason behind the low growth exhibited by both the microalgae grown in brown SLF may be due to the low nitrogen and iron content as well as the dark colour of the media. The presence of low nutrient content may cause nutrient deficiency thereby declining the microalgal growth whereas the darkness of the media may reduce the penetration of light thereby inhibiting the microalgal growth. It was also observed that low concentration of the SLF-3 supported growth to an extent whereas the high concentration inhibited growth in higher level. Similar result was also noted in the study of Malik et al. (2018) that the growth of *Chaetoceros gracilis* was reduced when grown at media added with higher concentration of seaweed extract of *Sargassum* sp. Another documentation by Alvarado et al. (2008) states that *Chaetoceros muelleri* grown in the brown seaweed macroalgal compost supplemented with di-silicate resulted in less growth in comparison with the microalgae grown in Walne's medium.

The maximum cell density of microalgae *N. oculata* grown under SLF-1, SLF-2 and SLF-3 was achieved on the 12th, 14th and 14th day respectively suggesting that SLF-1 has shorten the life cycle, whereas for *D. salina* maximum growth was achieved on the 12th, 10th and 14th day respectively showing that SLF-2 medium had reduced the life cycle of the microalgae. These results are in agreement with report of Zheng et al. (2016) that the organic media had shorten the life cycle of microalgae grown in KWE. The best medium to obtain the maximum microalgal production of *N. oculata* was SLF-2 at 8% concentration whereas for *D. salina* the medium was SLF-1 at 6% concentration. Different microalgal species reacts

differently when exposed with different organic media of different doses. This varies with microalgal species based on their level of tolerance level to the exposed organic compounds (Zhang et al. 2014).

The biochemical composition of the microalgae can be well correlated with the nutritional quality of the culture medium in which it was cultivated (Zhang, 1997). Selection of suitable culture media mainly focuses on the biomass production at the same time the biochemical composition shouldn't get affected. The present study concludes that the best organic medium for *N. oculata* to achieve the maximum protein content was SLF-2 medium followed by SLF-1 whereas for *D. salina* the best medium was SLF-1 followed by SLF-2. Both the microalgae showed low protein content when grown under SLF-3 suggesting the red seaweed media as the suitable and effective culture media. The present study emphasizes that the protein content of the microalgae was found to be increasing when grown under the SLFs in comparison with control. Similar results of increase in microalgal protein content by seaweed extracts was mentioned by Rohani-Ghadikolaei et al. (2012) that when seaweed extracts of *Gracilaria corticate, Ulva lactuca* and *Enteromorpha intestinalis* were added as supplement with the commercial media F/2, it resulted an increase of protein content of the microalgae than grown exclusively with F/2 media. Another report documents that there was a slight enhancement of protein concentration of the microalgae grown under BBM media amended with red seaweed extract of *Gracilaria corticata* and *Grateloupia lithophila* (Lakshmi and Sheeja, 2021). The decrease in protein content of microlage grown using brown seaweed medium was reported by Alvarado et al. (2008) that when *Chaetoceros muelleri* was grown under brown seaweed compost added with silicate it resulted lower protein content of 2–5% whereas in control the protein value was 12%. Earlier reports and present study results concludes that red seaweed media enhances the protein value of the microalgae whereas the brown seaweed decreases it significantly.

SLFs has enhanced the pigment synthesis in microalgae which resulted in higher chlorophyll and carotenoid production than the commercial F/2 medium. Both the red seaweed media ie., SLF-1 and SLF-2 resulted in higher chl-*a* production in *N. oculata* that was 1.2 and 1.22 times higher than the control respectively whereas in SLF-3 treatment the chl-*a* content was 1.26 times lower than the control. Similarly, carotenoid production was also enhanced to 1.04 and 1.19 times higher by SLF-1 and SLF-2 than by the commercial media. In the halophyte *D. salina*, chl-*a*, *b* and carotenoid production were significantly enhanced by the red seaweed SLFs. SLF-1 and SLF-2 resulted in 2 and 1.76 times higher chl-*a* production; 1.42 and 1.33 times higher chl-*b* production; 1.85 and 2.01times higher carotenoid production than the control respectively. SLF-3 has decreased the pigment synthesis ie., chl-*a*, *b* and carotenoid content by 1.03, 134 and 1.14 times than the control respectively. In comparing the efficiency of both red seaweed SLFs, SLF-2 enhanced the pigment synthesis of *N. oculata* in a higher level than the SLF-1. In *D. salina*, SLF-1 enhanced chl-*a* and *b* level higher than the SLF-2 but carotenoid production was more enhanced by the SLF-2 when compared to SLF-1. Enhancement of the pigment synthesis in microalgae *Chlorella vulgaris* was noted when the seaweed extracts of *Gracilaria corticata* and *Grateloupia lithophila* were added as supplements in the commercial media BBM (Lakshmi and Sheeja, 2021). The extracts of *Kappaphycus alvarezii and Turbinaria conoides* added as supplement with F/2 and Walne's media for culture of *C. muelleri* and *D. salina* resulted in increase of pigment synthesis (chl-*a* and *b* production) in both the microalgae (Raja et al. 2015). Our results and the previous reports also conclude that SWE has

the potential to increase the pigment synthesis in microalgae. The reason behind this potential may be due to the presence of high level of phytohormones like cytokinins in the SWE that will increase the nutrient absorption and utilization capacity of microalgae and thereby increasing the pigment production (Zhang, 1997). The present study also suggest that the brown seaweed medium has reduced the pigment synthesis of microalgae, the main reason for this may be due to the low content of iron available in the media which remains a main key factor in deciding the pigment synthesis potential of microalgae (Kong et al. 2014).

The red seaweed SLFs had a positive influence on the lipid synthesis of both microalgae. SLF-1 and SLF-2 had enhanced the lipid production of *N. oculata* by 1.19 and 1.13 times higher than the control whereas SLF-3 reduced the lipid synthesis by 1.3 times. In *D. salina*, the lipid production was increased by SLF-1 and SLF-2 to 1.4 and 1.6 times than the control and decreased by 1.42 times by SLF-3. In *N. oculata*, the lipid production was greatly enhanced by SLF-1 whereas in *D. salina*, lipid synthesis was highly increased by SLF-2. SLF-3 has reduced the lipid synthesis in both the marine microalgae. These results are comparable with the results of Alvarado et al. (2008) that there was an increase in lipid production of microalgae *C. muelleri* when grown in seaweed compost amended with di-sodium silicate. The lipid production in microalgae cultured in seaweed compost media was 42% whereas in control (cultured in Walne medium) it was only 28%. Another study documented by Gireesh[51] is also in agreement with the present study results, that there was a notable increase in lipid production of *D. salina* grown in seaweed liquid fertilizer than the microalgae cultured in commercial media (Conveyor Walne's media). Rohani-Ghadikolaei et al. (2012) has also given similar results of enhanced microalgal lipid production in microalgae *I. galbana* when cultivated in seaweed extracts as a supplement with the commercial media (f/2 medium). The potentiality of seaweed extracts in enhancing the lipid synthesis in microalgae could be due to the presence of abundant levels of carbon in seaweed extracts which are in convertible form that can stimulate the lipid synthesis and accumulation in microalgae (Liang et al. 2009).

EPA content of *N. oculata* grown using SLF 1 and 2 was increased by 19.85% and 6.35% respectively. In *D. salina*, methyl palmitoleate concentration was increased up to 21.46% when grown using SLFs as culture media. In both the microalgae using commercial media, DHA was not detected whereas it was found at trace levels in both red SLFs treatment. The fatty acid study results reveals the efficiency of SLFs in increasing the composition of fatty acids and its concentration. Both the SLFs prepared from red seaweeds had enhanced the EPA and palmitoleic acid content of *N. oculata* and *D. salina* respectively suggesting its potential application in aquaculture sectors. There are no studies reported on the effect of seaweeds as liquid fertilizer for the growth of microalgae. But the study by Zheng et al. (2016) on the effect of kelp waste extract on microalgae suggest that it has reduced the PUFA synthesis of Chlorella strains whereas increased the synthesis of SFA.

There are few reports stating the effect of seaweed extracts as microalgal culture media that were added as supplement with the commercial media. This study provides an eco-friendly and completely alternative culture medium that has a shelf life of more than one year. This report shall be considered as the first study that documents an effective and cheap cost culture media for microalgae with a longer

shelf life. The organic fertilizer reported by Lam and Lee (2012), used for microalgal growth stimulation cost around 1.20 USD/400 g whereas another study by Zheng et al. (2016) documented the cost of production of one litre Kelp Waste Extract (culture media for microalgae) cost around 0.28 USD. The present study documents the overall production cost of one litre of SLF with an estimate of 0.1 USD (10 g cellulase cost) by the enzymatic hydrolysis method, that is very much lower than the production cost of previously reported organic media and commercial media. Keeping this in view, it can be practically applicable in aquaculture and biofuel industries.

Conclusion

The present study reveals that the best SLF as culture media for *N. oculata* and *D. salina* to achieve maximum biomass and lipid production *was* SLF-2 (*SLF-2*) and SLF-1 (*SLF-1*) respectively. This study has provided an alternative and inexpensive culture media that has added advantage of eco-friendly nature. The standardized methodology developed for the production of SLFs, takes its practical applicability to the next level such as biofuel and aquaculture industries. The ease of preparation and very low-cost method makes it practically viable to be utilized in microalgal mass production sectors. In addition to it, the SLFs has enhanced the nutritional properties of the cultivated microalgae than the commercial medium, which makes it a far better way to be considered for application in industrial sectors that focus on the nutritional quality of microalgae such as pharmaceutical and cosmetical industries.

Declarations

Author contributions

Krishna Moorthy Abarna: Research conceptualization, Investigation, Writing - Original Draft, Review & Editing; Rani. V: Research conceptualization, Supervision, Review & Editing; Padmavathy. P: Writing – Review, Data Curation; Jawahar. P: Writing - Review & Editing, Data Curation; Uma. A: Formal Analysis, Data Curation; Kalidas. C: Formal Analysis, Investigation, Writing - review and editing, Manimozhi. N: Writing – Review, Data Curation

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Conflict of interest disclosure

There is no conflict of interest to disclose

Ethical Approval

Not applicable

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Figures

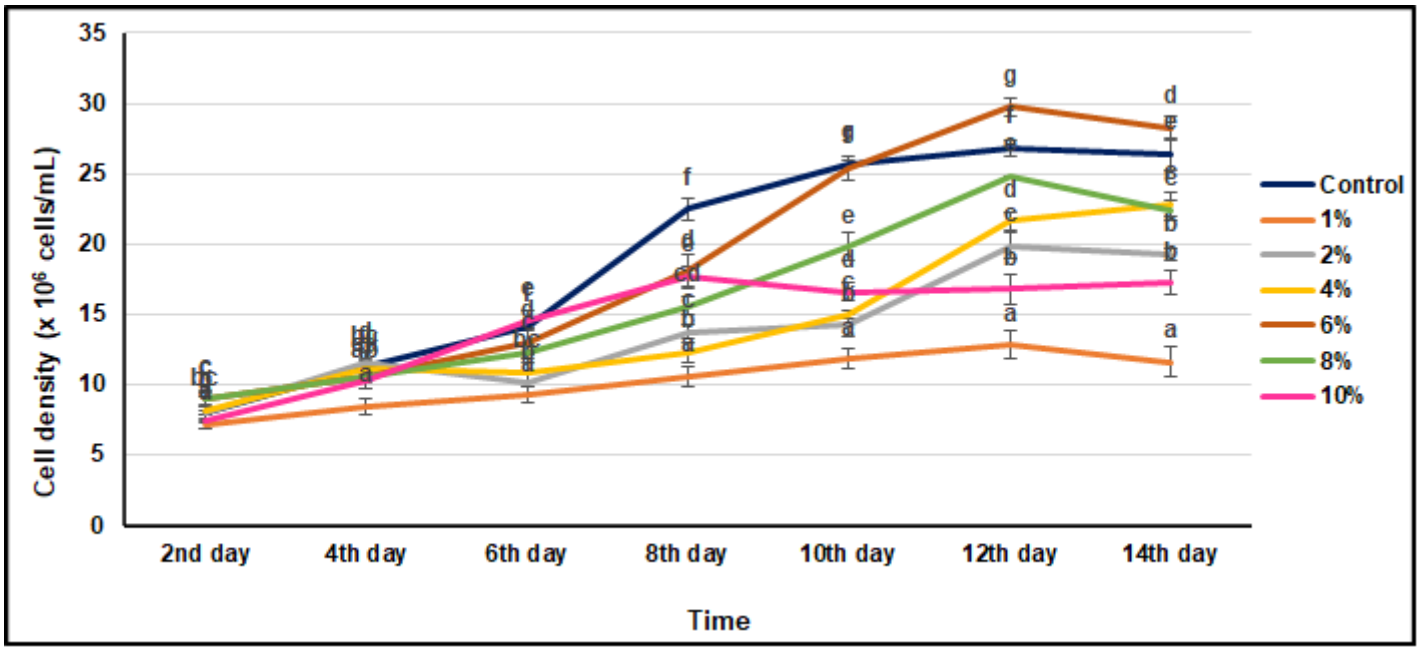


Figure 1

Growth curve of *N. oculata* in different concentrations of SLF-1

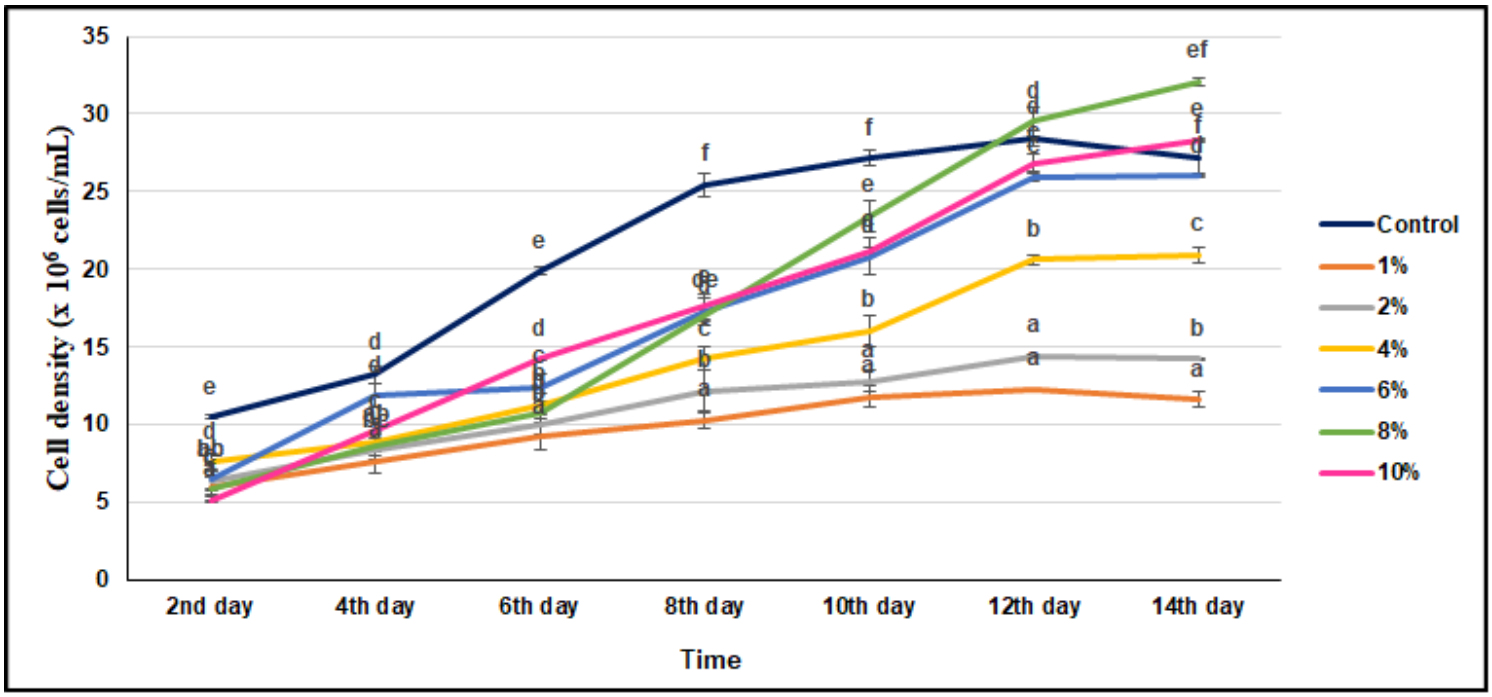


Figure 2

Growth curve of *N. oculata* in different concentrations of SLF-2

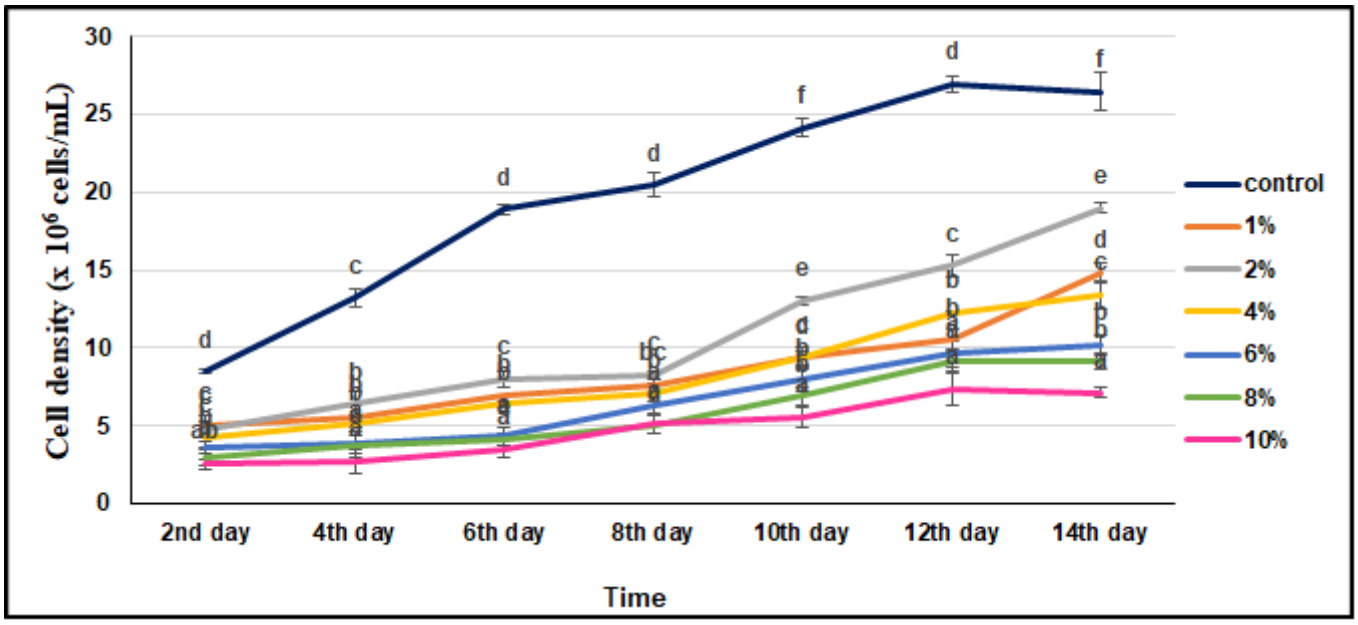


Figure 3

Growth curve of *N. oculata* in different concentrations of SLF-3

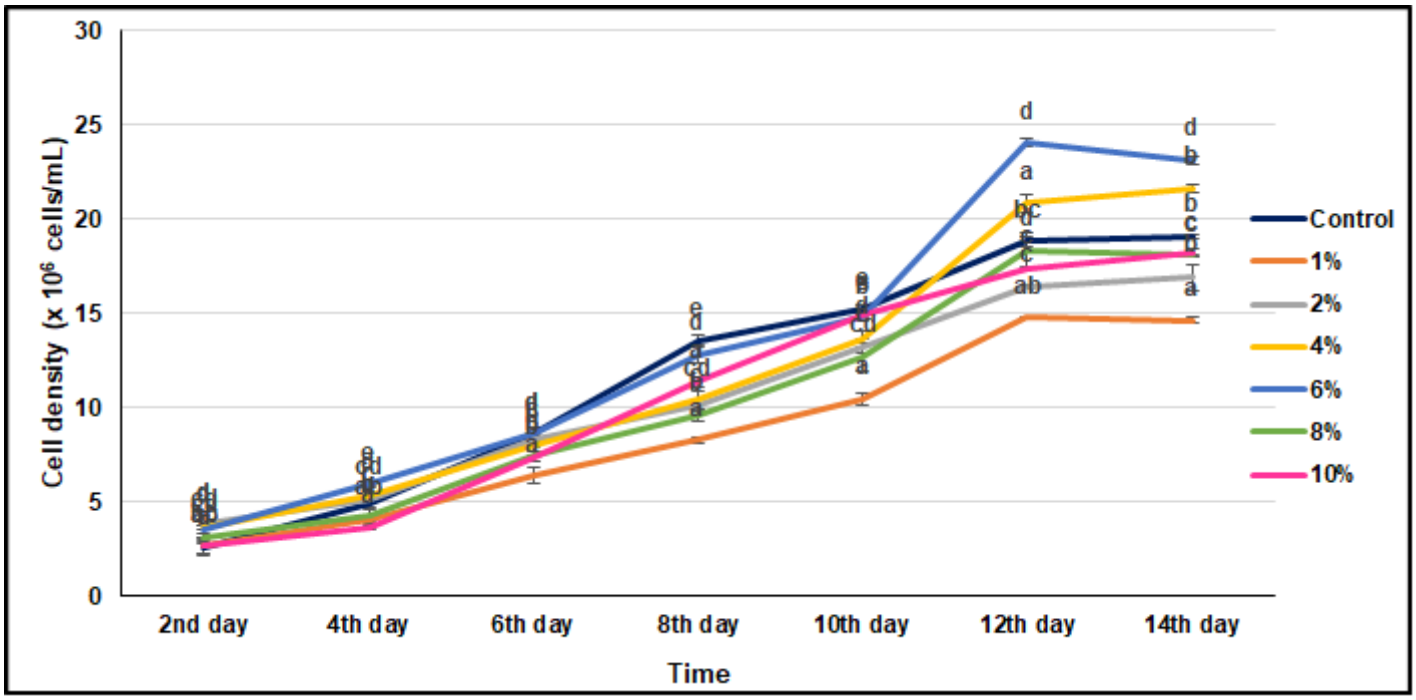


Figure 4

Growth curve of *D. salina* in different concentrations of SLF-1

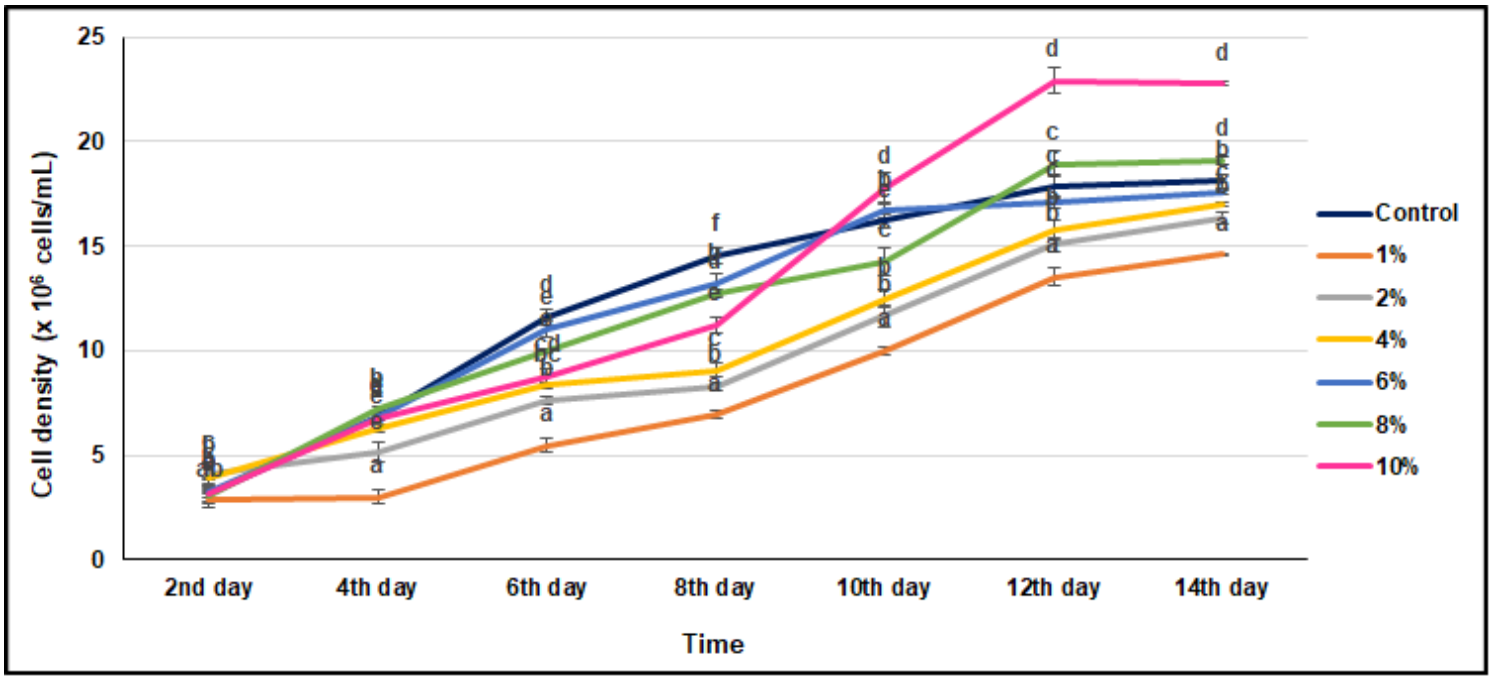


Figure 5

Growth curve of *D. salina* in different concentrations of SLF-2

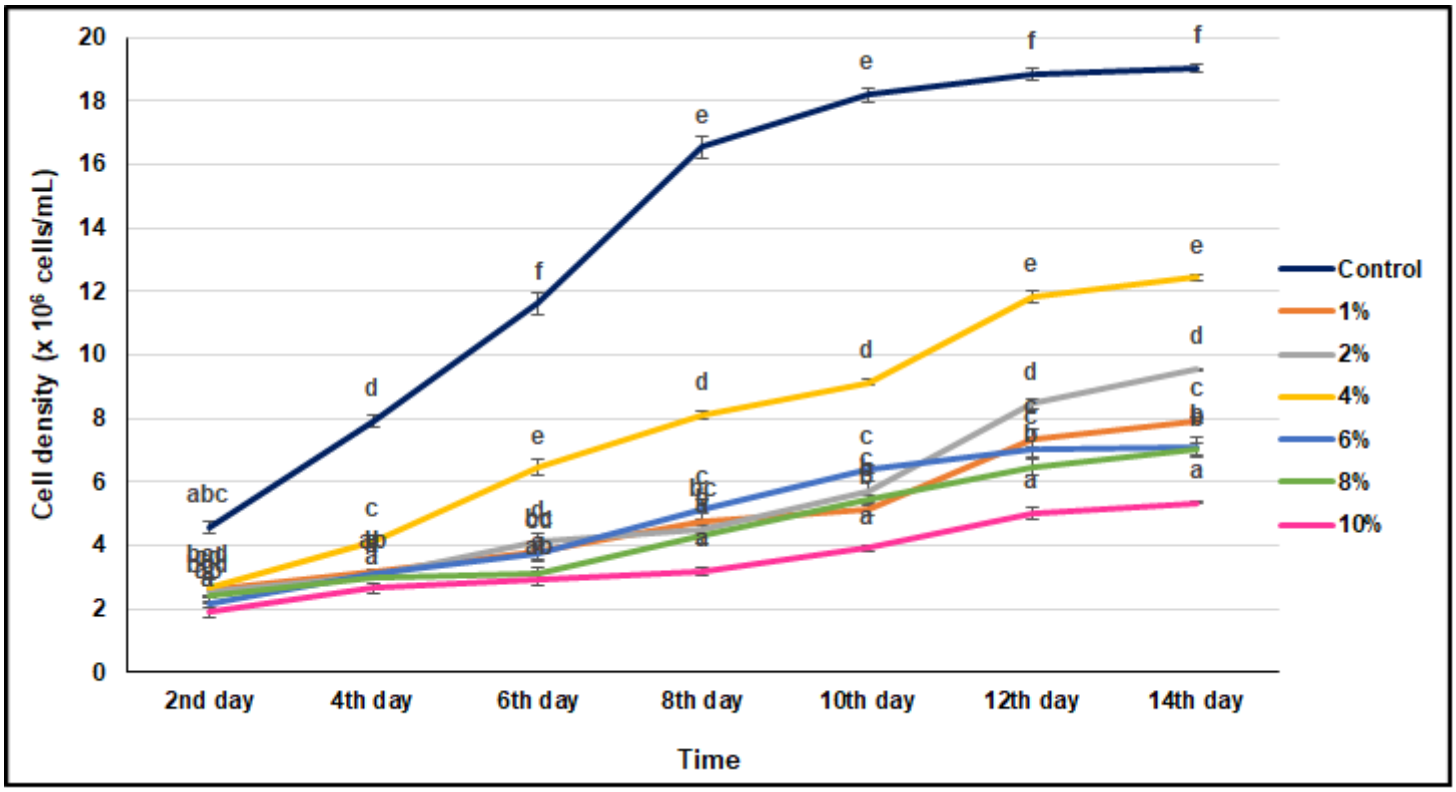


Figure 6

Growth curve of *D. salina* in different concentrations of SLF-3

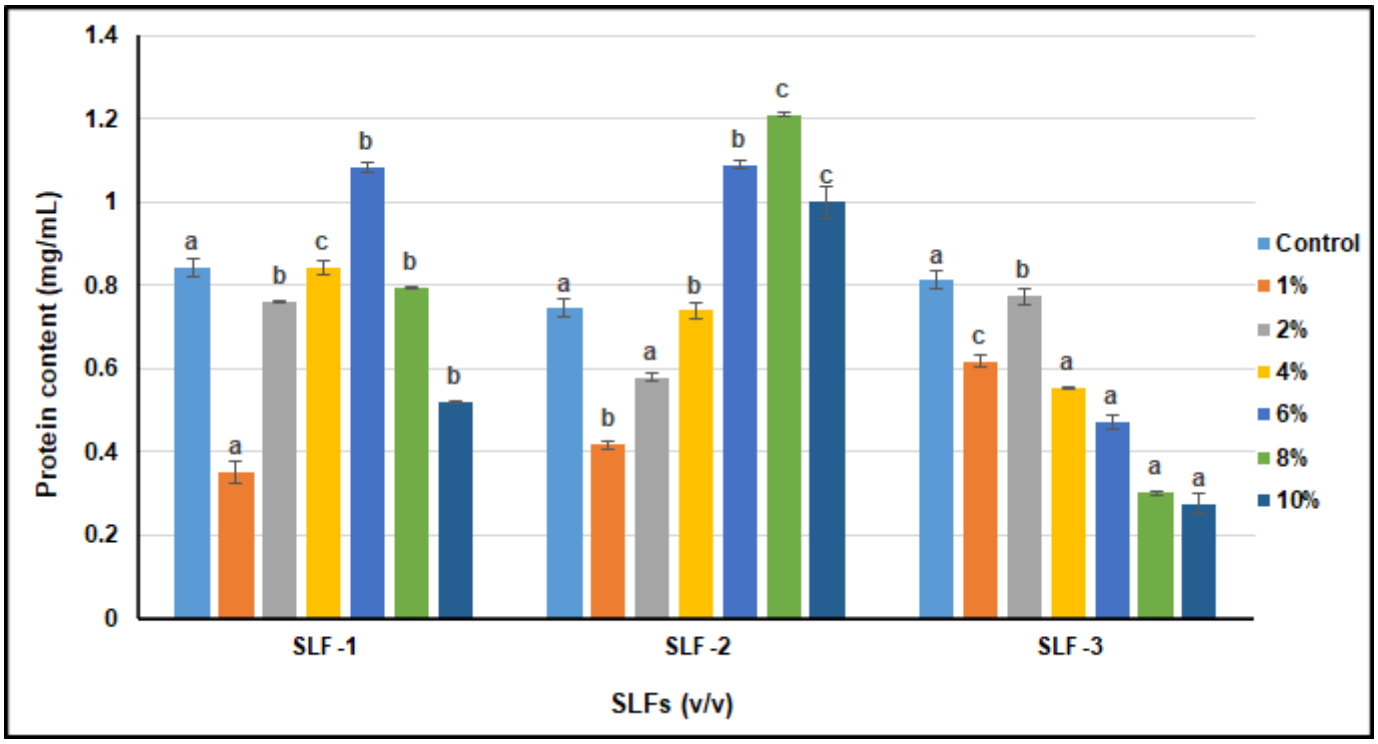


Figure 7

Protein content of *N. oculata* grown in three different SLFs at various concentrations

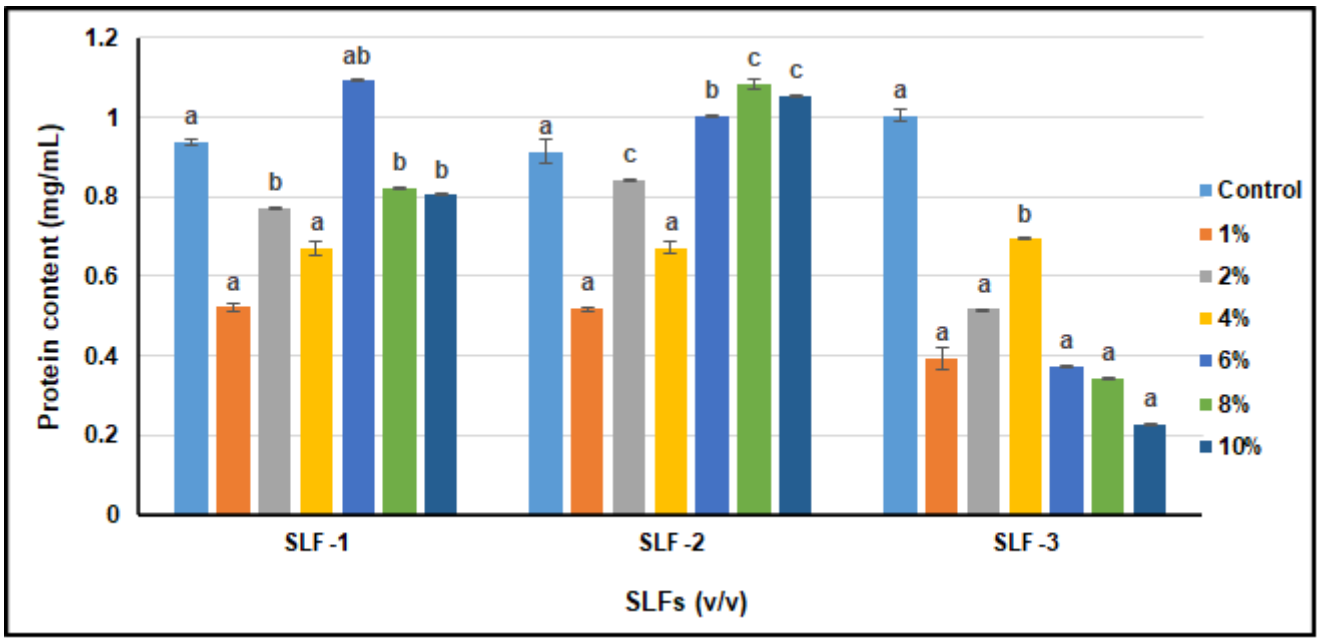


Figure 8

Protein content of *D. salina* grown in three different SLFs at various concentrations

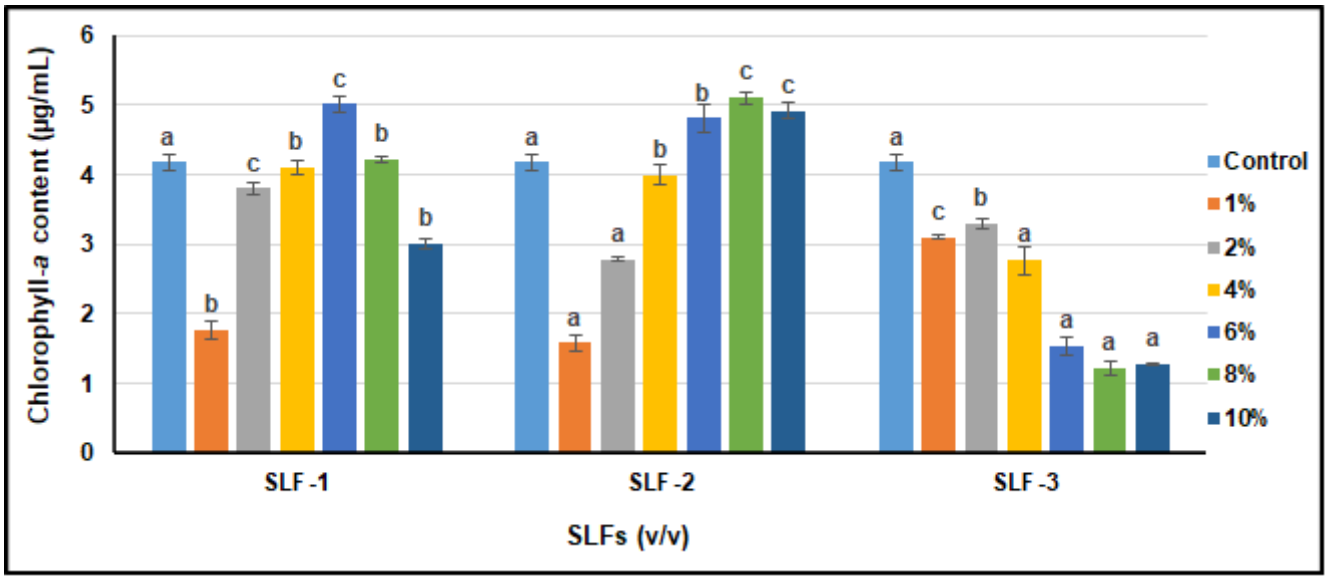


Figure 9

Chlorophyll- *a* content of *N. oculata*grown in three different SLFs at various concentrations

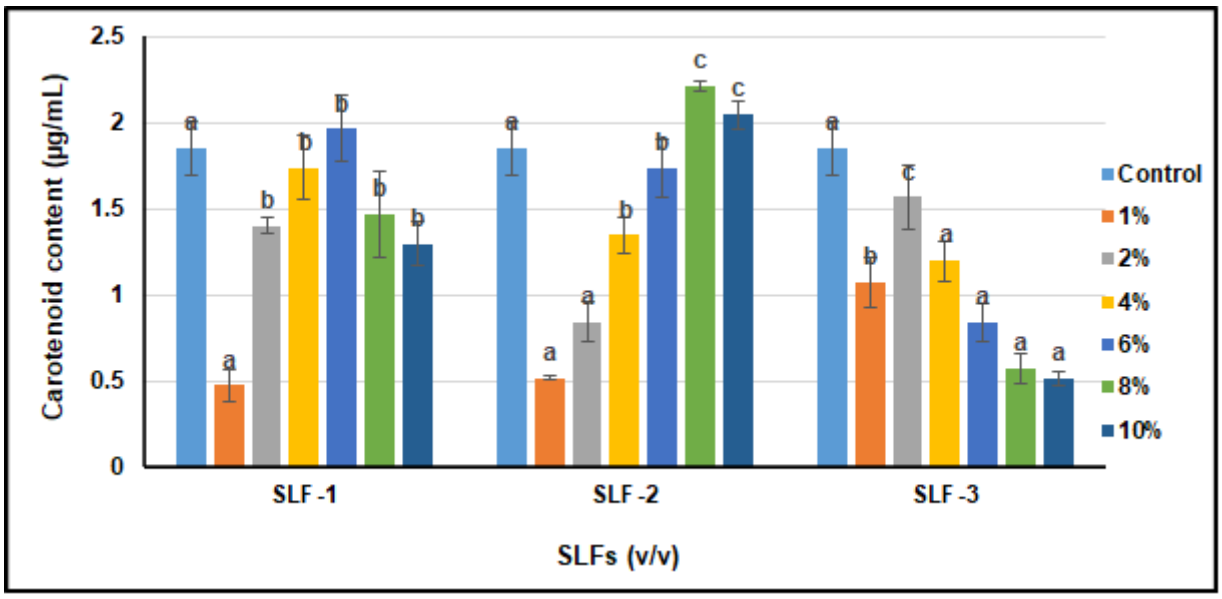


Figure 10

Carotenoid content of *N. oculata* grown in three different SLFs at various concentrations

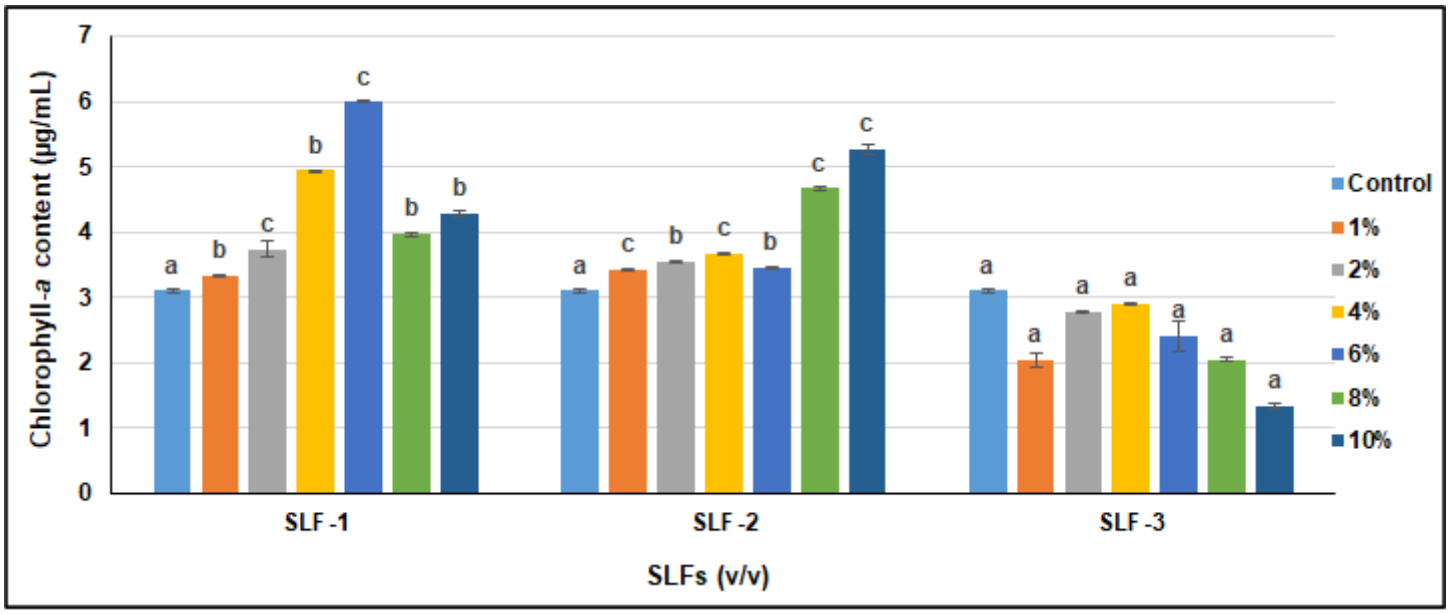


Figure 11

Chlorophyll- *a* content of *D. salina*grown in three different SLFs at various concentrations

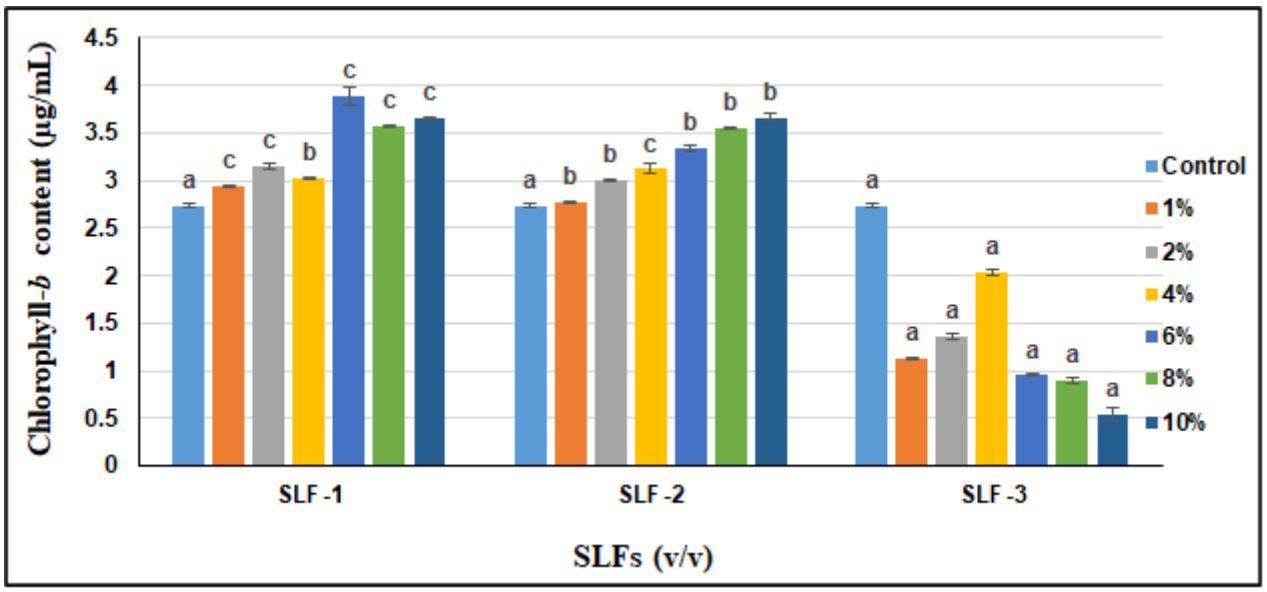


Figure 12

Chlorophyll- *b* content of *D. salina*grown in three different SLFs at various concentrations

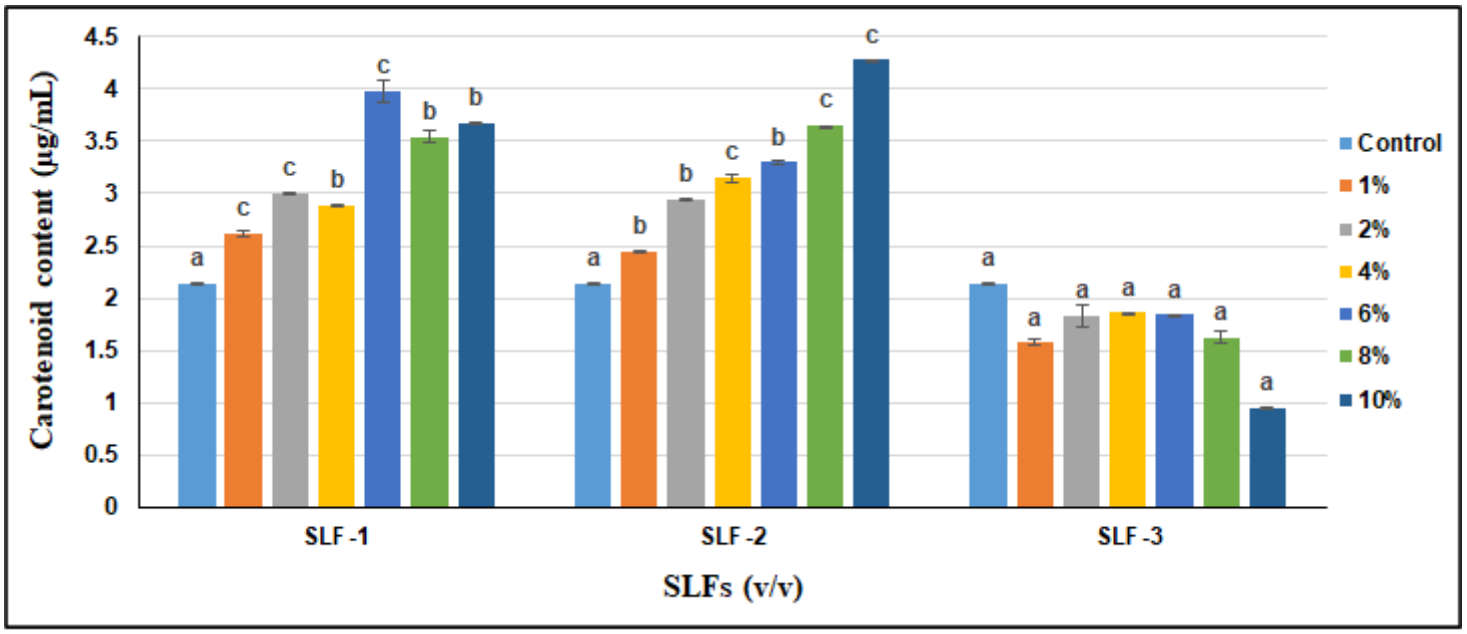


Figure 13

Carotenoid content of *D. salina* grown in three different SLFs at various concentrations

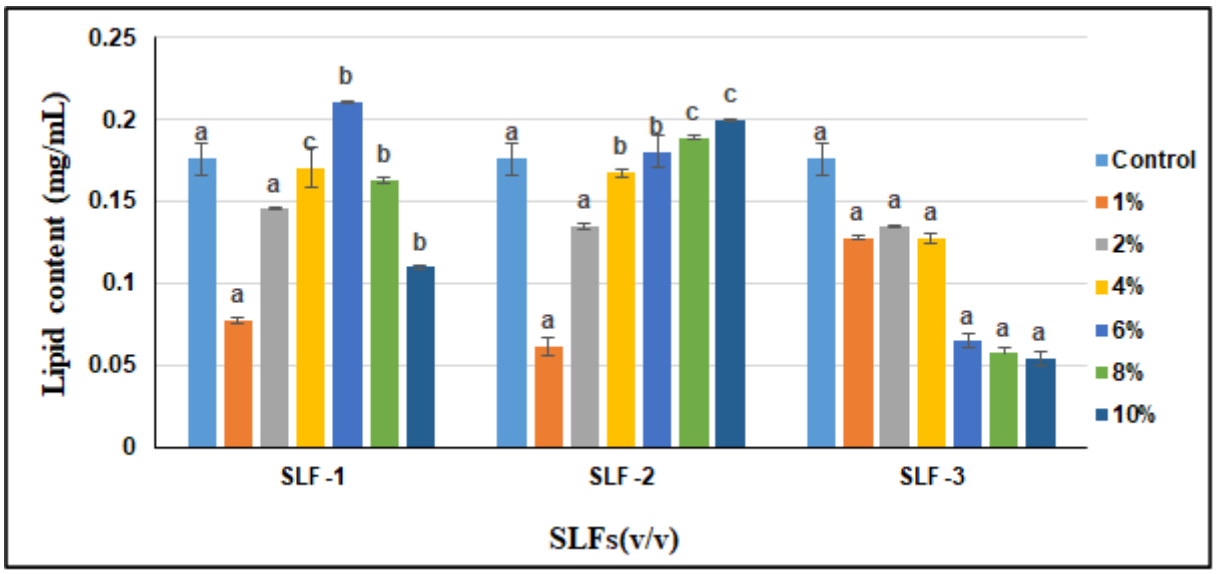


Figure 14

Lipid content of *N. oculata* grown in three different SLFs at various concentrations

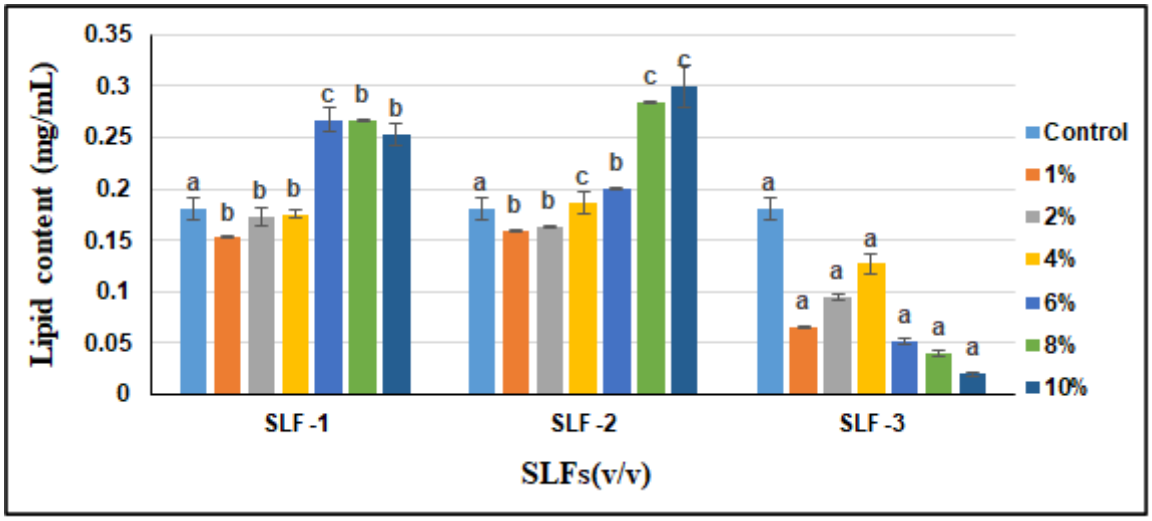


Figure 15

Lipid content of *D. salina* grown in three different SLFs at various concentrations