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Heterotrophic cultivation of *Nannochloropsis salina* for enhancing biomass and lipid production

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ABSTRACT

Response surface methodology (RSM) was used to enhance the biomass and lipid content in *Nannochloropsis* salina due to its economic importance. Preliminary screening results revealed that the heterotrophically cultivated *N. salina* with various carbon and nitrogen sources yielded higher biomass (0.91 ± 0.0035 g/L) and lipid content (37.1 ± 0.49 mg/L) than that of the photoautotrophical cultivation (0.21 ± 0.009 g/L and 22.16 ± 0.27 mg/L). Significant sources that greatly influenced on biomass and lipid content of the alga were optimized through RSM. The medium consisting of glucose (7.959 g/L), sodium acetate (1.46 g/L), peptone (7.6 g/L) and sodium thiosulphate (1.05 g/L) was found to be the optimal concentration for heterotrophic cultivation by response optimizer. Confirmation experiment results for the RSM optimized concentration yielded the biomass of 1.85 g/L and total lipid content of 48.6 mg/L. In this study, we provide with a strategy for enhancing the biomass and lipid content in *N. salina*.

1. Introduction

Polyunsaturated fatty acid (PUFA) plays an important role in human health and is often used in nutraceutical products. Several studies have suggested that EPA (20:5 n-3)-containing diets may increase resistance to cardiovascular diseases and cancer [1]. Intake of microalgae by fishes has been considered as the principal source of their EPA content [2]. Among the marine microalgae, *Nannochloropsis* sp., has been widely recognized as the most promising alternate sources to enhance the EPA content in fish oil and other valuable pigments [3]. Hence, several studies have been undertaken to enhance the biomass and lipid content of microalgae such as optimizing the medium composition, culture conditions and supplementing organic and inorganic nutrients [4–8].

Heterotrophic cultivation systems are a viable alternative to the more traditional photoautotrophic cultivation systems, which typically suffer from a deprived biomass, high production cost and low productivity. Several aquaculture algae species have been successfully cultivated under heterotrophic culture conditions. For examples, heterotrophic *Nitzschia laevis* [9], *Chlorella prototheocoides* [10] and *Galdieria sulphuraria* [11] were cultivated for the production of eicosapentaenoic acid, docosahexaenoic acid, biodiesel and c-phycocyanin. Additional benefits include the use of axenic culture for successive production of pure batches of algal products with consistent biochemical composition. In addition, heterotrophic culture not only can be used for improving the efficiency and also reducing the production cost of biomass and it can be used for efficient production of some metabolites such as lipids [12]. Moreover, heterotrophic cultivation has been considered as the potential method for reducing the production cost of economically viable products from microalgae [13].

To improve the biomass and lipid productivity, efficient medium composition for microalgae growth needs to be elucidated. Since, the media optimization using a one-factor-at-a-time approach is time consuming, expensive, and often leads to misinterpretation of results; it is replaced by response surface methodology (RSM), which is an efficient experimental strategy to seek optimal conditions for multivariable system. RSM has been successfully applied for the optimization of multiple variables in the fermentation processes with acceptable results [14]. With this background RSM was used in the present study for optimizing the medium composition to enhance biomass and lipid production of *Nannochloropsis* salina, under heterotrophic cultivation.

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2. Materials and methods

2.1. Microalgal culture

Stock culture of the marine microalga, *N. salina* was obtained from the Rajiv Gandhi Centre for Aquaculture, Marine Products Export Development Authority (MPEDA), Sirkali, Tamilnadu and maintained in 'Live Feed Stock Culture Facility' of the Marine Ornamental Fish Hatchery, CAS in Marine Biology, Annamalai University, using Walne medium [15].

N. salina was allowed to grow in photoautotrophic (control) and heterotrophic conditions using 300 mL (30 mL of inoculum + 270 mL of media composition) of sterile culture media with a pH of 7.8 ± 0.2, salinity 32 ± 1 g/kg and temperature 27 ± 1 °C in 500 mL of an Erlenmeyer flask. Mild aeration was provided continuously with an air pump to maintain the cultures and the flasks were kept under the light intensity of 3000 lux. Heterotrophic culture was allowed to grow in an orbital shaking incubator at 130 rpm without light exposure. Twenty micrograms per milliliter of chloramphenicol was added to the medium to prevent possible bacterial contamination during incubation. Each culture was maintained in triplicate.

2.2. Medium formulation

Screening of the best carbon and nitrogen sources for biomass and lipid production was done in one variable at a time manner. Briefly, two carbon sources, glucose (1, 2 and 4 g/L) and sodium acetate (0.25, 0.5 and 1 g/L) were added to Walne medium with 1 g/L of peptone as the basal medium. Subsequently, the nitrogen sources viz. peptone (1, 2 and 4 g/L), yeast extract (1, 2 and 4 g/L), meat extract (1, 2 and 4 g/L), malt extract (0.5, 1 and 2 g/L), urea (0.5, 1 and 2 g/L), sodium nitrate (0.5, 1 and 2 g/L) and ammonium nitrate (0.5, 1 and 2 g/L) were added to the Walne medium with 1 g/L of glucose as the basal medium. All microbiological grade carbon and nitrogen sources were recognized to be stable when subjected to sterilization at 121 °C, 103.4 kPa for 15 min.

2.3. Determination of biomass and lipid content

Biomass in terms of cell concentration of *N. salina* was measured, using UV–vis spectrophotometers (Thermoscientific, Evolution 201, USA) at an absorbance of 665 nm. Dry cell weight of microalga biomass was calculated by the method of Chiu et al. [16] where the algal cells were harvested in the late log phase and centrifuged at 6500 g for 5 min. Centrifuged samples were washed twice with distilled water and freeze–dried for 24 h. Absorbance of OD value was converted into dry weight from the calibration curves of the linear regression equation. These parameters corresponded with OD at 665 nm value analyzed by using regression equation: $y = 0.55x$ ($R^2 = 0.996$), where y ($g\,L^{-1}$) is the dry cell weight of the microalgae and x is an absorbance of suspension at 665 nm. Total lipid of the experimental microalga was extracted using Folch et al. [17] and according to the method suggested by Lepage and Roy [18].

2.4. Response surface methodology

There were 31 sets of experiments generated using central composite design (CCD) with variables such as glucose, sodium acetate, sodium thiosulphate and peptone. Variables were selected from the preliminary investigation results and published report by Feng et al. [19], which showed the considerable effects on biomass and lipid production. Experiments at the central points were run to determine the curvature and to compensate for the lack of fit values which indicate the significance of the model. The experimental results of RSM were fitted with the response surface regression procedure, using the following second order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where Y is the predicted response, X_i and X_j are independent factors, β_0 is the intercept, β_i denotes linear coefficient, β_{ii} denotes the quadratic coefficient and β_{ij} is an interaction coefficient. Analysis of experiments was performed using the design of experiment software MINITAB, version 16 (PA, USA). The same starting culture was used for the photoautotrophic control and optimal medium optimized for heterotrophic cultivation of *N. salina* by RSM.

2.5. Fatty acid analysis by gas chromatography (GC)

Freeze dried micro-algal photoautotrophic (control) and heterotrophic samples were ground finely with mortar and pestle. Preparation and analysis of fatty acid methyl esters (FAMEs) from the algal biomass were performed by the method described by Anon [20]; 50 ± 0.1 mg of powder samples were added to 1 mL of 1.2 M NaOH in 50% (V/V) of methanol in a screw-cap tube and then the solutions were maintained at 100 °C for 30 min in a water bath for saponification. Then, the saponified samples were allowed to cool at room temperature for 25 min and were methylated by adding 2 mL of a mixture of 54% (V/V) of 6 N HCl with 46% (V/V) of aqueous methanol. This solution mixture was maintained at 80 °C for 10 min. After rapid cooling, fatty acids were extracted with 1.25 mL of 50% (V/V) diethyl ether prepared with hexane. Then,

Table 1
Effect of different types and concentrations of carbon and nitrogen sources on biomass and lipid production of *N. salina* in heterotrophic conditions.

Sources	Concentration (g/L)	Biomass (g/L)	Lipid (mg/L)
Carbon			
Glucose	1	0.39 ± 0.023	26.6 ± 0.37
	2	0.57 ± 0.056	31.4 ± 0.21
	4	0.86 ± 0.062	37.1 ± 0.49
Sodium acetate	0.25	0.31 ± 0.036	23.5 ± 0.32
	0.5	0.36 ± 0.025	26.4 ± 0.24
	1	0.33 ± 0.032	25.5 ± 0.24
Nitrogen			
Peptone	1	0.57 ± 0.0027	26.3 ± 0.15
	2	0.73 ± 0.0029	23.2 ± 0.29
	4	0.91 ± 0.0035	22.5 ± 0.35
Yeast extract	1	0.24 ± 0.0071	21.4 ± 0.15
	2	0.28 ± 0.0052	19.5 ± 0.23
	4	0.31 ± 0.0083	17.4 ± 0.57
Meat extract	1	0.41 ± 0.0032	15.3 ± 0.1
	2	0.35 ± 0.0051	14.2 ± 0.26
	4	0.22 ± 0.0047	11.5 ± 0.39
Malt extract	0.5	0.23 ± 0.0003	22.3 ± 0.36
	1	025 ± 0.0065	19.4 ± 0.81
	2	0.42 ± 0.0061	16.6 ± 0.53
Urea	0.5	0.24 ± 0.0053	21.4 ± 0.31
	1	0.18 ± 0.0036	23.4 ± 0.22
	2	0.16 ± 0.0021	24.6 ± 0.25
Sodium nitrate	0.5	0.29 ± 0.0036	21.4 ± 0.27
	1	0.38 ± 0.0042	19.7 ± 0.36
	2	0.43 ± 0.0028	15.2 ± 0.69
Ammonium nitrate	0.5	0.29 ± 0.0029	22.8 ± 0.73
	1	0.25 ± 0.0025	21.4 ± 0.26
	2	0.18 ± 0.0038	20.2 ± 0.14

samples were allowed to phase separate for 10 min. The collected top phases were mixed with 3 mL of 0.3 M NaOH for 5 min and used for the analysis. Before analyzing the samples, FAMEs were cleaned with anhydrous sodium sulphate and then transferred to Gas chromatography sample vials. The FAMEs were separated by GC (HP 6890 N, Agilent Technologies, USA), using ultra–2 capillary columns (25 m × 2 mm × 0.33 μ film thickness). Oven initial temperature was 170 °C, ramp 1 and 2 maintained the temperature 5–260 °C and 40–310 °C respectively. Split injector ratio was 100:1, and Hydrogen used as carrier gas at a flow rate of 30 mL/min and Nitrogen used as makeup gas, at a flow rate of 30 mL/min. FAMEs profiles were detected by flame ionization detector (FID) and identified by comparing the commercial Eucary database with MIS Software package (MIS Ver. No. 3.8).

3. Results

3.1. Preliminary investigation of carbon and nitrogen sources on heterotrophic growth of N. salina

Preliminary investigations on various concentrations of carbon and nitrogen sources were employed to cultivate *N. salina* under heterotrophic conditions (Table 1). Maximum biomass (0.86 ± 0.062 g/L) and lipid (37.1 ± 0.49 mg/L) content of the heterotrophic culture was obtained at 4 g/L of glucose. In addition, 0.5 g/L of sodium acetate supplemented media showed the maximum biomass of 0.36 ± 0.025 g/L and lipid content of 26.4 ± 0.24 mg/L respectively. Higher biomass 0.91 ± 0.0035 g/L was obtained at 4 g/L of peptone supplementation, but the gradual increase in the peptone concentration marginally reduced the total lipid content. Whereas, photoautotrophic cultivated alga with Walne medium yielded maximum biomass of 0.21 ± 0.009 g/L and 22.16 ± 0.27 mg/L of lipid content.

3.2. Heterotrophic cultivation using central composite design

In this study, heterotrophic culture medium was further statistically optimized using RSM. Range of the variables, experimental designs and results obtained, for biomass and lipid content of *N. salina* are given in Table 2. The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation for biomass and lipid production, respectively. The response of biomass production (Y1) by *N. salina* can be expressed in terms of the following regression Eq. (1):

$$Y_1 = -3.149 + 0.956X_1 + 0.07562X_2 + 0.28055X_3 + 0.03906X_4 - 0.05884X_1^2 - 0.60052X_2^2 - 0.02125X_3^2 - 0.07819X_4^2 + 0.02108X_1X_2 - 0.00083X_1X_3 - 0.00439X_1X_4 + 0.09143X_2X_3 + 0.47364X_2X_4 - 0.07214X_3X_4 \quad (1)$$

Where X_1 is glucose, X_2 is sodium acetate, X_3 is sodium thiosulphate and X_4 is peptone. The statistical significance of Eq. (1) was checked by an *F* test, and by the analysis of variance (ANOVA) for the response surface model. It is clear from Table 3 that the model is highly significant, as demonstrated by the model *F* value and a very low probability value ($P < 0.001$). The goodness of fit of the model can be verified by the determination coefficient R^2 (0.981) and the value of lack-of-fit was insignificant ($P = 0.569$).

The response of lipid production (Y_2) by *N. salina* can be expressed in terms of the following regression Eq. (2):

$$Y_2 = -203.812 + 38.877X_1 + 73.658X_2 + 9.222X_3 + 25.616X_4 - 1.681X_1^2 - 21.166X_2^2 - 0.735X_3^2 - 4.144X_4^2 - 5.722X_1X_2 - 0.212X_1X_3 - 1.379X_1X_4 + 2.777X_2X_3 - 2.746X_2X_4 - 0.241X_3X_4 \quad (2)$$

Table 2
Response surface method design in actual level of variables and the predicted and observed responses functions for optimizing the media composition of *N. salina* under heterotrophic cultivation.

Run order	Glucose (g/L)	Sodium acetate (g/L)	Peptone (g/L)	Sodium thiosulphate (g/L)	Biomass		Lipid	
					Observed (g/L)	Predicted (g/L)	Observed (mg/L)	Predicted (mg/L)
1	6.25	1.25	6.5	0.5375	1.4763	1.53843	35.7370	35.9195
2	8.75	1.25	6.5	0.5375	1.7847	1.77049	46.0236	46.9064
3	6.25	1.75	6.5	0.5375	1.1792	1.16578	30.3100	31.4074
4	8.75	1.75	6.5	0.5375	1.3428	1.42419	33.9520	35.2420
5	6.25	1.25	9.5	0.5375	1.5534	1.57109	33.5662	34.3670
6	8.75	1.25	9.5	0.5375	1.7641	1.79691	42.7780	43.7658
7	6.25	1.75	9.5	0.5375	1.2548	1.33559	33.2910	34.0211
8	8.75	1.75	9.5	0.5375	1.6152	1.58776	36.3659	36.2677
9	6.25	1.25	6.5	1.5125	1.4629	1.51356	38.5140	39.3325
10	8.75	1.25	6.5	1.5125	1.7563	1.73493	46.7420	46.9585
11	6.25	1.75	6.5	1.5125	1.3452	1.37181	33.5230	33.4818
12	8.75	1.75	6.5	1.5125	1.6140	1.61953	34.0360	33.9555
13	6.25	1.25	9.5	1.5125	1.3572	1.33523	37.4173	37.0740
14	8.75	1.25	9.5	1.5125	1.5137	1.55034	43.4890	43.1119
15	6.25	1.75	9.5	1.5125	1.2932	1.33063	35.5520	35.3895
16	8.75	1.75	9.5	1.5125	1.5748	1.57209	33.5110	34.2751
17	5.00	1.50	8.0	1.0250	1.1836	1.10497	29.4460	28.7383
18	10.00	1.50	8.0	1.0250	1.5825	1.57850	39.5700	38.6108
19	7.50	1.00	8.0	1.0250	1.7647	1.73483	46.3136	45.5628
20	7.50	2.00	8.0	1.0250	1.4367	1.38393	33.1300	32.2139
21	7.50	1.50	5.0	1.0250	1.5730	1.52567	39.5320	38.1825
22	7.50	1.50	11.0	1.0250	1.5462	1.51090	37.2670	36.9496
23	7.50	1.50	8.0	0.0500	1.7240	1.65545	41.6330	39.5298
24	7.50	1.50	8.0	2.0000	1.6290	1.61492	40.5140	40.9503
25	7.50	1.50	8.0	1.0250	1.6380	1.70951	43.6230	44.1799
26	7.50	1.50	8.0	1.0250	1.6594	1.70951	43.6530	44.1799
27	7.50	1.50	8.0	1.0250	1.6856	1.70951	43.5380	44.1799
28	7.50	1.50	8.0	1.0250	1.6842	1.70951	45.6420	44.1799
29	7.50	1.50	8.0	1.0250	1.8290	1.70951	43.6585	44.1799
30	7.50	1.50	8.0	1.0250	1.7841	1.70951	43.1167	44.1799
31	7.50	1.50	8.0	1.0250	1.6863	1.70951	46.0279	44.1799

Table 3
Analysis of variance (ANOVA) for biomass and lipid production response surface quadratic model.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Biomass						
Regression	14	0.94212	0.942120	0.067294	14.55	0.000
Linear	4	0.52384	0.170770	0.042693	9.23	0.000
Square	4	0.30079	0.300788	0.075197	16.26	0.000
Interaction	6	0.11749	0.117494	0.019582	4.23	0.010
Residual error	16	0.07401	0.074008	0.004625	–	
Lack-of-fit	10	0.04479	0.044791	0.004479	0.92	0.569
Pure error	6	0.02922	0.029217	0.004870	–	–
Total	30	1.01613	–	–	–	–
Lipid						
Regression	14	786.680	786.680	56.191	36.32	0.000
Linear	4	418.795	261.058	65.265	42.19	0.000
Square	4	283.263	283.263	70.816	45.78	0.000
Interaction	6	84.621	84.621	14.103	9.12	0.000
Residual error	16	24.751	24.751	1.547		
Lack-of-fit	10	16.796	16.796	1.680	1.27	0.402
Pure error	6	7.955	7.955	1.326	–	–
Total	30	811.430	–	–	–	–

The model characteristics were measured to determine if it could be efficiently utilized. The model is under a high R^2 (0.953) value, a significant *F* value and an insignificant lack-of-fit *F* value (Table 3).

3.3. Mutual effects of variables on biomass and lipid content

Growth period of heterotrophic cultivation of *N. salina* was slow while comparing to photoautotrophic cultivation (Fig. 1). Foremost goal of the three-dimensional response surface plots (graphical representation of the regression equation) (Figs. 2 and 3) is to efficiently hunt for the predicted values of the variables. The optimal values of glucose (7.959 g/L), sodium acetate (1.46 g/L), peptone (7.6 g/L) and sodium thiosulphate (1.05 g/L) respectively, with a predicted biomass of 1.795 g/L and lipid (45%) content. The confirmation experiment conducted for the predicted optimum conditions and the results showed biomass and lipid content were 1.85 g/L and 48.6% respectively. The biomass and lipid content from the experiment was similar to the predicted value, thereby revealing the higher accuracy of the model.

3.4. Fatty acid compositions of N. salina cultivated under photoautotrophic and heterotrophic condition

Fatty acid profile of *N. salina* cultivated under photoautotrophic and heterotrophic conditions is given in Table 4. The results showed that *N. salina* fatty acid profile was altered under these culture conditions. Photoautotrophically cultivated alga exhibited higher amount of saturated fatty acid (SFAs), followed by mono unsaturated fatty acid (MUFAs) and poly unsaturated fatty acid (PUFAs). Whereas, heterotrophically grown algae with the optimized medium showed considerable reduction in SFAs content, but notable increases in PUFAs content compared to that of photoautotrophically cultivated alga. There was no such variation in MUFAs content between these two culture conditions. However, in the present study, the optimized medium for heterotrophic cultivation of *N. salina* showed a considerable increases in eicosapentaenoic acid (EPA) content ($30.54 \pm 1.19\%$), while it was $17.4 \pm 0.62\%$ in photoautotrophic cultivation, suggesting that the optimized medium for *N. salina* could be used for enhancing the EPA content in *N. salina*.

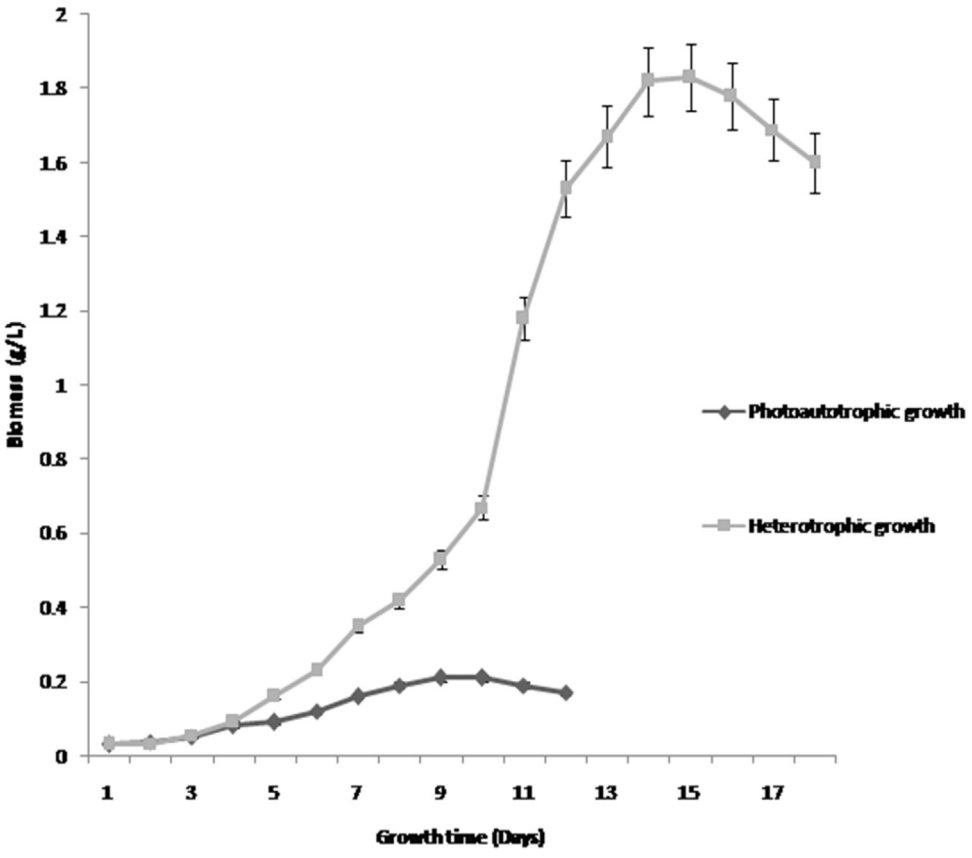

Fig. 1. Photoautotrophic and heterotrophic growth of *N. salina*.

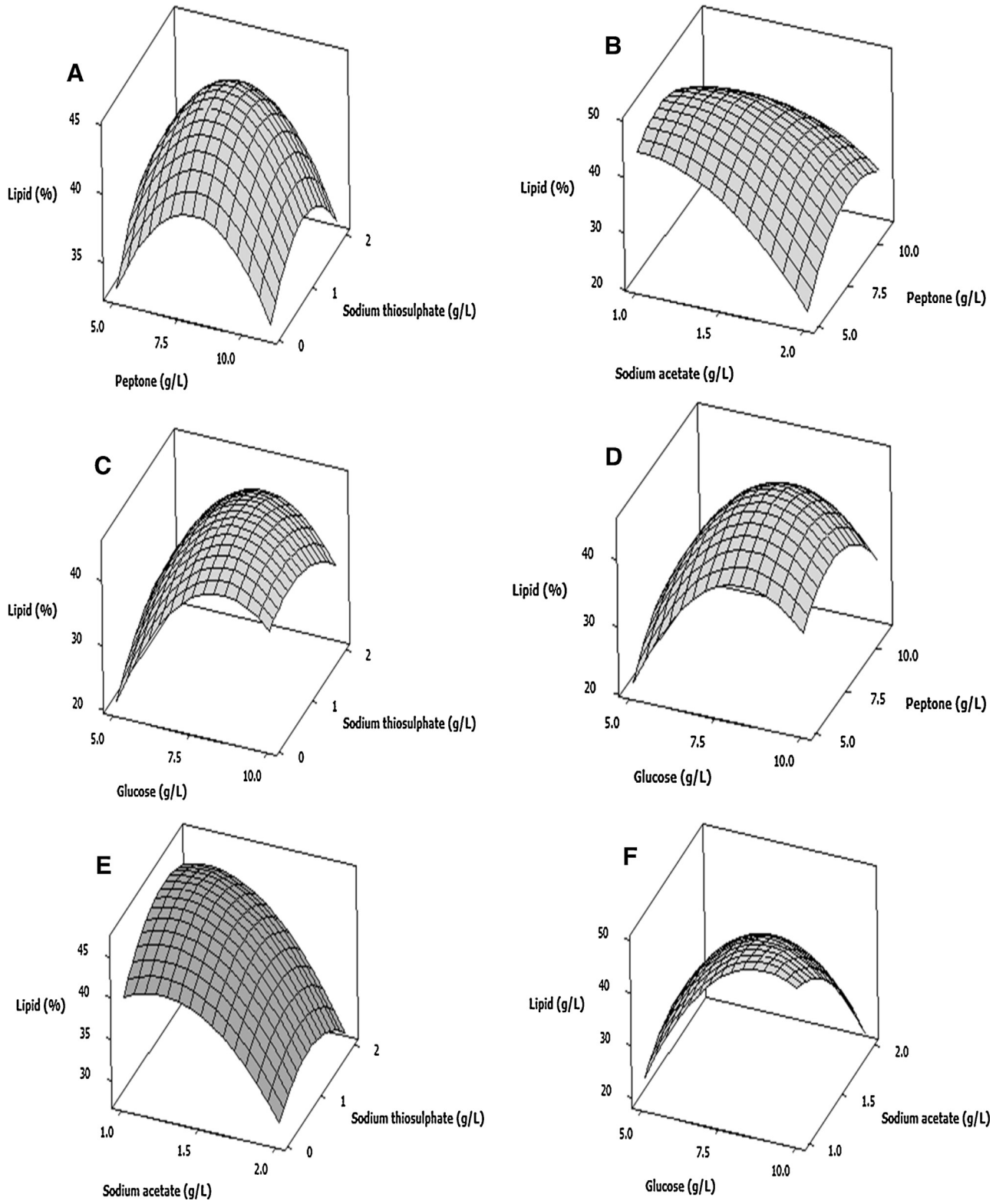

Fig. 2. Response surface plots showing the mutual effect of peptone and sodium thiosulphate (A), sodium acetate and peptone (B), glucose and sodium thiosulphate (C), glucose and peptone (D), sodium acetate and sodium thiosulphate (E), and glucose and sodium acetate (F) concentration of lipid production, when other variables are held at constant level.

4. Discussion

Nowadays, optimizing the media composition of microalgae for improving biomass and lipid content has been considered as the major factor for the sustainable product development. The algal lipids were widely used in several industrial applications and enhancing the biomass is also to be considered as the major bottleneck in economic concern. In the present study, glucose supplemented culture under the heterotrophic condition yielded the maximum biomass and lipid content than that of the sodium

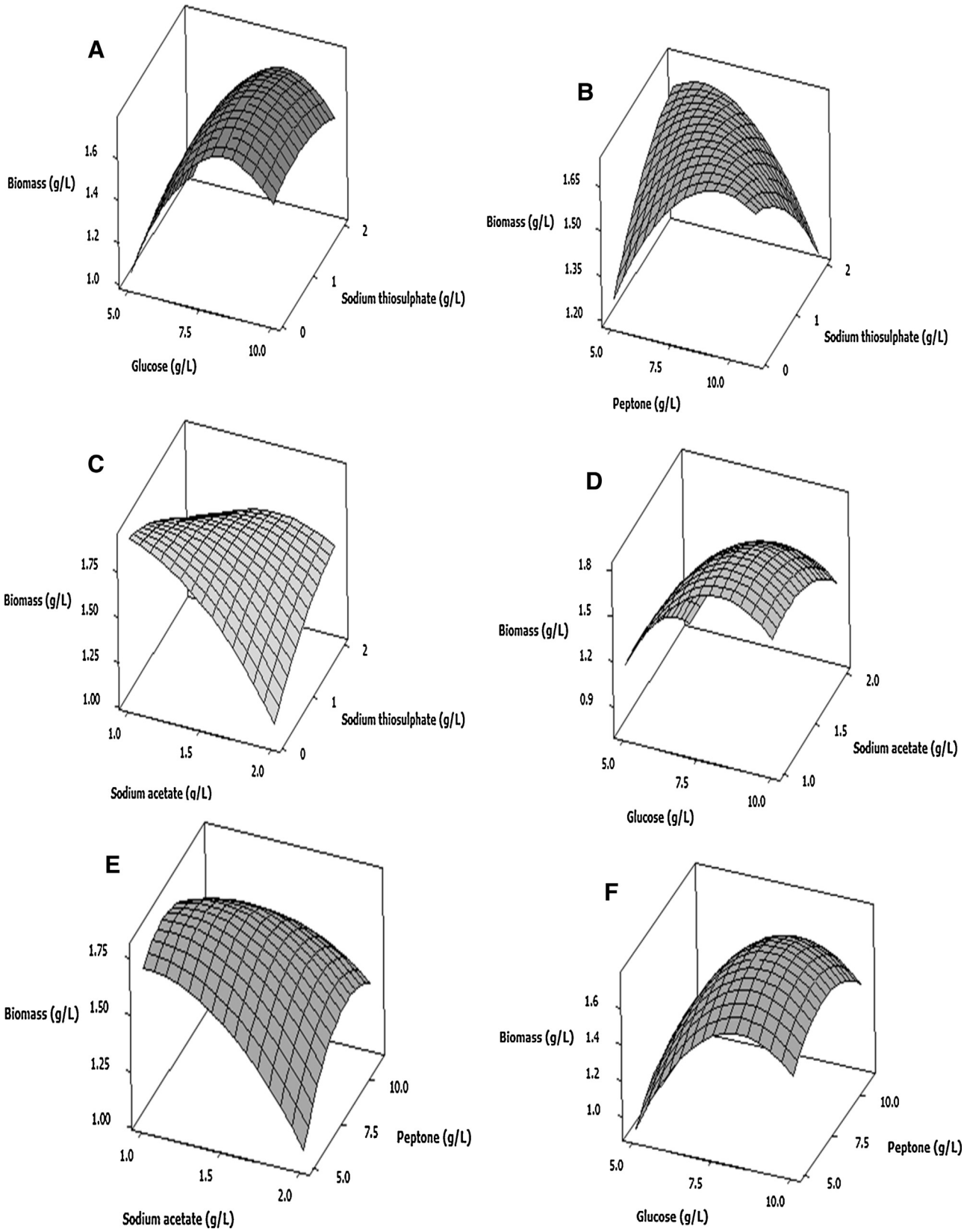

Fig. 3. Response surface plots showing the mutual effect of glucose and sodium thiosulphate (A), peptone and sodium thiosulphate (B), sodium acetate and sodium thiosulphate (C), glucose and sodium acetate (D), sodium acetate and peptone (E) glucose and peptone (F) concentration of biomass production, when other variables are held at constant level.

Table 4
Fatty acid composition of *N.salina* cultivated under photoautotrophic and heterotrophic condition.

Carbon chain	Fatty acids	Hetorotrophically cultivated alga fatty acid (%)	Photoautotrophically cultivated alga fatty acid (%)
12:00	Lauric acid	2.80 ± 0.037	1.3 ± 0.002
14:00	Myristic acid	8.01 ± 0.009	5.9 ± 0.01
16:00	Palmitic acid	22.84 ± 0.265	40.7 ± 0.55
18:00	Stearic acid	2.01 ± 0.005	0.78 ± 0.001
20:00	Arachidic acid	1.48 ± 0.015	0.3 ± 0.001
22:00	Behenic acid	0.86 ± 0.002	0.48 ± 0.002
24:00	Lignoceric acid	1.41 ± 0.173	1.16 ± 0.005
Σ of SFA	**Saturated fatty acid**	**39.41 ± 0.506**	**50.62 ± 0.571**
14:1 n-7	*Cis*-7 Myristoleic acid	1.75 ± 0.01	2.7 ± 0.023
16:1 n-7	*Trans*-7-Palmitoleic acid	4.71 ± 0.005	8.6 ± 0.36
18:1 n-7	*Cis*-7-Octadecenoic acid	4.65 ± 0.028	5.5 ± 0.15
18:1 n-9	Oleic acid	3.92 ± 0.01	4.9 ± 0.03
20:1 n-11	*Trans*-7-Eicosenoic acid	2.86 ± 0.007	1.83 ± 0.02
20:1 n-9	*Cis*-9-Eicosenoic acid	1.32 ± 0.015	0.26 ± 0.001
22:1 n-9	*Cis*-9-Docosenoic acid	0.72 ± 0.004	0.43 ± 0.008
24:1 n-9	*Trans*-9-Tetracosenoic acid	1.67 ± 0.027	1.32 ± 0.017
Σ of MUFAs	**Mono unsaturated fatty acid**	**21.6 ± 0.106**	**25.54 ± 0.60**
18:2 n-6	Linoleic	3.65 ± 0.21	1.87 ± 0.03
18:3 n-3	Alfa-linolenic	0.58 ± 0.56	0.3 ± 0.001
18:4 n-3	Stearidonic	1.62 ± 0.19	2.37 ± 0.026
20:5 n-3	Eicosapentaenoic	30.54 ± 1.19	17.4 ± 0.62
22:6 n-3	Docosahexaenoic	2.6 ± 0.04	1.9 ± 0.007
Σ of PUFAs	**Poly unsaturated fatty acid**	**38.99 ± 2.19**	**23.84 ± 0.68**

acetate supplementation. In agreement with the present study, Shi et al. [21] have reported that *C. protothecoides* has significantly enhanced the final cell concentration ranging from 4.9 to 31.2 g/L, while increasing the glucose concentrations in the culture medium with only a slight reduction in the cell yield (0.49–0.39 g/g). In addition, Fang et al. [22] have reported that *Nannochloropsis* sp., could utilize the carbon sources for their mixotrophic and heterotrophic growth. Moreover, glucose is the most commonly used carbon source in microalgae cultivation as it has enhanced the lipid production [23]. However, degradation of glucose in plant cells causes the generation of reactive oxygen (e.g. $^1O_2$ and O^{2-}), which can greatly damage the bio-macromolecule. Excess of glucose in the culture medium can produce more reactive oxygen than microalgae cells themselves can scavenge. Sodium thiosulphate is a reducing agent could scavenge the reactive oxygen effectively and protect cells against damage caused by the biodegradation of exogenous organic carbon in culture of zooblasts and bacteria [24]. Similarly, Wang et al. [25] have observed the preventing effects of sodium thiosulphate on the membrane lipid damage of *Synechocystis* sp. PCC 6803 caused by high concentration of glucose supplementation under heterotrophic culture condition. In addition, combination of different concentrations of glucose (2.5–5 mM) with sodium thiosulphate (2.5–5 mM) has increased the growth and fatty acid production in *Chlorella* sp. [19]. It is proved that the microalgae species *C. protothecoides* [26], *Crypthecodium cohnii* [27,28] are having the capacity for uptaking of carbohydrates (eg., glucose) directly and transforming them to lipid. This heterotrophic metabolism allows much higher cell density and lipid production in *C. protothecoides*, representing a promising approach for algal oil production [29].

The stimulated effects of acetate uptake on growth of several species of microalgae such as *Haematococcus lacustris*, *Navicula saprophila*, *Rhodomonas salina*, *Nitzschia* sp., *Nannochloropsis* sp., and *Haematococcus pluvialis* have been reported [30–33]. In this study, higher concentration of sodium acetate supplementation caused notable reduction on biomass and lipid content. It was in agreement with previous study reported by Hu and Gao [33] that supplementing acetate with CO_2 in *Nannochloropsis* sp., culture showed minimal effect on growth and lipid content. In addition, presence of a higher amount of amino acids and soluble nitrogenous materials in peptone has improved the formation of algal material and cell division [34]. Likewise, Hoffmann et al. [35] have found that the medium contained higher concentration of nitrate has improved biomass of *Nannochlorpsis salina* in mixotrophic cultivation. Further, they stated that lower concentration nitrogen sources in the media yielded poor biomass.

Statistical optimization for an experimental design provides with an accurate and elegant medium [36] and the most widely used statistical experimental design was the central composite design [37]. Advantages of these designs include simplicity and assessment of a large number of factors on the relative efficiency of the production process. In addition, optimized medium through RSM for *C. protothecoides* culture yielded maximum biomass (1.8 times) and lipid (3 times) content than that of the original medium [21]. Similarly, Azma et al. [38] have optimized the medium composition using different carbon and nitrogen sources through RSM for heterotrophic cultivation of *Tetraselmis suecica* for obtaining increased algal biomass. Besides, Hallenbeck et al. [39] have found that higher lipid productivity while investigating the interaction effects of selected anions and cations on *Chlorella* sp. EN1234 by RSM. Another study of Hallenbeck et al. [40] have revealed that optimizing light intensity, inoculums size and CO_2 concentration by RSM-DOE has maximized biomass and lipids production by the marine green alga *Nannochloropsis gaditana*. Likewise, Jeon et al. [41] have examined first factorial design (2^2) for investigating the combined effects of light intensity level (80–120 $\mu Em^{-2} s^{-1}$) and acetate concentration (40–70 mM) on the growth of unicellular microalga *H. pluvialis*. As a result, the optimum conditions were not found within the domain of the first experimental design. On the basis of ascent line, additional experiments was conducted by using central composite design and they have obtained the growth rate up to 0.243 $g L^{-1} day^{-1}$ at a light intensity of 170 $\mu Em^{-2} s^{-1}$ and acetate concentration of 30 mM using. Similarly, Karpagam et al. [42] used RSM for enhancing the effect of sugarcane industry effluent and citric acid on growth and lipid production in two fresh water microalgae, *Coelastrella* sp. M-60 and *Micractinium* sp. M-13. Moreover, Gladue and Maxey [43] have suggested that the low growth rate of *Nannochloropsis* sp., under heterotrophic cultivation is the main limiting factor for executing commercial level production. In addition, Beacham et al. [44] have reported six different *Nannochloropsis* sp., growth rates for a period of 27 days under

photoautotrophic condition. But, interestingly, the present study heterotrophic culture of *N. salina* has reached the late log phase on the 13th day of culture, revealing the fact that the statistically optimized heterotrophic medium considerably increased the growth rate. In addition, higher biomass and lipid content of the confirmation experiment than the predicted optimal value generated by software, depicting the higher accuracy of the model. At present, the only commercial source of EPA is marine fish oil. This has problems such as contamination, taste, odor, stability and purification of EPA from fish oil have lead to the investigations an alternative EPA sources [45]. In this context, species of *Nannochloropsis* are a potential source of EPA production and several attempts have been made to improve the algal EPA production by optimizing the medium composition [33,46]. Interestingly, in this study a notable increase in EPA content was recorded in heterotrophic culture condition and our study adds strength. In addition, improving fatty acids synthesis is a promising strategy for lipid accumulation in algal cells. Prominently, some acyl-CoA synthase, diacylglycerol kinase, and glyceraldehyde-3-phosphate dehydrogenase, which were played a major role for glycolysis, acetyl CoA synthesis, and TAG accumulation, respectively, were considered as key sites for controlling of lipid synthesis [47]. Higher PUFA content in heterotrophic cultivation indicates that a simple up regulation or enhancement of one or more of the desaturase and elongases in the omega 6 pathway and it would be sufficient to induce the FA profile change of *N. salina*. Similarly, the higher SFA content in *N. salina* might be the deregulation of the fatty acid synthase KASII is a possible candidate for substantial increase in conversion of palmitic acid to stearic acid, hence altering the available substrate pool for ensuing biosynthesis [48]. Moreover, Campos and their colleagues [49] have studied the effects of biomass and lipid production on *N. salina* using various nitrogen sources such as ammonium chloride, ammonium hydroxide, sodium nitrate, urea, and a mixture of all these sources. They observed highest percentages of SFA than the PUFA content in mixed treatment. In this study, higher DHA content of *N. salina* was observed in heterotrophic cultivation ($2.6 \pm 0.04\%$), it was higher than the finding of Fang et al. [22] reported in *Nannochloropsis* sp., cultivated under mixotrophic condition. Similarly, Eikassas, [50] have reported 0.15% of DHA content in *Pichochloruim* sp., cultivated under nitrogen starvation. Besides, Bea and Hur [51] have found the DHA content of 0.29% and 0.02% in *Nannochlorosis* sp., and *N. oculata* respectively.

5. Conclusion

In conclusion, results of this study have demonstrated the process of RSM used for optimizing heterotrophic culture medium to improve the biomass and lipid production in *N. salina*. The optimized media produced higher biomass and lipid content than the photoautotrophic cultivation. In addition, higher PUFA content also observed in heterotrophic culture condition, therefore optimized media by RSM for heterotrophic cultivation of *N. salina* was considered as a better option than the photoautotrophic cultivation.

Conflict of interest

The authors declare that they have no conflict of interest

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