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ORIGINAL ARTICLE

Culture media optimization of *Porphyridium purpureum*: production potential of biomass, total lipids, arachidonic and eicosapentaenoic acid

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Abstract *Porphyridium purpureum* a red marine microalga is known for phycobiliproteins (PB), polyunsaturated fatty acids and sulphated exopolysaccharides. In the present study, effects of media constituents for the production of different polyunsaturated fatty acids from *P. purpureum* were considered using a response surface methodology (RSM). A second order polynomial was used to predict the response functions in terms of the independent variables such as the concentrations of sodium chloride, magnesium sulphate, sodium nitrate and potassium dihydrogen phosphate. The response functions were production of biomass yield, total lipid and polyunsaturated fatty acids like arachidonic acid (AA 20:4) and eicosapentaenoic acid (EPA 20:5). Results corroborated that maximum Biomass (0.95 gL^{-1}) yield was at the concentrations of sodium chloride (14.89 gL^{-1}), magnesium sulfate (3.93 gL^{-1}) and sodium nitrate (0.96 gL^{-1}) and potassium dihydrogen phosphate (0.09 gL^{-1}). Optimum total lipid (17.9 % *w*/w) and EPA (34.6 % *w*/w) content was at the concentrations of sodium chloride (29.98 gL^{-1}), magnesium sulfate (9.34 gL^{-1}) and sodium nitrate (1.86 gL^{-1}). Variation in concentration of potassium dihydrogen phosphate for both lipid (0.01gL^{-1}) and EPA content (0.20 gL^{-1}) was observed. The optimum conditions for biomass, total lipid, AA and EPA varied indicating their batch mode of growth and interaction effect of the salt.

Keywords Porphyridium · Fatty acids · Response surface methodology (RSM) · Optimization · Eicosapentaenoic acid

Introduction

Porphyridium purpureum is a red marine microalga (Rhodophyta) with spherical cells that lack cell wall (Adda et al. 1986). *P. purpureum* is known to produce valuable compounds such as phycobiliproteins, extracellular sulfated polysaccharide and polyunsaturated fatty acids with potential food, pharmaceutical and nutraceutical applications (Gudin and Thepenier 1986; Kavitha et al. 2015). It produces phycoerythrin (PE), an accessory red-colored photosynthetic pigment (Velea et al. 2011). *P. purpureum* also accumulates lipids consisting of polyunsaturated fatty acids especially eicosapentaenoic acid (EPA, 20:5 ω3) and arachidonic acid (AA, 20:4 ω6) in higher proportion (Servel et al. 1994). Arachidonic acid is a precursor of eicosanoids while EPA is shown to have several beneficial physiological effects in cardiovascular and inflammatory systems (Ward and Singh 2005). The minimum recommended dietary intake of n-3 PUFAs as prescribed by WHO and FAO is 250 mg day^{-1} while American Heart Association has recommended up to 500 mg day^{-1} for a healthy adult (Lichtenstein et al. 2006). However, n-6 to n-3 ratio of fatty acids is essential in any food since these fatty acids usually compete for the same enzyme to synthesize prostaglandins, a pro-inflammatory marker. A lower ratio of n-6 to n-3 fatty acids is required for reducing the risk of chronic diseases (Simopoulos, 2008). In general, animals do not synthesize the long-chain unsaturated fatty acids such as EPA and DHA due to lack of requisite

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enzymes and animals have to get them from the diet (Certik and Shimizu 1999). Although fish and fish oil are considered as the common sources of long-chain PUFAs (Apt and Behrens 1996), but fish itself get their PUFA from microalgae. In addition, many toxic chemicals such as dioxins, methyl mercury, and polychlorinated biphenyl (PCB) are found in fish oil, due to the increasing pollution levels in the oceanic ecosystem caused by anthropogenic activities. These toxic contaminants are hydrophobic in nature and bind to the lipid deposits in fish, causing bioaccumulation down the food chain (Storelli et al. 2004). Further, fish oil has a high cholesterol levels and unpleasant odour (Melanson et al. 2005). For this reason, it is more relevant and logical to explore microalgae as a source of PUFA (Jiang et al. 1999). Microalgae have an advantage over other plant-based sources as they have higher photosynthetic and surface area productivity (ten-fold) than terrestrial crop plants (Rittmann, 2008; Mallikarjun Gouda et al., 2015). They can be cultivated on non-arable land in outdoor ponds throughout the year with minimal nutritional input requirement (Borowitzka 1999; Vidyashankar et al. 2015). In recent times, considerable interest has generated in microalgal production of PUFA, as it is considered an economical alternative to produce fatty acids (Spolaore et al. 2006). *P. purpureum* is one of the microalgae that produce PUFAs in considerable amounts. The growth of *P. purpureum* was reported to be slow in artificial seawater medium (Tao and Barnett 2004). Culture conditions such as light intensity and residence time were reported to influence the content and compositions of fatty acids in microalgae (Carvalho and Malcata 2000). The conventional method of medium optimization, one factor at a time, is time-consuming and may lead to misinterpretation of results due to interactions between different components present in the medium. Statistical experimental designs can minimize the error in determining the effect of parameters, which allows systematic and efficient variation of all parameters (Ooijikass et al. 1999). A commonly used tool for designing experiments to obtain optimal conditions is a response surface methodology (RSM). RSM is an efficient statistical technique for optimization of multiple variables with minimum number of experiments (Vohra and Satyanarayana 2002). Therefore, this study aimed at understanding the effects of media constituents and their optimization employing RSM for total lipids and PUFA production in *P. purpureum*.

Materials and methods

Microalga and culture conditions

P. purpureum (112.79) was obtained from Sammlung von Algen Kulturen, Pflanzen physiologisches Institut, Universitat Gottingen, Germany. The stock culture was maintained in Artificial Sea Water (ASW) medium with a pH of 7.4 (Tao and Barnett 2004). Four constituents namely sodium chloride, magnesium sulfate, sodium nitrate, and potassium dihydrogen phosphate were taken for optimizing their concentration using the RSM methodology. A set of 250 mL conical flasks with 100 mL medium containing different concentrations of the four variables (sodium chloride, magnesium sulfate, sodium nitrate, and potassium dihydrogen phosphate), as mentioned in Table 1, were taken and the flasks were inoculated. The inoculated culture was incubated on Orbiteck shaker with 60 rpm speed maintained at 25 ± 1 °C under 18.75 μmol m^{-2} s^{-1} light intensity. The ten-days-old culture was used as inoculum at 20 % (*v*/v) with an optical density of 0.5 at 560 nm for all the experiments. The cultures were harvested after 28 days of growth. All the experiments were carried out in triplicates.

Growth measurement

The cultures were harvested by centrifugation (Remi C-24) at 8000 rpm. The biomass obtained was washed with distilled water and centrifuged again at the same speed. The biomass was freeze dried, and biomass was estimated gravimetrically.

Lipid extraction and fatty acid analysis

The known quantity of biomass was extracted using chloroform–methanol (2:l) mixture. The solvent was then evaporated under reduced pressure, and the residue was dried under slight nitrogen current. The total lipid content was expressed on dry weight basis (gL^{-1}), and that of individual fatty acids were expressed as a percentage of total fatty acids. For fatty acid methyl esters (FAME) preparation, the total lipid sample was dissolved in benzene and 5 % methanolic hydrogen chloride (95 mL chilled methanol +5 mL of acetyl chloride) and shaken well. The mixture was refluxed for two h, then 5 % sodium chloride solution was added and the FAME was extracted with hexane. The hexane layer was washed with 2 % potassium bicarbonate solution and dried over anhydrous sodium sulfate (Christie, 1982). The FAME solution was analyzed by gas chromatography and identified by comparing the retention times with those of standards of Sigma chemicals using GC Fison 8000 series (Italy) chromatograph with a flame ionization detector. A capillary column of high polarity-fused silica was used (Supelco SPB 1; length: 30 m; internal diameter: 0.22 mm; thickness of the film: 0.25 μm). The flow of nitrogen carrier gas was 1.5 ml min^{-1}, and the split ratio of the injector was 20:1. The injector temperature was 250 °C, and that of a detector was 280 °C. The starting temperature of the oven was 120 °C (2 min hold), and it was increased at a

Table 1 Central composite experimental design in coded and actual level of variables and the response functions

Ex. No	Sodium chloride		Magnesium sulfate		Sodium nitrate		Potassium dihydrogen phosphate		Biomass dw (gL^{-1})	AA (%)	EPA (%)	Total lipid (%)
	Coded level (x_1)	Actual level* (X_1)	Coded level (x_2)	Actual level* (X_2)	Coded level (x_3)	Actual level* (X_3)	Coded level (x_4)	Actual level* (X_4)	Experimental	Experimental	Experimental	Experimental
1	1.00	25.60	1.00	8.536	1.00	1.707	1.00	0.171	0.34	9.1	26.0	18.4
2	−1.00	4.392	−1.00	1.464	1.00	1.707	1.00	0.171	0.50	19.0	29.9	19.4
3	1.00	25.60	−1.00	1.464	−1.00	0.293	1.00	0.171	0.64	10.5	9.2	8.0
4	−1.00	4.392	1.00	8.536	−1.00	0.293	1.00	0.171	0.51	27.6	26.1	17.8
5	1.00	25.608	−1.00	1.464	1.00	1.707	−1.00	0.029	0.60	25.4	11.8	11.8
6	−1.00	4.392	1.00	8.536	1.00	1.707	−1.00	0.029	0.40	4.7	7.5	6.5
7	1.00	25.608	1.00	8.536	−1.00	0.293	−1.00	0.029	0.48	10.4	20.8	11.1
8	−1.00	4.392	−1.00	1.464	−1.00	0.293	−1.00	0.029	0.50	7.9	4.6	10.0
9	1.00	25.608	−1.00	1.464	1.00	1.707	1.00	0.171	0.42	11.1	26.8	9.5
10	−1.00	4.392	1.00	8.536	1.00	1.707	1.00	0.171	0.55	7.1	21.4	8.1
11	1.00	25.608	1.00	8.536	−1.00	0.293	1.00	0.171	0.52	17.0	27.7	13.9
12	−1.00	4.392	−1.00	1.464	−1.00	0.293	1.00	0.171	0.53	15.9	24.8	12.2
13	1.00	25.608	1.00	8.536	1.00	1.707	−1.00	0.029	0.47	10.8	26.8	12.1
14	−1.00	4.392	−1.00	1.464	1.00	1.707	−1.00	0.029	0.74	5.7	16.4	6.3
15	1.00	25.608	−1.00	1.464	−1.00	0.293	−1.00	0.029	0.66	9.9	11.6	7.3
16	−1.00	4.392	1.00	8.536	−1.00	0.293	−1.00	0.029	0.50	11.3	13.9	10.7
17	0.00	15.00	0.00	5.00	0.00	1.00	0.00	0.10	0.93	4.9	19.8	4.0
18	1.414	30.00	0.00	5.00	0.00	1.00	0.00	0.10	0.75	5.2	18.3	6.0
19	−1.414	0.00	0.00	5.00	0.00	1.00	0.00	0.10	0.80	5.5	14.7	5.4
20	0.00	15.00	1.414	10.00	0.00	1.00	0.00	0.10	0.68	3.8	10.6	4.4
21	0.00	15.00	−1.414	0.00	0.00	1.00	0.00	0.10	0.84	2.7	8.6	5.3
22	0.00	15.00	0.00	5.00	1.414	2.00	0.00	0.10	0.57	6.6	14.9	3.1
23	0.00	15.00	0.00	5.00	−1.414	0.00	0.00	0.10	0.66	11.2	13.5	10.3
24	0.00	15.00	0.00	5.00	0.00	1.00	1.414	0.20	0.83	8.5	16.1	4.7
25	0.00	15.00	0.00	5.00	0.00	1.00	−1.414	0.00	0.73	4.6	7.0	5.6
26	0.00	15.00	0.00	5.00	0.00	1.00	0.00	0.10	0.95	2.8	11.3	4.0
27	0.00	15.00	0.00	5.00	0.00	1.00	0.00	0.10	0.94	2.9	11.2	4.0
28	0.00	15.00	0.00	5.00	0.00	1.00	0.00	0.10	0.94	2.9	11.2	4.0
29	0.00	15.00	0.00	5.00	0.00	1.00	0.00	0.10	0.93	2.9	11.1	4.0
30	0.00	15.00	0.00	5.00	0.00	1.00	0.00	0.10	0.95	2.9	11.3	4.0

*Actual level in g/L; dw - dry weight

rate of 5 °C/min until 250 °C (10 min hold). The injection volume of samples was 1 μL (Kavitha et al. 2013).

Experimental design and analysis of data

The experimental design employed was a 4-variable (5 levels of each variable), second-order central composite design. The statistical software Statistica'99 (StatSoft, Tulsa, Ohio, USA) was used to design the experimental plan, and for subsequent analysis of variables. The four independent variables were X_1 (concentration of sodium chloride), X_2 (concentration of magnesium sulfate), X_3 (concentration of sodium nitrate), and X_4 (concentration of potassium dihydrogen phosphate) in their coded levels of variables (−1.414, −1, 0, 1, 1.414) (Myers 1971). The experimental design in the actual (X) and coded (x) levels of variables is shown in Table 1. The response functions (Y_{ijk}), that is, yield of biomass, arachidonic acid (20:4), eicosapentaenoic acid (20:5) and total lipid content in the *P. purpureum* culture was approximated by a second-degree polynomial. The linear, quadratic, and interaction effects in

coded level of variables were considered and the equation describing the same was represented as follows (Little and Hills 1978):

$$y_{ijk} = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j + \in_{ijk} \quad (1)$$

$i \leq j$.

The number of variables is denoted by n, and j, while k and i are integers. b_o, b_i, and b_{ij} are the coefficients of the polynomials and $\in_{ijk}$ is the random error. The interaction effects of the variables x_i and x_j are represented by $i < j$, b_{ij}. From the regression equations in actual level of variables response surface graphs were obtained; keeping the response function on the Z axis, and X and Y axes representing the two independent variables, while keeping the third and fourth variables constant at their center (corresponding to 0 level in coded level) points. The effects of individual variables were found out from the analysis of variance (ANOVA) on coded level; to arrive at the final regression equation that is later converted to the actual level of variables.

Optimization

The optimization was performed by applying canonical analysis technique as detailed earlier (Bhattacharya and Prakash 1994; Myers 1971) by using a self developed software (Bhattacharya and Prakash 1994). The levels of the variables (x_1, x_2, x_3, and x_4) were determined to obtain the maximum yield of biomass AA, EPA, and total lipid production (Table) individually. Optimization of the response functions consisted of the translation of the response function (y_k) from the origin to the stationary points (Myers 1971). Further the roots (λ_1, λ_2, λ_3, λ_4) of the auxiliary equation were calculated initially to know the nature of an optimum. The response function was found to be at maximum if all the roots had negative values and at the minimum if all roots had positive values. If some of the roots were positive and some were negative, then it was the situation of a saddle point (Bhattacharya and Prakash 1994; Myers 1971; Sarada et al. 2002). Maximization of individual response functions (Y_1, Y_2, Y_3, and Y_4) had been performed. The developed program, based on Myers (1971), was used where a trial-and-error approach was employed to obtain the maximum value of the individual response functions within the range of the experimental variables. The results are reported in terms of an actual level of variables. The predicted optimized condition was validated by conducting experiments in triplicates.

Results and discussion

In this study, optimization of the medium constituents for increased production of total lipid and polyunsaturated fatty acids in batch mode was achieved by employing RSM. The effect of the four independent variables (concentrations of sodium chloride, magnesium sulfate, sodium nitrate, and potassium dihydrogen phosphate) on the response functions (biomass yield, AA, EPA, and total lipid production) are shown in Table 1. The condensed results on the analysis of variances (ANOVA) (in coded level of variables) are given in Table 2 for all the four response functions. It was found that second-order polynomials were suitable to fit the data for response functions as the multiple correlation coefficients (r) are ≥0.87 ($p \leq 0.01$). The response surfaces (Figs. 1 and 2) are presented to aid in visualizing the effect of the four variables on the response functions.

Yield of biomass

The biomass yield of 28 days culture varied between 0.34 and 0.95 gL^{-1} (Table 1) at different levels of independent variables; total quadratic effect dominated (significant at $p \leq 0.01$) over linear ($p \leq 0.01$) and interaction effects ($p \leq 0.05$) (Table 2) indicating curvilinear effects (Fig. 1a). Out of the individual variables, sulfate had the maximum negative linear effect ($p \leq 0.01$) on biomass yield followed by nitrate ($p \leq 0.05$) while on the contrary chloride and phosphate concentrations had non-significant effects on the biomass yield. The quadratic effects of all the variables were also found to be significant ($p \leq 0.01$). Amongst the various interaction effects, chloride × nitrate concentration had a predominant negative effect on biomass yield ($p \leq 0.01$) (Fig. 1a). It suggested that the lower level of their concentration had a marginal impact on biomass yield but at higher concentration, they tend to decrease the biomass production markedly (Fig. 1a). For this reason, all these variables are required to be kept at lower levels in order to have maximum biomass in the experimental ranges used in this study. When *P. purpureum* culture was grown in artificial sea water medium, the biomass was found to be 0.48 gL^{-1}. The major difference between optimized medium constituents for high biomass yield and ASW medium was the higher concentration of sodium chloride in ASW medium (27.0 gL^{-1}) while the optimized sodium concentration in the present study was 14.89 gL^{-1} therefore lower biomass yield of *P. purpureum* in ASW medium.

Arachidonic acid (AA) content

Arachidonic acid content (Y_2) varied between 2.7 % and 27.6 % *w*/w (Table 1). Among the variables, total quadratic effect dominated ($p \leq 0.01$) over the interaction effects while the total linear effect was found to be marginal ($p \leq 0.05$) indicating non-linear behavior. For AA content, maximum linear effect was observed for phosphate concentration ($p \leq 0.05$) and highest positive quadratic effects were observed for nitrate, followed by phosphate ($p \leq 0.01$) and sodium

Table 2 Analysis of variance (ANOVA) for production of biomass (Y_1), AA (Y_2), EPA (Y_3) and total lipid production (Y_4) in coded level of variables

Source of variation	Coefficient of polynomial Y_1	F-value	Coeffi of polyno Y_2	F-value	Coeffi of polyno Y_3	F-value	Coeffi of polyno Y_4	F-value
Constant	0.94 ± 0.01	_	2.33 ± 0.51	_	10.59 ± 0.58	_	3.05 ± 0.55	_
x_1	−0.01 ± 0.00	0.46^{NS}	0.23 ± 0.06	0.13^{NS}	1.21 ± 0.37	2.63^{NS}	0.09 ± 0.04	0.02^{NS}
x_2	−0.05 ± 0.00	17.29^{c}	−0.29 ± 0.07	0.21^{NS}	1.75 ± 0.37	5.48^{b}	0.64 ± 0.35	0.84^{NS}
x_3	−0.02 ± 0.00	3.16^{b}	−1.21 ± 0.06	3.54^{a}	1.64 ± 0.37	4.86^{b}	−0.46 ± 0.34	0.43^{NS}
x_4	−0.01 ± 0.00	0.62^{NS}	1.84 ± 0.07	8.21^{b}	4.42 ± 0.37	35.09^{c}	1.51 ± 0.34	4.72^{b}
x_1^2	−0.08 ± 0.01	20.41^{c}	2.18 ± 0.41	5.43^{c}	3.75 ± 0.55	11.76^{c}	2.06 ± 0.51	4.06^{b}
x_2^2	−0.09 ± 0.01	24.25^{c}	1.13 ± 0.41	1.46^{NS}	0.29 ± 0.17	0.73^{NS}	1.64 ± 0.51	2.59^{a}
x_3^2	−0.16 ± 0.01	78.54^{c}	3.96 ± 0.41	17.83^{c}	2.59 ± 0.54	5.64^{c}	2.55 ± 0.51	6.27^{c}
x_4^2	−0.08 ± 0.01	19.20^{c}	2.78 ± 0.42	8.82^{c}	1.27 ± 0.54	1.35^{NS}	1.80 ± 0.51	3.12^{b}
x_1x_2	−0.01 ± 0.00	0.79^{NS}	−0.74 ± 0.12	1.06^{NS}	3.23 ± 0.42	15.01^{c}	1.48 ± 0.39	3.59^{b}
x_1x_3	−0.04 ± 0.00	7.59^{c}	2.18 ± 0.12	9.22^{c}	0.83 ± 0.42	0.99^{NS}	1.38 ± 0.39	3.15^{b}
x_1x_4	−0.02 ± 0.00	1.13^{NS}	−3.05 ± 0.36	18.13^{c}	−2.38 ± 0.41	8.15^{c}	−1.03 ± 0.39	1.76^{NS}
x_2x_3	−0.01 ± 0.00	0.64^{NS}	−3.22 ± 0.36	20.27^{c}	−2.41 ± 0.41	8.32^{c}	−1.13 ± 0.39	2.09^{NS}
x_2x_4	0.03 ± 0.00	4.55^{b}	1.00 ± 0.36	1.95^{NS}	−1.07 ± 0.41	1.64^{NS}	0.24 ± 0.39	0.09^{NS}
x_3x_4	−0.03 ± 0.00	4.18^{b}	−1.98 ± 0.36	7.70^{c}	0.48 ± 0.42	0.33^{NS}	0.36 ± 0.39	0.21^{NS}
TLE	_	5.39^{c}	_	3.02^{b}	_	12.02^{c}	_	1.50^{NS}
TQE	_	67.32^{c}	_	15.37^{c}	_	7.78^{c}	_	7.90^{c}
TIE	_	3.15^{b}	_	9.71^{c}	_	5.74^{c}	_	1.82^{NS}
R	0.98^{c}		0.95^{c}		0.94^{c}		0.87^{c}	
R^2 (adjusted)	0.96^{c}		0.90^{c}		0.88^{c}		0.76^{c}	

Values for coefficient are reported as mean ± standard error (SE)

Variables: x_1: Concentration of sodium chloride, x_2: Concentration of magnesium sulfate, x_3: Concentration of sodium nitrate and x_4: Concentration of potassium dihydrogen phosphate

TLE Total linear effect

TQE Total quadratic effect

TIE Total interaction effect

[a] Significant at $p \leq 0.10$

[b] Significant at $p \leq 0.05$

[c] Significant at $p \leq 0.01$

[NS] Non-significant at $p \geq 0.10$

chloride ($p \leq 0.05$). The positive quadratic effect of nitrate and its negative linear effect indicated that an initial increase in nitrate decreases AA, but increases latter with an increased level of nitrate (Fig. 1b). GCMS profile confirmed the presence of AA (Fig. 2). Among the various interactions, the sulfate × nitrate and chloride × phosphate concentration had the maximum negative effect on AA production ($p \leq 0.01$) while chloride and nitrate concentration had a positive impact ($p \leq 0.01$). These results indicated that the effect of nutrient on AA production is interdependent on the concentration of other nutrients. The positive quadratic effects of chloride, nitrate, and phosphate are more pronounced compared to their negligible linear effect. All this indicated that high concentration of these nutrients in combination facilitate more production of this fatty acid. Even Cohen (1990) also reported high AA production from *P. cruentum* under nitrogen starvation with slow growth.

Eicosapentaenoic acid content

The eicosapentaenoic acid content (Y_3) (4.6 to 29.9 % *w*/w, Table 1) mostly depended on the linear effects of four independent variables (Table 2); though the total quadratic ($p \leq 0.01$) and interaction effects were also significant at $p \leq 0.01$. The high positive linear effect was observed for phosphate ($p \leq 0.01$) followed by sulfate and nitrate concentration ($p \leq 0.05$). High positive quadratic effects were observed for chloride followed by nitrate concentration indicating the curvilinear effect on EPA production (Fig. 3a). Among the interaction effects, chloride × sulfate had the maximum positive impact followed by negative effects of sulfate × nitrate and chloride × phosphate concentrations. The effect of chloride on the yield of EPA production was largely dependent on the level of sulfate and phosphate concentrations. The present study indicated that chloride, sulfate, and phosphate

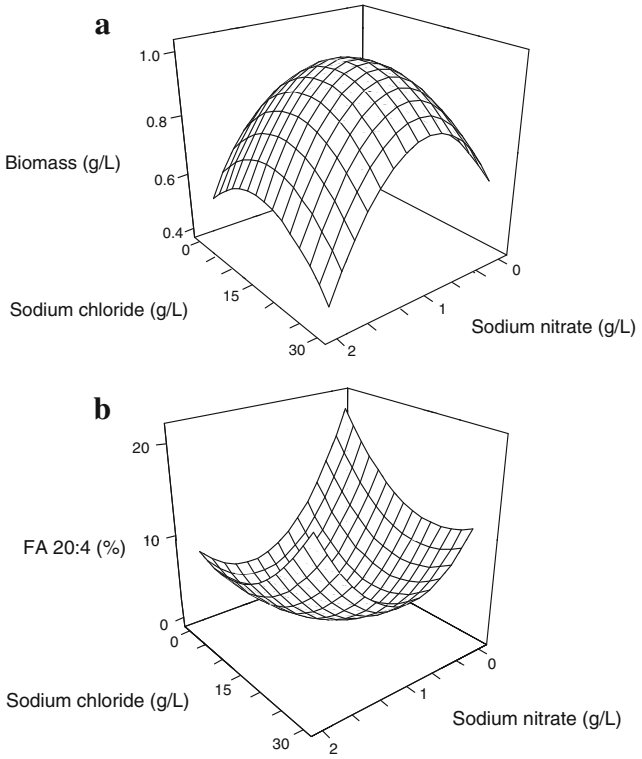

Fig. 1 Response surface plot for (**a**) biomass production and (**b**) AA production when magnesium sulfate and potassium dihydrogen phosphate concentration kept constant

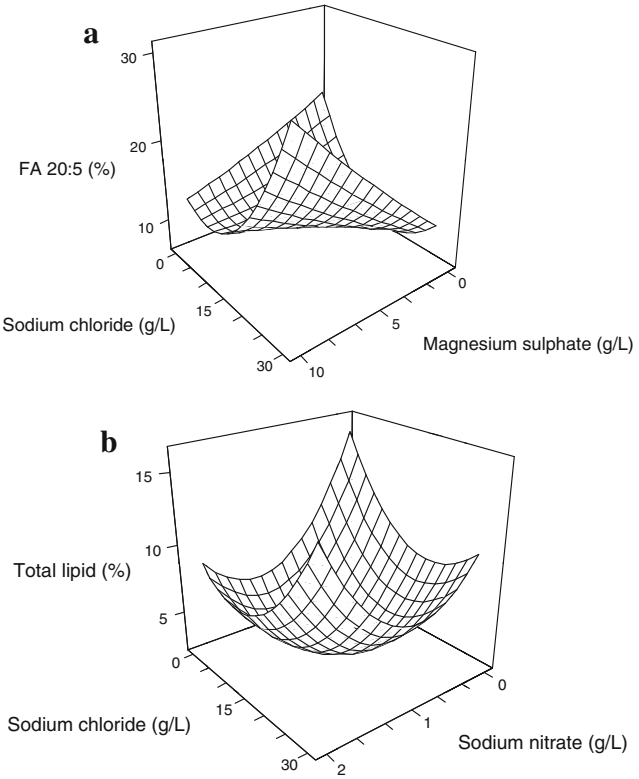

Fig. 3 Response surface plot for (**a**) EPA when phosphate and nitrate concentration kept constant and (**b**) total lipid production when sulfate and potassium dihydrogen phosphate concentration kept constant

are essential for growth and EPA content. Ohta et al. (1992) observed an increase in the growth and EPA content of *P. purpureum* by changing the concentrations of phosphate and NH_4Cl. Yongmanitchai and Ward (1991), also reported an increase in growth and EPA production in *Phaeodactylum*

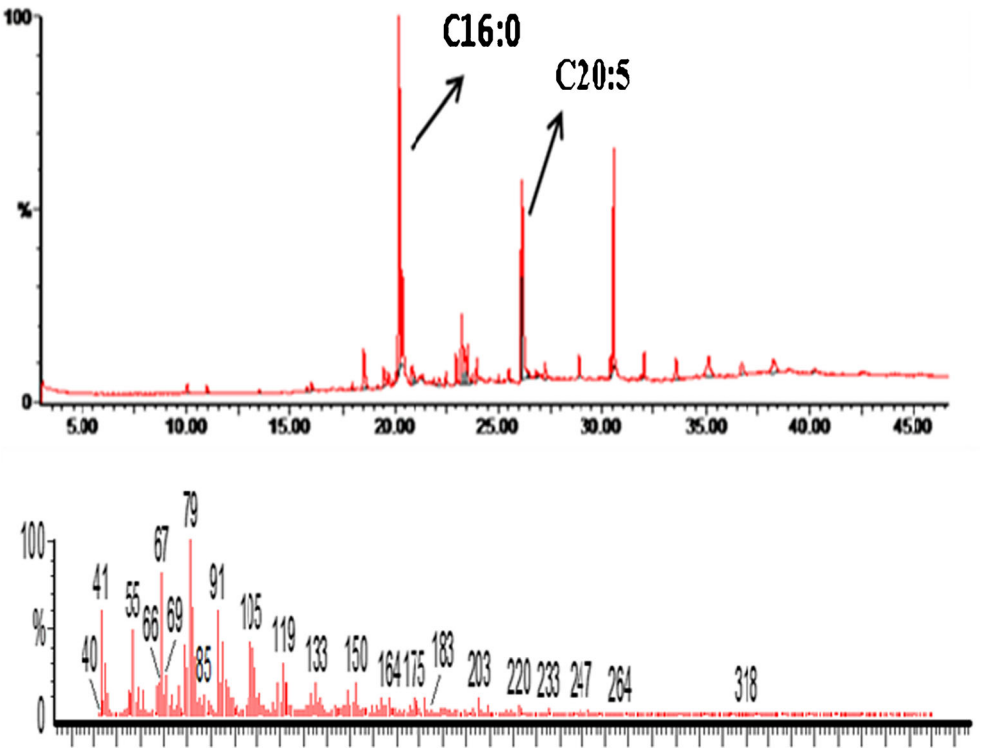

Fig. 2 GCMS profile of FAME of *P. purpureum* biomass indicating presence of AA

tricornutum by nitrogen sources such as nitrate and urea. The interaction between the chloride and phosphate concentrations was found to be negative; the same pattern of results was also observed when there was an increase in phosphate concentration in the medium. Even though the medium contained a higher concentration of nitrogen and phosphate at the initial stage, their levels gradually decreased due to the utilization of nutrients by the alga for growth; concomitantly leading to more EPA accumulation. Similarly, the marine diatom *Nitzschia laevis* also shown increased production of EPA as the culture aged in heterotrophic condition (Wen and Chen 2001). In general, the major lipids present in microalgae are polar lipids and triacylglycerols. The polar lipids constitute more content of PUFA, where as triacylglycerols have proportionally more saturated fatty acids (Dunstan et al. 1993). If cells are starved for nitrogen, amino acid synthesis cannot occur and the protein-rich chloroplasts will be reduced, leading the cells to store energy and thus increasing the amounts of saturated fatty acids. Similarly, if phosphate is limiting, the capacity of cells to metabolize phospholipids will be reduced. Ultimately, this leads to a decrease in the membrane polar lipids available for cell division (Carvalho and Malcata 2000). Thus, it was found that the interaction between both nitrogen and phosphate plays a role in the synthesis of the EPA. In this study, culture medium containing varying amounts of nitrogen and phosphate available for cell uptake was used, which resulted in an optimum production of EPA.

Total lipid content

The total lipid content (Y_4) showed a broad variation between 3.1 % and 19.44 % due to the changes in experimental conditions (Table 1). Among these variables, total quadratic effects were predominant ($p \leq 0.01$) over the non-significant interactive and linear effects. The quadratic effect of nitrate ($p \leq 0.01$) was the most dominating curvilinear effect (Fig. 3b). Sodium chloride also imparted a curvilinear effect ($p \leq 0.05$). The interaction terms for chloride × sulfate and chloride × nitrate indicated that the effect of chloride on total lipid production depended on the concentration of sulfate and nitrate. However in this study, the inoculum density was constant for all the combinations. It has been reported widely that an availability of nutrients plays a major role in lipid accumulation by microalgae (Harwood and Jones 1989). The nutrient-sufficient conditions promotes growth, while nutrient-deficient conditions specifically nitrogen limitation and other abiotic stress induces lipid accumulation (Harwood and Jones 1989). Under these stressed or nutrient deficient conditions microalgae channelize their metabolism toward an accumulation of lipid as a preliminary storage material (Guschina and Harwood 2009).

Optimization

The process of optimization (maximization) of the biomass yield (Y_1), arachidonic (20:4) content (Y_2), EPA (20:5) content (Y_3), and total lipid content (Y_4) have been conducted individually (Table 3). The roots (λ_1, λ_2, λ_3, and λ_4) of the auxiliary equations were determined. The roots of the auxiliary equation for arachidonic and total lipid indicated minimization of response function, maximization for the yield of biomass while saddle point was obtained for EPA content. Thus, canonical search method was applied to get the maximum production of all these four response functions separately as maximization of these response functions is desirable. The corresponding values of the independent variables were obtained at actual levels of variables (Table 3) within the range of the present experimental variables. Accordingly, the maximum value for the yield of biomass was 0.95 gL^{-1}, arachidonic acid content was 31.4 %, for EPA it was 34.6 %, and for the maximum total lipid content was 17.9 %. However, the levels of chloride, sulfate, nitrate, and phosphate were different for different response functions. Results indicated that in general, the levels of chloride and nitrate should be moderate-to-high concentration to maximize all the four response functions. The levels of all the independent variables should be high to maximize the EPA production. The production of AA and EPA from *P. cruentum* was reported by Cohen (1990). Gupta et al. (2012) reported major PUFA producing microorganisms. Velea et al. (2011) reported enhanced production of biomass and Phycoerythrin through culture medium optimization, but no study was conducted to optimize the medium condition for AA, EPA and total lipids production. Our study indicated an increased production of the AA, EPA, and total lipids under optimized condition. Hence, optimization was the best way to identify the exact value of the variables for the production of the metabolites.

The optimization studies indicated that moderate levels of chloride, sulfate, nitrate, and phosphate favored maximum biomass yield, while higher levels of chloride, sulfate, and nitrate resulted in maximum production of total lipid and EPA content while more elevated chloride and nitrate, moderate phosphate, and lowest sulfate levels gave maximum arachidonic acid (Table 3). In other words, the optimum conditions for biomass, total lipid, AA, and EPA varied and this may be due to the batch mode of growth and interaction effects of the salts. During the validation of some of the combinations of the variables, it was observed that low biomass is associated with high PUFA content and the optimum conditions are found to be different for AA and EPA. The combination of salts, which favored production of more EPA was found to lower the AA content. It is rather difficult to compare the present

Table 3 Results of the optimization study

Parameters		Biomass Y_1	AA Y_2	EPA Y_3	Total lipid Y_4
Roots of the auxiliary equation	λ_1	−0.06	5.98	4.87	3.06
	λ_2	−0.08	2.75	3.08	2.64
	λ_3	−0.10	0.95	0.78	1.85
	λ_4	−0.17	0.38	−0.82	0.51
Type of optimization obtained from auxiliary equations		Maximization	Minimization	Saddle point	Minimization
Type of optimization desirable		Maximization	Maximization	Maximization	Maximization
Optimum conditions in coded level	x_1	−0.01	1.33	1.41	1.41
	x_2	−0.30	−1.41	1.22	1.41
	x_3	−0.05	1.33	1.22	−0.38
	x_4	−0.11	−0.67	1.40	−1.37
Optimum conditions in actual level	X_1 (gL^{-1})	14.89	29.09	29.99	29.98
	X_2 (gL^{-1})	3.93	0.003	9.34	9.34
	X_3 (gL^{-1})	0.96	1.94	1.86	1.86
	X_4 (gL^{-1})	0.09	0.05	0.20	0.01
Maximum production		0.95 gL^{-1}	31.42%*w*/w	34.62%*w*/w	17.9 %*w*/w
Experimental values*		0.90 ± 0.12 gL^{-1}	26.9 ± 3.5%*w*/w	31.0 ± 3.0 % *w*/w	16.5 ± 1.1%*w*/w

X_1, X_2, X_3 and X_4 are same as mentioned in Table 1

*Experimental values are obtained from the validation experiment and are reported as mean ± standard deviation of 3 repetitions

results with the earlier reports. Oh et al. (2009) reported effect of light-dark cycle, temperature, carbon dioxide levels, glucose and glycerol as carbon source under autotrophic as well as heterotrophic growth conditions on lipid production and fattyacid profile. The culture conditions studied favoured more of saturated and monounsaturated (C16:0 - C18:1) fattyacids compared to polyunsaturated fattyacids suitable for biofuel application. Nuutila et al. (1997) studied effect of pH, salinity, nitrate and temperature on EPA production and the results were expressed on cell number and culture volume. However, there is a consensus for the general observations among the reports about growth, PUFA levels, EPA and AA content variations with culture conditions.

Conclusions

Experimental results illustrated that RSM was an efficient method to get optimized parameters for maximum production of arachidonic acid, EPA, and total lipids production from *P. purpureum*. RSM provided insight into the interaction and identified the optimum combination of variables (within a specified range) for the maximized biomass, lipid, AA, and EPA production with the help of a relatively small number of experiments, thus reducing the time and cost of the study. The optimization studies indicated that moderate levels of chloride, sulfate, nitrate, and phosphate favoured maximum biomass yield; while higher levels of chloride, sulfate, and nitrate resulted in maximum production of total lipid and EPA content.

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Compliance with ethical standards

Conflict of interest The authors have declared that there is no conflict of interest.

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