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ORIGINAL ARTICLE

Response surface methodology as a statistical tool for optimization of physio-biochemical cellular components of microalgae *Chlorella pyrenoidosa* for biodiesel production

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Abstract

The current study utilized Box–Behnken design of the response surface methodology, aimed at identifying the best levels of particular variables. An experimental design was performed on *Chlorella* sp. considering initial nitrogen, phosphorus and iron concentration as independent variables. Lipid, biomass, chlorophyll, carbohydrate and protein contents were analysed as response variables. Various physio-biochemical attributes were studied under varied concentration of nutrients in BG11 media, nitrate ($NaNO_3$, 10–750 mg mL^{-1}), phosphate (K_2HPO_4, 40–120 mg mL^{-1}), iron (ferric ammonium citrate, 3–9 mg mL^{-1}) with an objective to establish the actual potential of *Chlorella pyrenoidosa* under phototrophic nutrient stress conditions were obtained lipid percentage (35.4 dcw%) and biomass yield (1.89 g L^{-1}). A Prob > F value of < 0.05 and > 0.05 indicated model terms which were significant and non-significant, respectively. The fit of model yielded R^2 values up to 96.25% for lipids and 94.12% for biomass; similar values were obtained for proteins, carbohydrate and chlorophyll. FAME profile of *Chlorella pyrenoidosa* contained palmitic (C16:0, stearic acid), (C18:0) oleic (C18:1), linoleic C18:2 and linolenic (C18:3) which showed that *Chlorella pyrenoidosa* possessed a favourable fatty acids profile that can be successfully utilized for biodiesel production.

Keywords Microalgae · *Chlorella pyrenoidosa* · Lipid · Biomass · RSM · Box–Behnken design (BBD) · Transmethylation · FAME and biodiesel

Introduction

Biofuels, the sustainable energy system, may be regarded as a cost-effective, trustworthy and environmental-friendly system that efficiently makes use of local resources (Shahare et al. 2017). Biodiesel comprises fatty acid alkyl esters of long-chain fatty acids with short-chain alcohols, primarily methanol and ethanol, and is generally produced from the trans-esterification of triglycerides by alkali acids or enzymes in vegetable oils or animal fats (Ma and Hanna 1999; Zhang et al. 2003; Noureddini et al. 2005; Wang et al. 2007; Fadhil et al. 2016). Microalgal cultivation is environmentally friendly because the microalgal biomass can be utilized for the productions of biofuels, food and feed supplements, pharmaceuticals, nutraceuticals and cosmetics (Show et al. 2017). Microalgal biodiesel appeared to be the most promising renewable biofuel that has the potential to entirely substitute petroleum-derived transport fuel (Chisti 2008; Harun et al. 2010; Bajwa and Bishnoi 2016). Some advantages such as easily cultivable, devoid of any seasonal changes, high lipid production capability, superior photosynthetic efficiency, no competition with food crops for land and water, nutrients uptake from wastewater, ability to produce substantial amount of triacylglycerides (TAG) and shorter doubling time make them better than other available feedstocks (Kiran et al. 2014; Sharma et al. 2012; Wang et al. 2010; Chisti 2008; Bajwa and Bishnoi 2016; Sharma et al. 2015). Among the numerous algal species, *Chlorella pyrenoidosa* is identified as a potential strain for fuel production because of its rapid growth and enormous amount of lipid content (Chelladurai and Perumalsamy 2017). Mixotrophic

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and heterotrophic cultivation has been suggested as feasible alternative for higher biomass yield and lipid productivity, which is a key step in the reduction in biodiesel cost (Yu et al. 2009; Cheirsilp and Torpee 2012; Ren et al. 2013). Several factors influencing algal growth both qualitatively and quantitatively include light, temperature, media composition, nitrogen and phosphate source, carbon sources, nutrient limitation, aeration, photoperiod (dark/light) condition, oxygen, CO_2, pH, salinity, heterotrophic cultivation and toxic chemicals as abiotic factors (Mata et al. 2010; Singh and Singh 2015). Nutrient availability has a substantial impact on growth and culturing of microalgae and broad effects on their lipid content and fatty acid composition (El-Kassas 2013). So far, various studies have been carried out to demonstrate that the nitrogen source was the important nutrition in the medium affecting the growth and lipid accumulation (Li et al. 2008). Previous studies have demonstrated that lipid content in some microalgae increases under nitrogen-deprived condition (Illman et al. 2000; Hsieh and Wu 2009). Similarly, phosphorus and nitrogen are essential nutrients for metabolic growth of microalgae (Sharma et al. 2015). Studies have shown that the phosphorus-deprived conditions are responsible for significant lipid accumulation in *Chlorella* sp. *Chaetoceros* sp. High lipid accumulation was reported under nitrogen-deprived conditions in microalgal species, viz. *Neochloris oleoabundans*, *Nannochloris* sp., *Chlorella muelleri* and *Scenedesmus* sp. (Courchesne et al. 2009; Radakovits et al. 2010; Gao et al. 2013; Blinová et al. 2015).

RSM is a novel arithmetical design employed to evaluate problems which in the response is dependent on several independent variables with an objective to maximize the process variables for achieving optimum response (Box and Behnken 1960). The main objective of RSM is determination of the optimized operational condition of the process or to determine a region that satisfies the operating specifications (Myers and Montgomery 2002). RSM uses quantitative data from appropriate experiments to determine and simultaneously solve multivariate equations (Tokcaer et al. 2006; Silambarasan et al. 2017). RSM reduces the number of experiments eventually saving chemicals, time and labour. Furthermore, it offers a rapid and unfailing prediction of response, making it a beneficial option for experimental design. The Box–Behnken design was taken as it fulfilled most of the requirements for interaction study for various factors (Molina Grima et al. 1999; Su et al. 2009). Trans-esterification process is a very important step for biodiesel production at industrial scale. Homogeneous alkali catalysts like potassium hydroxide or sodium hydroxide are utilized by the common industrial practices (Mata et al. 2010; Yu et al. 2015). Physico-chemical properties of biodiesel are assessed by fatty acids profile such as saturated, monounsaturated and polyunsaturated fatty acid (Kumar and Chauhan 2013). The biodiesel parameters, viz. cetane number, iodine value and oxidation stability, were correlated with degree of unsaturated fatty acids, whereas the cold filter plugging was correlated with long-chain saturated fatty acids (Chuah et al. 2016). Gas chromatography (GC) is the most popular method to characterize fatty acid profiles of lipids in biological materials. In fatty acid profile analysis, lipids need to be converted into fatty acid methyl esters (FAME) in order to improve their volatility and hence ensure better GC peak shape (Masurkar and Vakil 2015). The FAME elute at different retention times, due to their different degrees of interaction. FAME can be determined by comparing their retention time provided in the literature (Quehenberger et al. 2011; Renita and Joshua 2011). Thus, the aim of the present study is to evaluate simultaneously the effect of nitrogen, phosphorus and iron in BG-11 medium on growth and other physio-biochemical attributes of *Chlorella pyrenoidosa* by using Box–Behnken model and fatty acids detection by using GCMS.

Materials and methods

Isolation and molecular characterization

The freshwater microalgal species was isolated from the freshwater pond at Shahidawaali village, Dist. Sirsa (Haryana), India. Genomic DNA from microalgal sample was extracted by using cetyl trimethyl ammonium bromide (CTAB) method (Rogers and Bendich 1994). Polymerase chain reaction (PCR) was analysed to amplify 18S rRNA gene of microalgae using forward (5″GGGACCCGTTACCGTAGGTGAACCTGC-3″) and reverse primers (5″-GGGATCCATATGCTTACGTTC CGCGGAT-3″). The purified PCR products were sequenced by Amnion Biosciences Pvt. Ltd. (Bengaluru, India). Comparisons of nucleotide sequences and statistical significance of matches were carried out with the National Centre for Biotechnology Information (NCBI) nucleotide BLAST program.

Analytical methods for physio-biochemical parameters

Extraction of lipid

The extraction of total lipid was carried out by mixing methanol–chloroform (2:1.5 v/v) with the algal samples using slightly modified version of Bligh and Dyer's method (Bligh and Dyer 1959). According to Suganya and Renganathan (2012), the oil extraction yield (%w/w) was determined by the following formula:

$$\text{Oil extraction yield (dcw\%)} = \frac{\text{Weight of extracted oil}}{\text{Weight of biomass}} \times 100$$

Estimation of dry Biomass

Dry cell biomass was measured as the cell density (dcw) at OD625 of an 11-day-old culture at dilutions ranging from 0.2 to 1.0. The dry biomass was calculated using the regression equation as the linear relationship given by Yount (2006)

$$y = 0.137x + 0.1766, \quad R^2 = 0.9859$$

Extraction and determination of photosynthetic pigment

Chlorophyll content of the algae was estimated spectrophotometrically at 650 and 665 nm, MacKinney (1941). The concentration of chlorophyll was calculated using the formula:

$$\text{Total chlorophyll}\left(\text{mgmL}^{-1}\right) = 2.55 \times 10^{-2}E650 + 0.4 \times 10^{-2}E665 \times 10^{3}$$

Extraction and determination of total soluble carbohydrate by Anthrone reagent

Glucose was determined at 625 nm using Anthrone reagent method by Dubois et al. (1956). The sugar content was calibrated against standard curve prepared by using graded conc. of glucose dilution ranging from 0.2 to 1 and expressed in terms of mg mL^{-1} $y = 0.636x + 0.0592$, $R^2 = 0.9595$ where y is the concentration of glucose and x the optical density.

Estimation of total protein

The protein content was estimated using Lowry's method. Protein concentration was calculated from the standard curve prepared with bovine serum albumin (BSA) (Lowry et al. 1951):

$$y = 0.1097x - 0.0005, \quad R^2 = 0.9989$$

Optimization of nitrogen, phosphorus and iron concentrations in BG-11 media using Box–Behnken model

The statistical experiments were carried out using Design Expert 7.0.0 Box–Behnken model (Miao et al. 2006). The experimental design consisted of three nutrient stress factors: sodium nitrate concentration (nitrogen source), dipotassium phosphate concentration (phosphorous source) and ferric ammonium citrate concentration (iron source) (Table 1). Seventeen experiments were designed by Design Expert 7.0.0 Box–Behnken model and conducted at 25 °C as per experimental setup. The relationship between independent variables and responses was evaluated by using second-order polynomial equation. The adequacy of designed model was evaluated by coefficient of determination regression (R^2) and model P value. The analysis of variance (ANOVA) of various responses was obtained after applying independent variables for lipid productivity (dcw%), biomass (g L^{-1}), total chlorophyll (µg mL^{-1}), carbohydrate (mg ml^{-1}), protein (mg mL^{-1}) by using response surface methodology.

Table 1 Levels of three independent variables used in RSM in terms of actual and coded factors of Box–Behnken design

Process variable (mg L^{-1})	Low (− 1)	Medium (0)	High (+ 1)
$NaNO_3$	10	388	750
K_2HPO_4	20	40	60
Ferric ammonium citrate	30	60	90

ANOVA analysis of predicted model was carried out to estimate its statistical significance. The quality of the fit polynomial model was expressed by the coefficient of determination regression (r^2), and its statistical significance was tested by the Fisher F-test. Model terms were accepted or rejected based on the p value (probability) with a 95% of confidence level. BBD responses were fitted by a second-order polynomial in order to correlate response with independent factors. The polynomial equation is in the following form: $Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_i X_i X_j + \Sigma\beta_{ii} X_{i2} \ldots \Sigma\beta_{ii} X_n$ where X_1, X_2 and X_3 represent the coded levels of the independent variables as described in Table 1 and β_0, β_i and β_j ($i, j = 1, 2, 3, 4$) are the coefficient estimates, where β_0 is the interception term, β_i is the linear term, β_{ii} is the quadric term and β_{ii} is the interaction term. The overall second-order polynomial mathematical relationship of response Y and the three variables, i.e. $NaNO_3$, K_2HPO_4 and ferric ammonium citrate, can be approximated by quadratic equation.

Extraction of diesel hydrocarbons and analysis of biodiesel using gas chromatography mass spectroscopy (GCMS)

Direct trans-methylation method

The lipid samples were methylated by heating under reflux using methanolic HCl (5%) at 60 °C as shown in Fig. 1a. Extraction of hydrocarbon was carried out by using petroleum ether 60 °C in hot water bath. The ether extract was washed three times with deionized water then dried over anhydrous sodium sulphate, filtered off and collected sample (Abdo et al. 2014) and subjected to GCMS analysis.

The analysis of FAME produced from microalgal oil was performed on gas chromatograph mass spectrometer (PerkinElmer Clarus-580) equipped flame ionization detector with two narrow bore capillary columns Agilent, DB-225, (30 m × 250 µm, film thickness 0.25 µm). The pressure of the carrier gas (helium) was 6.08 Psi at the primary oven temperature with flow rate 67 mL min^{-1}. The temperature of the

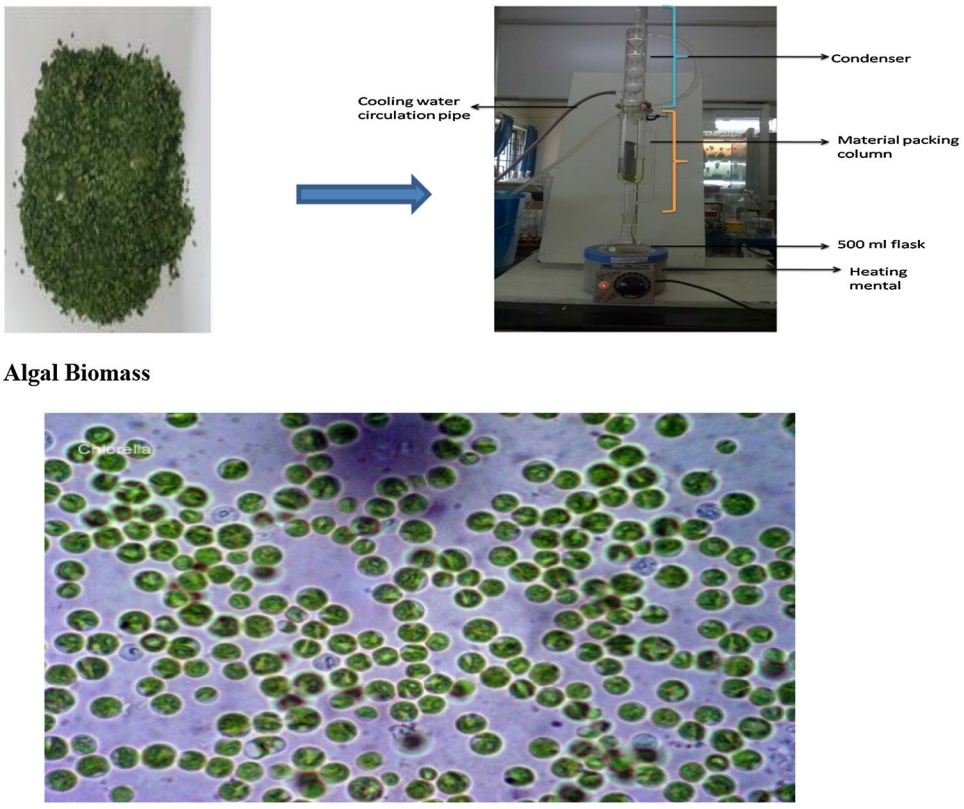


Fig. 1 Extraction of hydrocarbons. **a** Heating under reflux method and **b** microscopic image of *Chlorella pyrenoidosa* (× 100X)

transfer line and of the ion source was set to a value of 320 and 280 °C, respectively. The 1.0 µL volume of sample was injected in column. Peak identification of algal oil was performed, and an oil compound was identified with retention times. The mass spectra obtained are compared with Wiley and NIST libraries (Wiley Registry TM, 8th Edition Mass Spectral Library and the NIST 08 Mass Spectral Library (NIST/EPA/NIH) 2008 version) with an acceptance criterion of a match above a critical factor of 80% (Guan et al. 2011).

Results and discussion

Partial sequencing of microalgae

The freshwater microalgal species was isolated from the freshwater pond and preliminarily identified as *Chlorella* sp. on the basis morphological features and habitat (Fig. 1b). The 18S rRNA partial sequence of a gene amplified from the strain *Chlorella* sp. was 610 bp. The molecular phylogenetic tree analysis indicated that this strain had a close relationship with *Chlorella pyrenoidosa* and confirmed as *Chlorella pyrenoidosa* (Fig. 2b). The partial gene sequence of 18S rRNA of *Chlorella pyrenoidosa* was deposited to NCBI GenBank under accession no. KU236002.1.

Optimization of nitrogen, phosphorus and iron concentrations in BG-11 media using Box–Behnken model

The Box–Behnken design (BBD) (Design Expert 7.0.0 trial version; Stat-Ease, Minneapolis, MN) of five variables and three levels, each with three concentric point combinations were used to identify the optimum variable level for the indigenous isolate of *Chlorella pyrenoidosa*. The minimum, central and maximum levels for each design variable used in the Box–Behnken for various responses was listed in Table 2. The experimental design was applied for each variable maximum and minimum level for each design variable. The independent factors that exhibited higher influence on the response variables were listed hierarchically in Table 3. Analysis of variance was used in order to ensure a good model as explained in Table 4. A Prob > F value of < 0.05 and > 0.05 indicated model terms which were significant and non-significant, respectively. The fit of model yielded R^2 values up to 96.25% for lipid and 94.12% for biomass; similar values were obtained for proteins, carbohydrate and chlorophyll (Table 4). The predicted R^2 and adjusted R^2 values closer to 1.0 were in a reasonable agreement indicating the better fitness of model to obtain experimental data. Adequate precision determined the signal-to-noise ratio, and numerical values greater than 4 indicated that model can be used to navigate the designed space. A very small

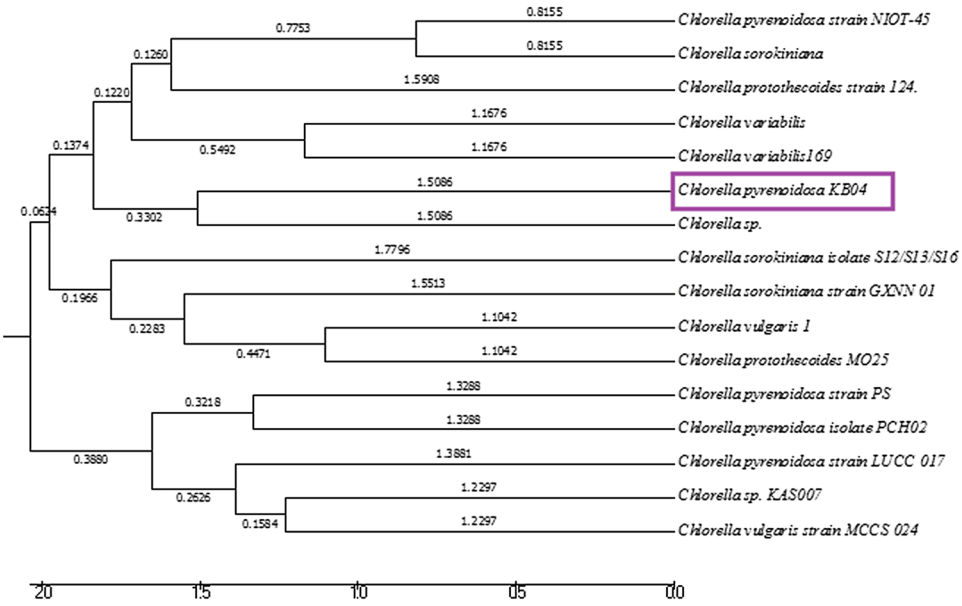


Fig. 2 Phylogenetic tree analysis based on 18s rRNA sequencing of *Chlorella pyrenoidosa* showing relationship with other universal identified species

Table 2 The various variable levels used in the Box–Behnken design for *Chlorella pyrenoidosa*

Std.	Sodium nitrate (mg L^{-1})	Di-potassium hydrogen phosphate (mg L^{-1})	Ferric ammonium citrate (mg L^{-1})	Lipid yield (dcw%)	Biomass (g L^{-1})	Total chlorophyll (µg mL^{-1})	Carbohydrate (mg L^{-1})	Protein (mg L^{-1})
1	380	40	6	35.4	1.89	20.16	0.354	0.0342
2	750	40	9	26.159	1.68	12.8	0.265	0.024
3	10	60	6	23.118	1.17	8.929	0.231	0.023
4	380	20	3	24.278	1.07	13.47	0.247	0.028
5	380	20	9	30.35	1.11	16.438	0.305	0.0305
6	380	40	6	35.4	1.89	20.16	0.354	0.0345
7	750	60	6	22.98	1.44	10.96	0.229	0.0229
8	10	40	3	17.279	1.36	13	0.177	0.018
9	380	40	6	35.41	1.89	20.16	0.354	0.034
10	750	40	3	18.251	1.8	15.1	0.185	0.0185
11	380	60	9	27.968	1.8	11.478	0.278	0.0276
12	10	40	9	24.193	1.54	12	0.249	0.0249
13	380	60	3	19.218	1.4	17.879	0.198	0.0198
14	380	40	6	35.4	1.89	20.16	0.354	0.0348
15	380	40	6	35.4	1.89	20.16	0.354	0.0356
16	10	20	6	25.218	1.31	9.2	0.258	0.028
17	750	20	6	28.308	1.42	10.539	0.28	0.028

All the experiments were run at central value

value of coefficient variation in lipid, biomass, chlorophyll, carbohydrate and protein (0.016%, 10.42%, 0.77%, 0.71%, 2.16%) and low standard deviation value (4.29, 0.16, 0.77, 1.96, 5.92), respectively, clearly indicated very high degree of precision and good reliability of the experimental data.

The second-order polynomial equation was used to evaluate the relationship between various variables and responses. The regression equation coefficients were calculated, and data fitted to a second-order polynomial equation. A flat surface of three-dimensional response graphs indicated an optimum condition for responses such as lipid accumulation, biomass production, carbohydrate consumption and chlorophyll and protein contents were shown in Fig. 4a–e. The experimental details were provided by Design Expert from the analysis of all the data points of normal probability, and the studentized residual was approximately linear and found

Table 3 Independent factors influence on the response variables

Response variables	Equation terms (descendant order of influence)
Lipids	$A, B, C, AB, AC, BC, A^2, B^2, C^2$
Biomass	$AB, AC, BC, A^2, B^2, C^2$
Chlorophyll	$A, C, AB, AC, BC, A^2, B^2, C^2$
Carbohydrates	$A, B, C, AB, BC, A^2, B^2, C^2$
Protein	$AB, AC, BC, A^2, B^2, C^2$

Factors: A-$NaNO_3$, B-K_2HPO_4, C-ferric ammonium citrate

to be lie around zero line, indicating the suitability of model and showing that the variance was associated with prediction changes over the design space as shown in Fig. 5a–e that clearly indicated the adequacy of model and have adequated harmony with predicted and actual data obtained by applying BBD.

The lipid yield, three linear (A, B, C), interactive (AB, AC, BC) and quardratic (A^2, B^2, C^2) model terms were found to be significant. The chlorophyll contents, two linear (A, C), interactive (AB, AC, BC) and quardratic (A^2, B^2, C^2) model terms were significant, whereas for carbohydrate content three linear (A, B, C), interactive (AB, BC) and quardratic (A^2, B^2, C^2) model terms were significant. In case of biomass yield, quardratic B^2 term was significant. The protein content, two linear (B, C) interactive BC and quardratic A^2, B^2, C^2 model terms were significant. All of the significant terms were found to contribute more towards the responses than non-significant terms. The multiple correlation coefficient R^2 value for all responses suggested that the quadratic polynomial model was suitable for revealing the mutual relationship of factors and predicting the response values.

Mutual interactive effect of nitrogen, phosphorus and iron on lipid accumulation, biomass yield, chlorophyll, carbohydrate and protein content in *Chlorella pyrenoidosa*

In orthodox one-factor time experiments, a single factor varies, kept other constant factors and the effect of interaction among the variables is ignored. The RSM systematic statistical design tool intended at exploring the relationships between design variables and responses in order to provide a better overall understanding with a minimal number of experimentations (Kirrolia et al. 2014; Myers and Montgomery 2002). The Box–Behnken design creates desirable statistical properties but most importantly with only a fraction of the trials required for a 3-level factorial so the quadratic model is suitable for data evaluation. The sensitivity of the responses of two interacting variables can be excluded by the three-dimensional graphs by holding the other variable at the central values were shown in Fig. 3a–e. The final response for biomass production, lipid accumulation, total chlorophyll, carbohydrate and protein contents in terms of coded factors is presented in Eqs. (1–5):

Model equations in terms of coded factors

$$\begin{aligned}\text{Biomass gL}^{-1} = {} & +1.89+0.12*A+0.1*B \\ & +0.06*C+0.040*A*B-0.075*A*C \\ & +0.090*B*C-0.15*A2 \\ & -0.40*B2-0.14*C2\end{aligned} \tag{1}$$

$$\begin{aligned}\text{Lipid (\%)} = {} & +35.40+0.74*A-1.86*B \\ & +3.71*C-0.81*A*B+0.25*A*C \\ & +0.67*B*C-7.24*A2 \\ & -3.2*B2-6.69*C2\end{aligned} \tag{2}$$

$$\begin{aligned}\text{Chlorophyll } \left(\mu\text{gmL}^{-1}\right) = {} & +20.16+0.78*A-0.050*B \\ & -0.84*C+0.17*A*B \\ & -0.33*A*C-2.34*B*C \\ & -5.92*A2-4.33*B2-1.01*C2\end{aligned} \tag{3}$$

$$\begin{aligned}\text{Carbohydrate}\left(\text{mgmL}^{-1}\right) = {} & +0.35+5.500E \\ & -003*A-0.019*B+0.03*C \\ & -6.000E-003*A*B+2.000E-00*A*C \\ & +5.500E-003*B*C-0.071*A2 \\ & -0.033*B2-0.064*C2\end{aligned} \tag{4}$$

$$\begin{aligned}\text{Protein}\left(\text{mgmL}^{-1}\right) = {} & +0.035-6.250E \\ & -005*A-2.650E-003*B \\ & +2.838E-003*C-2.500E-005*A*B \\ & -3.500E-00*A*C+1.325E-003*B*-7.115E \\ & -003*A2-1.990E-003*B2-6.115E-003*C2\end{aligned} \tag{5}$$

The flat bowl-shaped bottom surface of the standard error is appropriate for an RSM design with no sign of data problems were shown in Fig. 3. Statistical results showed that increase in lipid accumulation was observed with increasing the nitrogen ($NaNO_3$) concentration from 10 mg to 380 mg L^{-1} in the culture medium with simultaneous increase in phosphorus and iron concentration supported biomass yield and declined with further increase in concentration of these selected variables were shown in Fig. 4a. The lipid accumulation and biomass yield of *Chlorella pyrenoidosa* under different initial $NaNO_3$, K_2HPO_4 and ferric ammonium citrate was shown in three-dimensional graphs (Fig. 4a) which indicated higher lipid accumulation under

Table 4 Analysis of variance for the response surface quadratic model of growth parameters in *Chlorella pyrenoidosa*

Source	*df*	Response			Response 2				Response 3			
Model	9	Sum of squares	Mean square	*p* value	Sum of squares	Mean square	*F* value	*p* value	Sum of squares	Mean squares	*F* value	*p* value
A-$NaNO_3$	1	647.17	71.91	<0.0001	1.25	0.138	5.223	0.02	282.36	31.37	2406.81	<0.0001
B-K_2HPO_4	1	4.34	4.34	<0.0001	0.11	0.115	4.347	0.07	4.91	4.91	376.97	<0.0001
C-Ferric ammonium citrate	1	27.64	27.64	<0.0001	0.10	0.101	3.820	0.09	0.02	0.02	1.54	0.2543
AB	1	109.84	109.85	<0.0001	0.03	0.031	1.179	0.31	5.66	5.66	434.70	<0.0001
AC	1	2.60	2.60	<0.0001	0.0064	0.006	0.241	0.63	0.11	0.11	9.18	0.0191
BC	1	0.24	0.247	<0.0001	0.0225	0.022	0.849	0.38	0.42	0.42	32.41	0.0007
A^2	1	1.79	1.79	<0.0001	0.0324	0.032	1.222	0.30	21.94	21.94	1683.43	<0.0001
B^2	1	220.67	220.67	<0.0001	0.097	0.0979	3.695	0.09	147.66	147.66	11328.24	<0.0001
C^2	1	44.65	44.65	<0.0001	0.68	0.6826	25.740	0.001	78.97	78.97	6058.41	<0.0001
Residual	7	188.55	188.55	<0.0001	0.085	0.0855	3.226	0.115	4.31	4.31	331.37	<0.0001
Lack of fit	3	0.0001	1.84		0.185	0.0265			0.09	0.013		
Pure error	4	4.9.05	1.63	0.5481	0.185	0.06			0.09	0.03		
Cor total	16	8-05	2-05		0	0			0	0		
		647.17			1.43				282.45			

Source	*df*	Response 3				Response 4				Response 5			
Model	9	Sum of squares	Mean squares	*F* value	*p* value	Sum of squares	Mean square	*F* value	*p* value	Sum of square	Mean square	*F* value	*p* value
A-$NaNO_3$	1	0.061	0.0068	1775.79	<0.0001	0.06	0.006	1775.79	<0.0001	0.0005	6.117	174.38605	<0.0001
B-K_2HPO_4	1	0.0002	0.0002	62.74	<0.0001	0.0002	0.0002	62.74	<0.0001	3.1258	3.125	0.0890857	0.7740
C-Ferric ammonium citrate	1	0.003	0.0029	768.57	<0.0001	0.002	0.0029	768.57	<0.0001	5.618	5.618	160.15475	<0.0001
AB	1	0.010	0.0105	2725.46	<0.0001	0.0105	0.0105	2725.46	<0.0001	6.441	6.441	183.61993	<0.0001
AC	1	0.00014	0.00014	37.33	0.0005	0.0001	0.0001	37.33	0.0005	2.5-09	2.59	0.0071269	0.9351
BC	1	1.6-05	1.6-05	4.14	0.0811	1.60	1.65	4.14	0.0811	4.9-07	4.90	1.3968642	0.2758
A^2	1	0.00012	0.0001	31.37	0.0008	0.0001	0.0001	31.37	0.0008	7.022	7.022	20.019344	0.0029
B^2	1	0.021	0.021	5541.66	<0.0001	0.021	0.021	5541.66	<0.0001	0.0002	0.0002	607.63712	<0.0001
C^2	1	0.004	0.0046	1206.85	<0.0001	0.0046	0.004	1206.859	<0.0001	1.66	1.66	47.533593	0.0002
Residual	7	0.017	0.017	4436.40	<0.0001	0.017	0.017	4436.40	<0.0001	0.0001	0.0001	448.83572	<0.0001
Lack of fit	3	0.00002	3.857			0.00002	3.85			2.45	3.508		
Pure error	4	0.00002	9-06			0.00002	9E−06			6.87	2.292	0.5184766	0.6918
Cor total	16	0	0			0	0			1.76	4.42		
		0.061				0.0616				0.0005			

Response 1: R^2-96.25%, Adj. R^2 91.43; Response 2: R^2-94.12%, Adj. R^2-86.56%, Response 3: R^2-99.76, Adj. R^2-99.46; Response 4: R^2 99.12%, Adj. R^2 97.99; Response 5: R^2-99.13, Adj. R^2-98.02

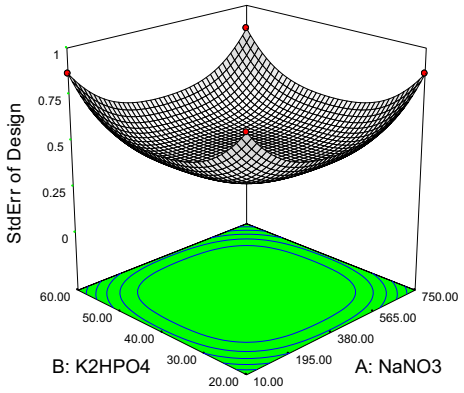


Fig. 3 Three-dimensional graph of standard error design of the model at $NaNO_3$, K_2HPO_4 and ferric ammonium conc

limited supply of nutrients of three variables (Guan et al. 2011). The higher levels of nitrate, phosphate and iron have inhibitory effects on lipid production, biomass yield, total chlorophyll, carbohydrates as well as protein concentration in experimental culture was shown in Fig. 4a–e. The highest lipid yield was obtained at 380 mg mL^{-1} $NaNO_3$, 40 mg mL^{-1} K_2HPO_4 and 6 mg mL^{-1} ferric ammonium citrate, i.e. 35.4% with corresponding biomass 1.89 g L^{-1}. $NaNO_3$ and K_2HPO_4 were the most important variables impacting lipid production and growth, with p value less than 0.0001. With increasing phosphate, nitrate concentrations from 20 to 40 mg L^{-1} and 10 to 388 mg L^{-1}, the cellular lipid content in microalgae *Chlorella pyrenoidosa* increased evidently, where the p value was less than 0.001. However, increased positive value (3.71, 0.67) of linear coefficients of phosphate and ferric ammonium citrate has much significant effects on lipid production capacity (Eq. 1). The negative values of quadratic coefficient for biomass, lipid, chlorophyll, carbohydrate and chlorophyll content (− 0.14, 6.69, 1.01, 0.0064, 6.11), respectively, infer towards an optimum value for phosphate concentration beyond which the decrease in biomass yield, lipid content, chlorophyll content, carbohydrate and protein, respectively, was observed (Eqs. 1–5). The positive linear coefficients in the model equations indicated that response values are increased with increasing the variable level and vice versa (Eqs. 1–5). Three-dimensional plots were included to the sensitivity of multiple responses of two interacting variables by holding other variables at the central values. The experimental results of BBD model are presented in Table 4 used to estimate not only main effects of variables but also their mutual interaction on growth and other variables in *Chlorella pyrenoidosa*. Nitrogen and phosphate are two essential macronutrients for algal growth and metabolism. Additionally, in this study simultaneous interactive effect of different factors, i.e. sodium nitrate, di-potassium hydrogen phosphate, ferric ammonium citrate in BG-11 media, was optimized. Dahmen et al. (2014) reported that *Picochlorum* sp. achieved the highest lipid contents under phosphate starvation condition. As reported by Ho et al. (2014), nitrogen starvation triggered the accumulation of carbohydrates in the *Chlorella vulgaris.*

Homogeneously spread data around either side of zero line indicated the suitability of the model for our present study. The residuals from the least squares fit also play an important role in judging the model adequacy (Myers and Montgomery 2002). The constructing a normal probability plot of the residuals, a check was made for the normality assumption. The normality assumption was satisfied as the residual plot approximated along a straight line. The general impression is that the residuals distribute randomly on the display, suggesting that the variance of the original observation is constant for all values of Y. All residuals plots were found to be satisfactory, thus representing the reliability of the model (Fig. 5a–e). Anand and Arumugam (2015) revealed that the 2.27-fold lipid yield was enhanced in nitrogen-depleted condition (226 mg L^{-1}) when compared to nitrogen-rich condition (99.33 mg L^{-1}). Although nitrogen limitation plays an important role in cell growth, the presence of other macronutrients (e.g. Fe, K and Mg) also affects biomass yield which might be one reason for the cell growth observed during nitrogen restriction (Converti et al. 2009; Chen et al. 2011). Nutrient availability has a substantial impact on growth and culturing of microalgae and wide effects on their lipid content and fatty acid composition (El-Kassas 2013). So far, various studies have been carried out to demonstrate that the nitrogen source was the important nutrition in the medium affecting the growth and lipid accumulation (Li et al. 2008). There was evidence to suggest that nitrogen deficiency could stimulate lipid accumulation (Mandal and Mallick 2009; Welter et al. 2013).

Validation of the model

The accuracy of the model was validated with experiments under the aforementioned optimal conditions. The Box–Behnken model for the growth media with varying concentration of nitrate ($NaNO_3$, 10–750 mg mL^{-1}), phosphate (K_2HPO_4, 40–120 mg mL^{-1}), iron (ferric ammonium citrate 3–9 mg mL^{-1}) in BG-11 media and lipid percentage (35.4 dcw%) and biomass yield (1.89 g L^{-1}) under nutrient stress condition of nitrogen, phosphorus and iron, which confirmed the validity of the predicted model. The residuals from the least squares fit also play an important role in judging the model adequacy. The normality assumption was satisfied as the residual plots approximated along a straight line (Singh et al. 2015). Relating to the enhancement of lipid accumulation in *Nannochloropsis oculata*, the effect of nitrogen inadequacy on lipid content in microalgal cultures has been earlier reported (Rodolfi et al. 2008; Fakhry and El Maghraby 2015; Kirrolia et al. 2014). The collective effect

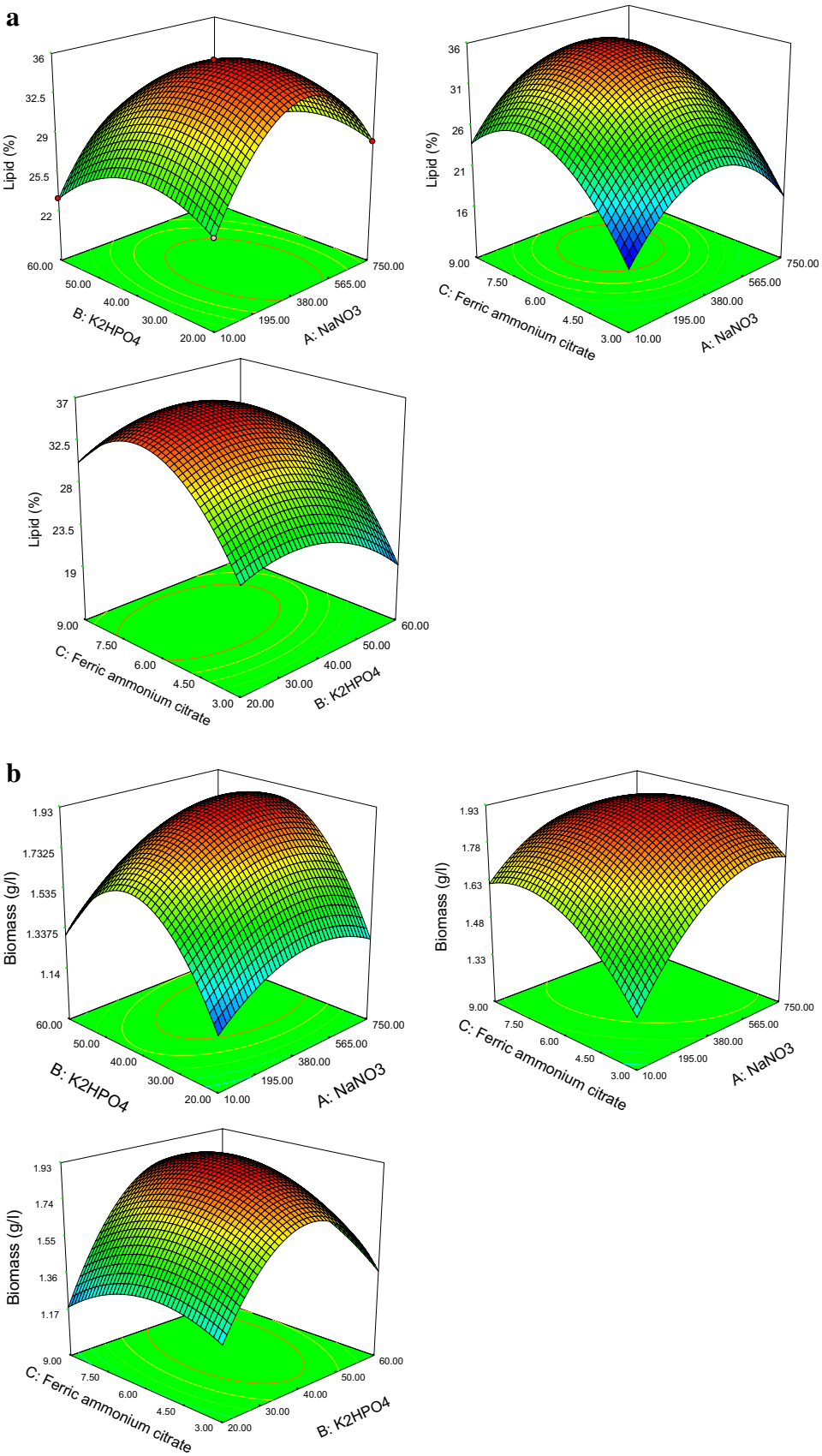
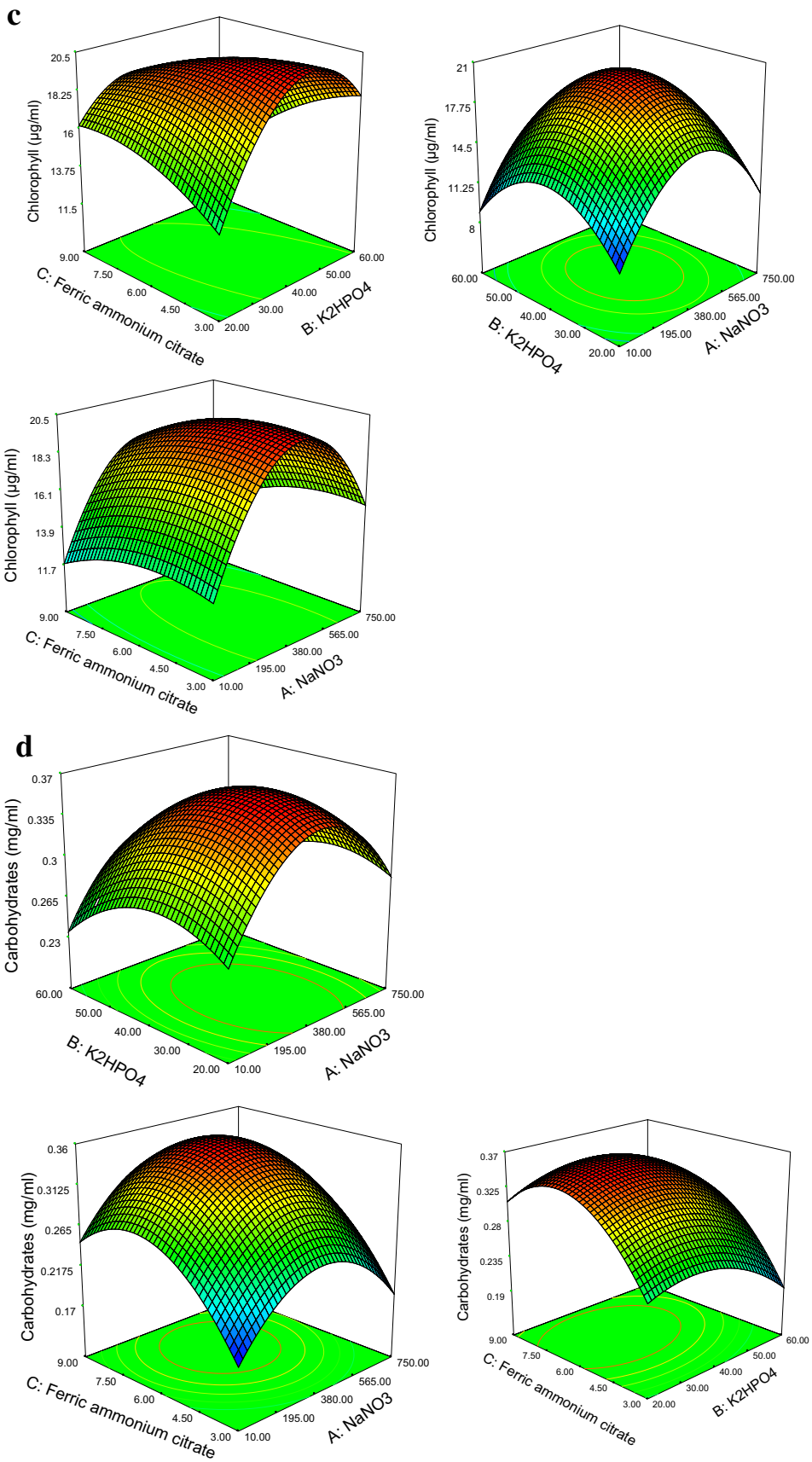


Fig. 4 Normal plots of studentized residuals verses normal percentage probability biomass. Three dimensional plot for **a** biomass (g L^{-1}), **b** lipid accumulation (%), **c** chlorophyll (µg mL^{-1}), **d** carbohydrate (µg mL^{-1}) and **e** protein (µg mL^{-1}). **A**, **B** Response surface graphs for the interactive effect of initial concentration sodium of nitrate, di-potassium hydrogen phosphate and ferric ammonium citrate on lipid % and biomass yield g L^{-1} in *Chlorella Pyrenoidosa*. **C**, **D** Response surface graphs for the effect interactive of initial sodium nitrate concentration, di-potassium hydrogen phosphate and ferric ammonium citrate on carbohydrate and total chlorophyll content (µg mL^{-1}) in *Chlorella pyrenoidosa*. **E** Response surface graphs for interactive the effect initial concentration of Sodium nitrate, di-potassium hydrogen phosphate and ferric ammonium citrate on protein content mg mL^{-1} in *Chlorella Pyrenoidosa*

Fig. 4 (continued)



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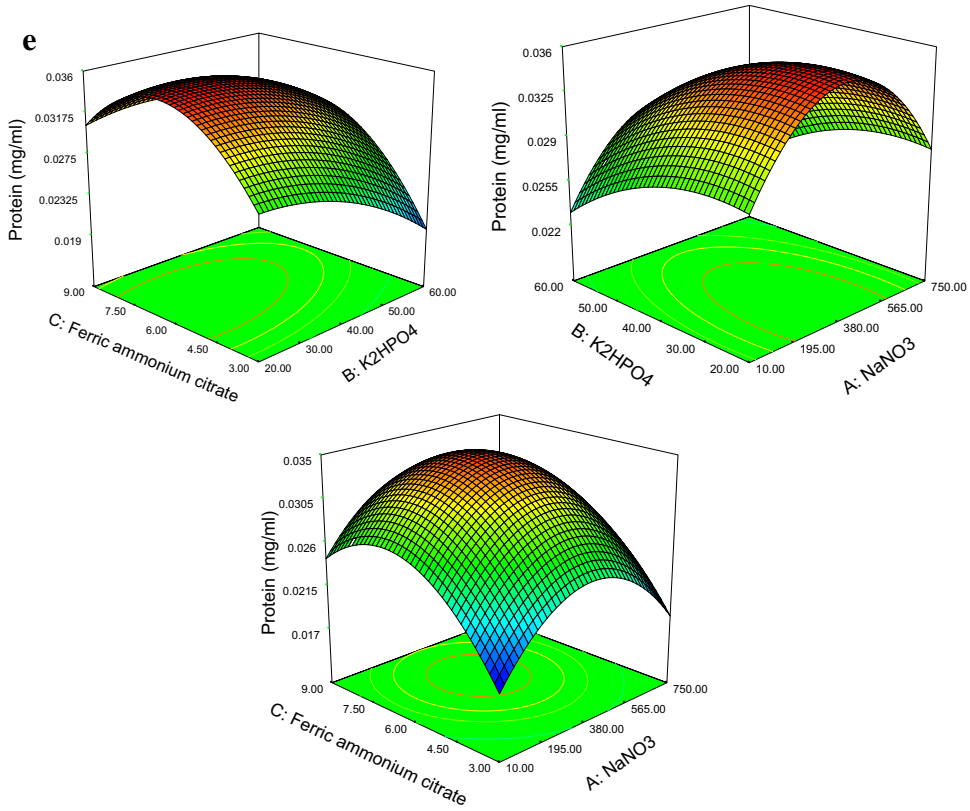
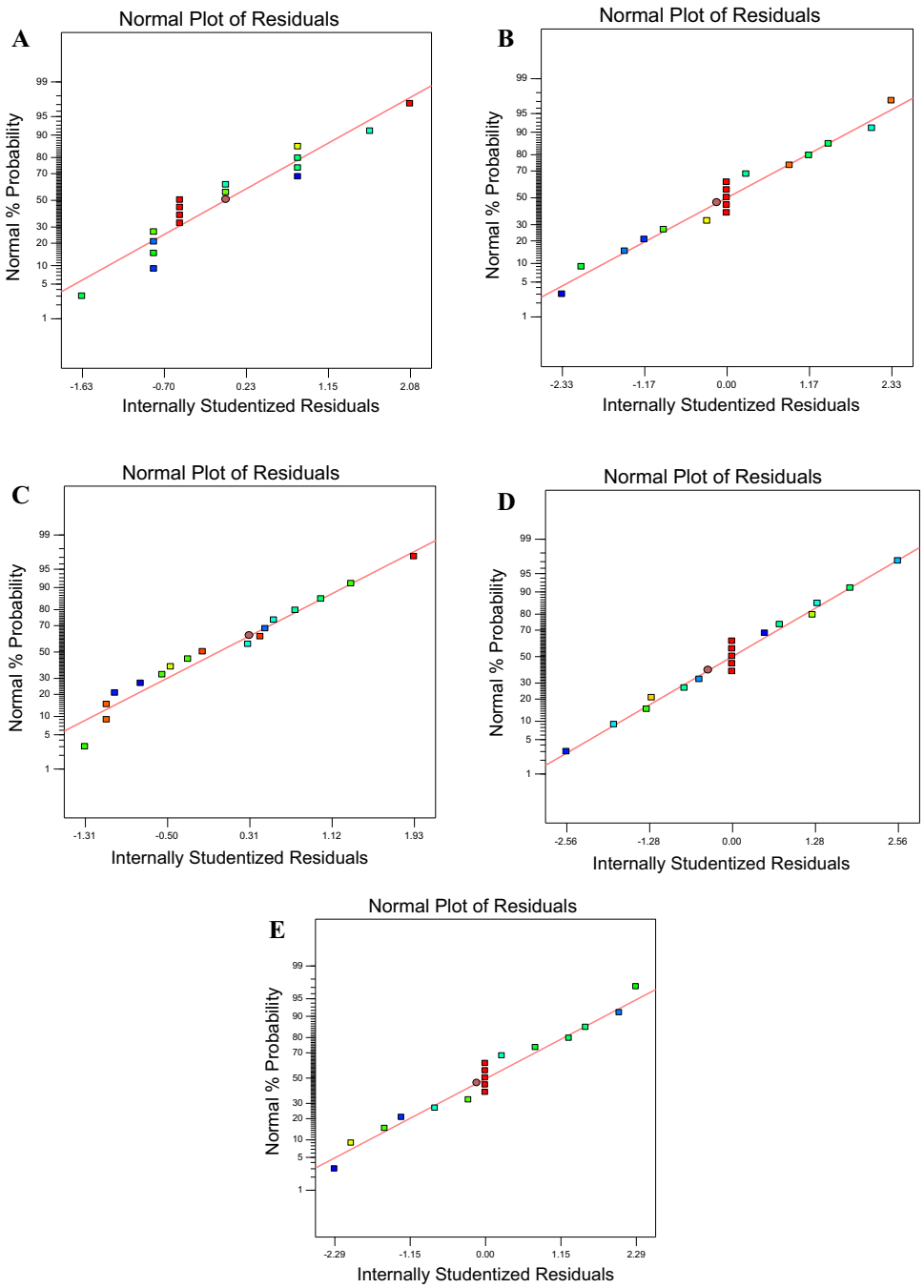


of nutrient, viz. nitrogen, phosphorous and iron stresses, on lipid productivity of selected strain was studied by response surface methodology, and the highest lipid content of 59.6% and lipid productivity of 74.07 mg L^{-1} per day was obtained in *Ankistrodesmus falcatus* KJ671624 (Myers and Montgomery 2002; Singh et al. 2015) as we obtained lipid percentage (35.4 dcw%) and biomass yield (1.89 g L^{-1}) under nutrient stress condition of nitrogen, phosphorus and iron.

Analysis of free fatty acids (FFAs) in *Chlorella pyrenoidosa*

In the present study, trans-esterification process developed by Abdo et al. (2014) was employed after optimization of reaction conditions, temperature, extraction time for preparation of FAME analysis from algal biomass and after analysis, and detection of diesel hydrocarbons. Description of typical relative percentage free fatty acids detected by GCMS in fatty acids methyl esters samples obtained through trans-esterification process was shown in Table 5 and Fig. 5. FAME composition of fresh water algal strain, *Chlorella pyrenoidosa,* was calculated as percentage of total esters present in the sample, determined from the peak areas. The GCMS chromatograph and free fatty acids profile of *Chlorella pyrenoidosa* oil individual FAME was shown in (Table 5, Fig. 5). Palmitic 16:0, palmitoleic C16:1, stearic acid C:18, oleic acid C:18:1 were detected in FAME samples. *Chlorella pyrenoidosa* consist of short-, medium- and long-chain diesel hydrocarbons. In *Chlorella pyrenoidosa*, both saturated and unsaturated fatty acids were identified. The polyunsaturated FA (4 double bond) was absent in *Chlorella* strains. GC–MS results revealed that the hydrocarbons such as heptacosane and 2-ethyl-2methyl tridecanol were detected among the hydrocarbons produced by *Chlorella pyrenoidosa*. The saturated fatty acids were identified mainly as palmitic acid methyl ester (C16:0, 33.91%) by trans-methylation trans-esterification process. As per previous study, short-chain hydrocarbons (< C20) were commonly present in *Chlorella pyrenoidosa* than compared to long-chain hydrocarbons (> C19) (Matsumoto et al. 2010). As per one study, *Chlorella* sp. possessed maximum linolenic acid (C18:3) (14.20%). On the other hand, the unsaturated fatty acids have been detected as palmitoleic methyl ester (C16:1, 12.05%), 18:1 oleic acid methyl esters, (22.72%), C18:2 (9.26%) linoleic acid have been detected in direct *trans*-methylation process (Abdo et al. 2014). Similar fatty acids profile has been detected in *Chlorella* sp. and *Scenedesmus* sp. by direct trans-esterification process (Kirrolia 2015). Hence, the above results stated that *Chlorella pyrenoidosa* possessed a approving fatty acids profile that can be successfully utilized for biodiesel production.

Fig. 4 (continued)



Conclusion

Microalgal biodiesel appeared to be the most promising renewable third-generational biofuel. *Chlorella* microalgae is considered as a promising for industrial-scale biodiesel production as it possessed elevated biomass, rapid growth lipid content and fatty acid productivity. In conventional one-factor time experiments, a single factor varies, keeping other factors constant, and the effect of interaction among the variables is disregarded. The RSM is a logical statistical design methodology aimed at exploring the interactions between design variables and responses in order to provide a better overall understanding with less number of experiments for optimum biomass and cellular lipid content. Lipid content, biomass, carbohydrate, chlorophyll, protein and biomass produced were studied under varied concentration of nutrients in BG11 media, nitrate ($NaNO_3$, 10–750 mg mL^{-1}), phosphate (K_2HPO_4, 40–120 mg mL^{-1}), iron (ferric ammonium citrate 3–9 mg mL^{-1}) with an aim to establish the actual potential of *Chlorella pyrenoidosa* under phototrophic nutrient stress conditions; we obtained lipid percentage (35.4 dcw%) and biomass yield (1.89 g L^{-1})

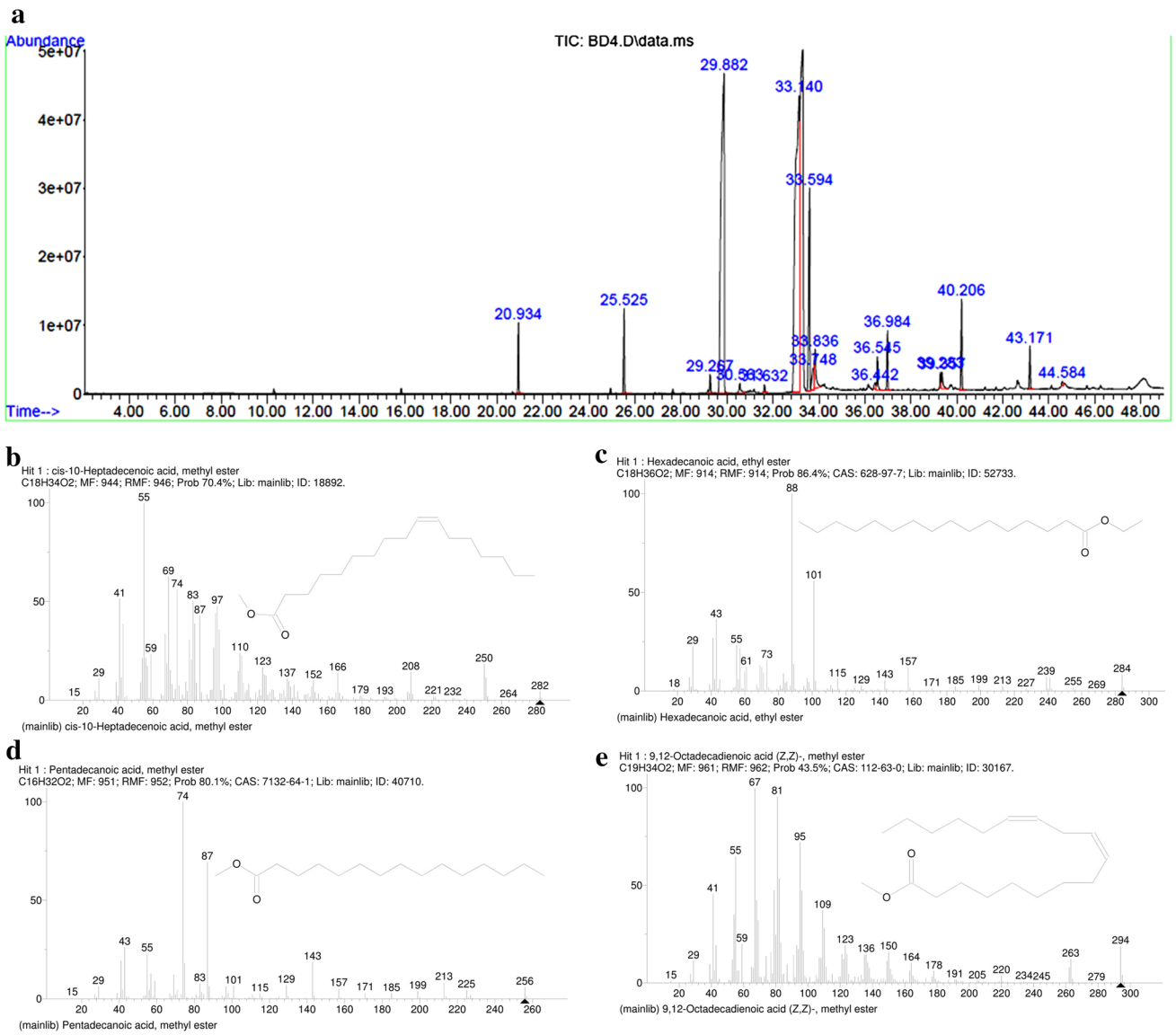


Fig. 5 GCMS mass spectra of *Chlorella pyrenoidosa* **a** palmitic acid, **b** stearic acid, **c** oleic acid and **d** linoleic acid

under nutrient stress condition of nitrogen, phosphorus and iron, which confirmed the validity of the predicted model. GCMS study revealed that palmitic 16:0, palmitoleic C16:1, stearic acid C:18, oleic acid C:18:1 were detected in FAME samples of *Chlorella pyrenoidosa* which makes it potential biodiesel-producing strain. Further, large-scale production of microalgal biodiesel at industrial applications prerequisites to resolve several environmental, economically and technological issues and microalgal oil productivity per acre can be improved by acquainting synthetic biology techniques, as well as refining the light source systems (Arudchelvam and Nirmalakhandan 2013).

Table 5 Analysis of free fatty acids (FFAs) of *Chlorella pyrenoidosa*

S. no	Common name	IUPAC name	Abbreviation	Molecular formula	Free fatty acids detected in *Chlorella pyrenoidosa* oil samples (FAME) (%)
1.	Caproic acid	Hexaonic acid	C6:0	$C_6H_{12}O_2$	ND
2.	Caprylic acids	Octanoic acid	C8:0	$C_8H_{16}O_2$	ND
3.	Undecanoic acid	Undecanoic acid	C11:0	$C_{11}H_{22}O_2$	5.21
4.	Lauric acid	Dodecanoic acid	C12:0	$C_{12}H_{24}O_2$	1.51
5.	Lauric acid	Dodecanoic acid	C12:1	$C_{13}H_{26}O_2$	0.12
6.	Myristic acid	Tetradecanoic acid	C14:0	$C_{14}H_{28}O_2$	0.31
7.	Pentadecanoic acid	Pentadecanoic acid	C15:0	$C_{15}H_{30}O_2$	1.65
8.	Pentadecenoic acid	*Cis*-10-heptadecenoic acid	C15:1	$C_{15}H_{28}O_2$	ND
9.	Palmitic acids	Hexadecanoic acid	C16:0	$C_{16}H_{32}O_2$	33.912
10.	Palmitoleic acids	*Cis*-9-*Cis*-10-heptadecenoic acid	C16:1	$C_{16}H_{30}O_2$	12.05
11.	Margaric acids	Heptadeconic acids	C17:0	$C_{17}H_{34}O_2$	0.53
12.	*Cis*-10-heptadecenoic acid	*Cis*-10-heptadecenoic acid	C17:1	$C_{17}H_{32}O_2$	ND
13.	Stearic acid	Octadecanoic acid	C18:0	$C_{18}H_{36}O_2$	1.782
14.	Oleic acid	*Cis*-9-octadecanoic acid	C18:1	$C_{18}H_{34}O_2$	22.72
15.	Linoleic acid	*Cis*-9,12,octadecadienoic acid	C18:2	$C_{18}H_{32}O_2$	9.286
16.	Linolenic acid	*Cis*- 9,12,15-octatetradecoic acids	C18:3	$C_{18}H_{30}O_2$	11.58
17.	Nonadecanoic acid	Nonadecyclic acid	C19:0	$C_{19}H_{88}O_2$	0.585
18.	Arachidic acid	Eicosanoic acid	C20:0	$C_{20}H_{40}O_2$	7.395
19.	Behenic acid	Docosenoic acid	C22:0	$C_{22}H_{44}O_2$	ND
20.	Erucic acid	*Cis*-13-docosenoic acid	C22:1	$C_{22}H_{42}O_2$	ND
21.	Heptacosylic acid	Heptacosanoic acid	C27:0	$C_{27}H_{56}O_2$	0.166
				SFAs%	44.37
				MUFA%	34.77
				PUFA%	20.86

Relative percentage of free acids detected by GCMS

SFA Saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

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