Formulation of a minimal nutritional medium for enhanced lipid productivity in *Chlorella* sp. and *Botryococcus* sp. using response surface methodology

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ABSTRACT

Chlorella sp. MCC 7 and *Botryococcus* sp. MCC 31 were investigated to enable large-scale biodiesel production from minimal constituents in the growth medium. Response surface methodology (RSM) was used to maximise the biomass productivity and lipid yield using only nitrogen (N), phosphorus (P) and potassium (K) as urea, single super phosphate and muriate of potash. The optimum values were 0.42 g/L nitrogen; 0.14 g/L phosphorus and 0.22 g/L potassium for *Chlorella* sp.; and 0.46 g/L; 0.14 g/L and 0.25 g/L for *Botryococcus* sp. Lipid yield of 42% for *Chlorella* sp. and 52% in *Botryococcus* sp. was observed. An enhancement in lipid yield by approximately 55% for *Chlorella* sp. and 73% for *Botryococcus* sp. was registered as compared to original nutrient medium. Fourier transform infrared (FTIR) analysis of extracted lipids revealed characteristic bands for triglycerides. This study provided utilisation of a practicable nutrient recipe in the form of N, P, K input for enhanced lipid yield from the selected microalgal strains.

Key words | biomass, *Botryococcus*, *Chlorella*, lipids, optimisation, RSM

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INTRODUCTION

Development of alternative fuel technologies have primarily focussed on the production of biodegradable, renewable and non-toxic fuel due to the instability of petroleum fuels, cost involved and dangers of CO_2 emission (Chisti 2007). Microalgae as well as macroalgae have been considered as possible sources for biofuel since decades. Microalgae are reported to store energy-rich compounds such as triacylglycerol (TAG) and starch, hence are considered as suitable feedstocks in the area of biodiesel. Cultivation of microalgae is ecofriendly and they can be grown under a wide range of conditions, including non-arable or marginal lands, using waste and saline water. Therefore, production of biodiesel from microalgae may not have competition with food and feedstocks (Chisti 2007; Amaro *et al.* 2011; Huang *et al.* 2010).

Extensive research has revealed that environmental conditions can modify the lipid metabolism of microalgae efficiently. In particular, nutritional factors such as nitrogen, phosphorus, carbon and iron are recognized as one of the most vital factors influencing the lipid yield and biomass (Yeesang & Cheirsilp 2011; White *et al.* 2013). Some microalgae can be grown in simple nutrient media, whereas other species require more complex media compositions containing essential nutrients (nitrogen, phosphorus, sulphur, carbon, iron and trace elements) to sustain growth (Ernst *et al.* 2005). It is crucial to select the most appropriate nutrients and their quantities to have the maximum biomass productivity and lipid yield. The lipid production is the product of lipid content and biomass productivity, and is considered to be an important indicator for evaluating microalgal biomass for biodiesel production.

Generally, nutrient starvation, such as nitrogen and phosphorus deficiency, can stimulate lipid accumulation (Reitan *et al.* 1994; Dean *et al.* 2010), and the lipid content of *Nannochloris* sp. UTEX LB1999 increased by 83.08%, with nitrogen concentration decreasing to 0.9 mM. However, the deficiencies in nutrients may limit the growth of microalgae; hence, the overall lipid production may be lower (Li *et al.* 2008; Griffiths & Harrison 2009). Moreover, these studies have been carried out by using single-factor optimization and such studies may result in unsatisfactory or incorrect results, if the interaction studies between factors are not carried out. Response surface methodology (RSM) is an effective and convenient tool to screen key factors rapidly from multiple factors for optimizing cultural conditions, and this may avoid the erroneous results achieved by single-factor optimization (Zhang *et al.* 2012; Qin *et al.* 2013).

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There are few reports available regarding the application of RSM for optimizing the autotrophic mode of nutrition in lipid-rich microalgae. Lipid production is enhanced by a two-step strategy with initial optimization of microalgal growth and final optimization of lipid accumulation (Cheng *et al.* 2013; Karemore *et al.* 2013). Central composite design (CCD) allows estimating of the polynomial regression between independent variables and dependent variables that optimizes the estimation of a second-order model, allowing for reduced costs and lesser time for the experimentation (Zheng *et al.* 2008).

In the present study, the interactive effect of nitrogen, phosphorus and potassium was evaluated, through RSM using CCD, on the lipid yield and biomass productivity of *Chlorella* sp. MCC 7 and *Botryococcus* sp. MCC 31. This study emphasizes the role of major nutrients, viz. nitrogen, phosphorus and potassium, and provides a minimal growth formulation in the form of urea, single super phosphate and muriate of potash. This kind of study has probably been used for the first time to understand the influence of these nutrients for maximum biomass productivity and lipid yield.

MATERIALS AND METHOD

Cultural conditions

Microscopically identified *Chlorella* sp. (MCC 7) and *Botryococcus* sp. (MCC31) were procured from the culture collection of CCUBGA, IARI, New Delhi. These were incubated and maintained in a modified medium containing NPK fertilizers (*i.e.* urea, single super phosphate and muriate of potash, in an appropriate ratio) at a temperature of $28 \pm 2\,^{\circ}C$, light intensity of $95\,\mu E\,m^{-2}\,s^{-1}$ with 16: 8 h light: dark cycle. The inoculum size, pH of the medium and the concentrations of the essential nutrients were used as per previous experiments (Rakesh *et al.* 2013, 2015). Statistical programs were used to design the experiment in order to obtain the best nutrient composition for growth and lipid yield of microalgal strains.

Experimental design and statistical analysis

CCD was used to develop Response Surface models to understand the interactive effects of nitrogen, phosphorus and potassium on growth and lipid yield of *Chlorella* sp. and *Botryococcus* sp. (Zheng *et al.* 2008).

To determine the optimum response regions for the observed parameters, and to study the combined effect of each independent variable, three-level, three-factor factorial CCD was created with a set of three variables, namely nitrogen, phosphorous and potassium, designated as N, P and K. Each variable was studied at three different levels (−1, 0, +1) and their respective responses (Y) as biomass productivity and lipid yield are depicted in Tables 1 and 2. With the CCD chosen, there were six replicates of the central point, six axial points, and eight factorial points (Tables 1 and 2).

The experimental data (biomass (mg/L), lipid (%)) were analyzed with regression, fitted with the RSRegress command of Minitab (v. 16, Minitab Inc.) in the following second-order polynomial equation:

$$Y = \beta_0 + \Sigma_{i=1}\beta_i x_i + \Sigma_{i=1}\beta_{ii} x_i^2 + \Sigma_{i=1}\Sigma_{j=i+1}\beta k_{ij} x_i x_j \quad (1)$$

On the basis of the initial results, a range of concentrations for nitrogen (0.4–0.5 g/L), phosphorus (0.12–0.24 g/L) and potassium (0.15–0.25 g/L) were tested for optimizing the nutrient medium for *Chlorella* sp. and *Botryococcus* sp. respectively.

In this equation, Y is the response of lipid yield (%) and biomass production (mg/L); β, β_0, β_i, β_{ii} and β_{ij} are constant coefficients and x_i, x_j are the coded independent variables, namely N, P and K, which influence the response variable Y. This response is preferred because a relatively few experimental combinations of variables are sufficient to estimate potentially complex response functions and their interrelations.

Biomass determination and lipid extraction

Microalgal biomass was harvested from the experimental flasks during the exponential phase (14th day of incubation). For dry weight measurements, aliquots of 20 mL homogenised microalgal suspension were filtered by preweighed GF/C filter paper (Whatman, Poole, UK), dried at 80 °C to a constant weight and after cooling to room temperature, the final dry weight of biomass was calculated. The pre-treated dried biomass with microwave (6 minutes), was used for the extraction of lipids (Bligh & Dyer 1959), using a mixture of chloroform-methanol in a ratio of 2:1(v/v) with vigorous shaking for three hours, and the collected lipid content was calculated from the equation, as below:

$$\text{Lipid content (\%)} = \frac{\text{weight of lipid extracted (mg)}}{\text{sample weight (mg)}} \times 100 \quad (2)$$

Table 1 | Lipid and biomass yield with predicted and obtained values in *Chlorella* sp. and *Botryococcus* sp

				Chlorella				*Botryococcus*			
				Lipid (%)		Biomass (mg/L)		Lipid (%)		Biomass (mg/L)	
Run order	N (g/L)	P (g/L)	K (g/L)	Obtained	Predicted	Obtained	Predicted	Obtained	Predicted	Obtained	Predicted
1	0.40	0.12	0.15	32.4	32.9	316	335.014	37.8	38.8	216	197.768
2	0.50	0.12	0.15	36.8	37.6	386	391.614	44.4	45.2	236	226.768
3	0.40	0.24	0.15	38.4	38.5	442	442.214	48.4	48.6	280	284.968
4	0.50	0.24	0.15	44.2	43.8	522	531.814	37.6	36.2	272	268.968
5	0.40	0.12	0.25	42.8	43.6	496	486.214	40.2	41.1	194	194.168
6	0.50	0.12	0.25	39.8	40.2	448	447.814	62.3	61.6	190	182.168
7	0.40	0.24	0.25	38.9	38.6	478	472.414	38.9	37.6	252	258.368
8	0.50	0.24	0.25	36.0	35.9	486	467.014	40.8	39.3	186	201.368
9	0.40	0.18	0.20	40.7	39.6	474	470.145	43.4	42.3	256	262.727
10	0.50	0.18	0.20	41.3	40.6	492	495.745	43.6	46.3	244	248.727
11	0.45	0.12	0.20	42.6	40.1	422	407.345	50.0	47.9	248	283.127
12	0.45	0.24	0.20	39.9	40.7	456	470.545	37.8	41.6	360	336.327
13	0.45	0.18	0.15	41.1	40.1	468	433.345	44.8	44.1	216	241.527
14	0.45	0.18	0.25	42.3	41.5	442	476.545	44.4	46.8	220	205.927
15	0.45	0.18	0.20	40.2	41.2	476	465.036	45.2	45.5	290	281.182
16	0.45	0.18	0.20	41.8	41.2	470	465.036	46.0	45.5	274	281.182
17	0.45	0.18	0.20	39.8	41.2	458	465.036	45.6	45.5	294	281.182
18	0.45	0.18	0.20	40.3	41.2	460	465.036	46.6	45.5	286	281.182
19	0.45	0.18	0.20	41.3	41.2	454	465.036	47.2	45.5	278	281.182
20	0.45	0.18	0.20	40.1	41.2	472	465.036	46.2	45.5	288	281.182

Fourier transform infrared (FTIR) analysis of extracted lipid

The extracted and weighed lipid was subjected to FTIR analysis for identification (FTIR spectrometer, model Alpha Bruker). In FTIR, the lipid sample was mixed with KBr in a ratio of 5:100 to make the KBr discs for spectrum analysis. The spectrum was obtained over a range of 400 cm^{-1} to 4,000 cm^{-1} with a spectral resolution of 0.5 cm^{-1} and the functional groups present in the lipids were identified (Schmitt & Flemming 1998).

RESULTS AND DISCUSSION

Optimisation of cultural conditions

Amongst the various bioprocess parameters affecting lipid productivity, cultural conditions are known to play a major role in growth, biomass production, and lipid accumulation. The quantity and quality of the lipids from algal biomass can be regulated and improved by varying the composition of the nutrient medium. A number of other studies have reported the effects of different cultivation conditions, in particular nitrogen sources and levels on growth (biomass) and lipid production (Ren *et al.* 2013; Ren & Ogden 2014). The growth response was influenced by all investigated factors (N, P, K), and their effects were either individual or interactive. The highest lipid yield (42%) was obtained in runs 11 and 5 at a medium concentration of nitrogen (0.45 g/L) and potassium (0.2 g/L) and minimum concentration of phosphorus (0.12 g/L) for *Chlorella*. However, for *Botryococcus,* a lipid yield of 62% was obtained in run 6 with maximum nitrogen and potassium concentrations (0.5 g/L, 0.25 g/L) and minimum concentration of phosphorus (0.12 g/L). Nitrogen is a key factor, since its depletion can lead to drastic metabolic remodeling and the increased production of lipids relevant to fuel production (Simionato

Table 2 | Experimental design matrix showing CCD for RSM

Run order	N	P	K
1	−1	−1	−1
2	1	−1	−1
3	−1	1	−1
4	1	1	−1
5	−1	−1	1
6	1	−1	1
7	−1	1	1
8	1	1	1
9	−1	0	0
10	1	0	0
11	0	−1	0
12	0	1	0
13	0	0	−1
14	0	0	1
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

et al. 2013). Hallenbeck *et al.* (2015) have also carried out an RSM–DOE study of the influence of important culture variables and examined the conditions that maximize biomass production and lipid content. Mandal & Mallick (2009) reported that $NaNO_3$ had a positive effect on lipid production in *Scenedesmus* sp., where experiments were limited to nine days of culture, and the nitrogen level was set in the range of 250 and 1,000 mg/L. Experiments by Yang *et al.* (2014) have identified $NaHCO_3$ as a significant factor in lipid production in addition to $NaNO_3$ in *Scenedesmus* sp. The lipid yield of *Picochlorum* sp. increased under nitrogen starvation and was in line with the results obtained by Li *et al.* (2008), who also reported that nitrogen is the most common nutrient limiting factor in lipid accumulation of microalgae. Tornabene *et al.* (1993) reported that higher lipid content was obtained at lower $NaNO_3$ concentration for green algae. Illman *et al.* (2000) observed that the lipid content of *Chlorella vulgaris* increased under low nitrogen level. The effect of media composition on the growth of *B. braunii* LB572 was examined by Tran *et al.* (2010) using fractional factorial design and CCD for faster growth and enhancement of lipid content.

CCD to evaluate the significant nutrient factors

The *p* values were used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The more significant coefficient corresponded to the smaller value of *p*. Positive sign in front of the terms indicates a synergistic effect, whereas negative sign indicates an antagonistic effect. When the *p*-value of the variable was less than 5%, it represented that the variable had significant effects on the response value. To further assess the effect of variables, coefficient estimate was applied. Lipid production enhanced with increasing concentrations of the variable if the coefficient estimate was positive; conversely, if the value was negative, it indicated that lipid production was negatively correlated with the variable levels.

The responses of the CCD design were fitted with a second-order polynomial equation (Equation (1)). The statistical significance of the model equation was evaluated by the F- test for analysis of variance (ANOVA), which showed that the regression was statistically significant. The 'Prob > F' value for the model was <0.0001, which indicated that the model was statistically significant with a confidence interval of 95%. The coefficient of determination (R^2) of the model was >85%, which further indicated that the model was suitable for adequately representing the real relationships among the selected reaction variables. The overall second-order polynomial equation for lipid yield and biomass productivity for *Chlorella* sp. and *Botryococcus* sp. in terms of uncoded units can be written as follows:

$$Y_1 = -168.534 + 555.535\,N + 235.438\,P - 595.93\,K - 440\,N^2 - 230.556\,P^2 - 156\,K^2 + 61.25\,N \times P - 805.5\,N \times K - 873.75\,P \times K$$

$$Y_2 = 227.75 - 4,786.27\,N + 3,914.92\,P + 8,136.55\,K + 7,163.64\,N^2 - 7,247.47\,P^2 - 4,036.36\,K^2 + 2,750\,N \times P - 9,500\,N \times K - 10,083.3P \times K$$

$$Y_3 = -109.62 + 455.636\,N + 950.121\,P - 399.073\,K - 461.818\,N^2 - 209.596\,P^2 - 21.818\,K^2 - 1,566.67\,N \times P + 1,410\,N \times K - 1,108.33\,P \times K$$

$$Y_4 = -3,130.35 + 10,518.6\,N - 340.38\,P + 11,026.7\,K - 10,181.2\,N^2 + 7,929.3\,P^2 - 22,981.8\,K^2 - 3,750\,N \times P - 4,100\,N \times K - 1,916.67\,P \times K$$

where Y_1 is lipid yield (%) and Y_2 is biomass production for *Chlorella* sp., and Y_3 is lipid yield (%) and Y_4 is biomass production for *Botryococcus* sp. The 3D response surface plots were obtained by plotting the response (percentage conversion) on the Z-axis against any two variables while keeping the other variable at its '0' level.

The analysis of variance for the experimental results of the CCD is shown in Table 2. All the linear terms, three quadratic terms, and three interaction terms were significant. The results of variance analysis, estimation of parameters and the regression coefficients for the lipid content and biomass yield are shown in Tables 3 and 4. The quality of the model developed was evaluated based on the correlation value. The R^2 value was relatively high, indicating that there was good agreement between the experimental and the predicted growth uptake from this model. The regression coefficients and the interaction between each independent factor can be considered statistically significant at 95%. All three linear coefficients, squared coefficients, and the interaction coefficient (AC and BC) were significant, as evidenced from low *p* and high F values.

The ANOVA result of the biomass production shows the quadratic model with F- value and *p*-value <0.005 to be significant (Table 5). For *Chlorella* sp., the goodness of the fit of the model was checked by the determination of correlation coefficient (R^2) which was calculated to be 86.72%, indicating that 86.72% of variables fit the response. The Adjusted R^2 of 74.76% indicated the number of predictors in the model. In *Botryococcus* sp., the R^2 and Adj R^2 values were 88.97% and 79.04%. The lack-of fit F-value of 9.81 for *Chlorella* sp. and 12.55 for *Botryococcus* sp. is not significant. R^2 cannot determine whether the coefficient estimates and predictions are biased, which is why the residual plots were assessed. This thus confirms that the model is statistically sound and can be used to navigate the design space.

The ANOVA of the lipid yield summarized in Table 6 showed a coefficient of determination (R^2) as 88.32%, which means that the model explains 88% of the variability in the data for *Chlorella* and the adjusted R^2 value was 77.8%. However, in *Botryococcus* sp. the R^2 and adjusted R^2 values were 91.77% and 84.73%. The lack of fit *p*-value for *Chlorella* sp. and *Botryococcus* sp. indicates that the linear predictors are not sufficient to explain the variation in the data. Minitab uses the Wherry formula to calculate the adjusted R^2, which is also related to R^2 shrinkage. R^2 shrinkage refers to the fact that the sample R^2 is systemically higher (a positive bias) than the corresponding population R^2 due to the optimizing process for multiple regression.

Table 3 | Analysis of Variance for lipid (%)

		Chlorella sp.				Botryococcus sp.			
Source	**DF**	**SS**	**MS**	**F**	***p***	**SS**	**MS**	**F**	***p***
Regression	9	115	12.87	7.25	0.002	532	59.15	12.35	0.000
Linear	3	7.977	2.65	1.50	0.274	154	51.51	10.75	0.002
N	1	2.237	2.23	1.26	0.28	39.60	39.60	8.27	0.01
P	1	0.992	0.99	0.56	0.47	96.72	96.72	20.2	0.00
K	1	4.747	4.74	2.67	0.13	18.22	18.22	3.8	0.08
Square	3	20.25	6.74	3.80	0.04	15.58	5.192	1.08	0.40
N × N	1	16.78	3.32	1.87	0.20	13.61	3.695	0.77	0.40
P × P	1	3.050	1.89	1.07	0.32	1.953	1.585	0.33	0.58
K × K	1	0.418	0.41	0.24	0.64	0.010	0.010	0.00	0.96
Interaction	3	87.68	29.22	16.4	0.00	362	120	25.2	0.00
N × P	1	0.270	0.27	0.15	0.70	175	175	36.7	0.00
N × K	1	32.44	32.44	18.2	0.002	98.70	98.7	20.6	0.001
P × K	1	54.97	54.96	30.96	0.000	87.78	87.7	18.32	0.002
Residual error	10	17.755	1.7755			47.904	4.79		
Pure error	5	3.155	0.6311			2.533	0.507		
Total	19	133				580			

DF: Degrees of Freedom; SS: Sequential Sum of Squares; MS: Adjusted Mean Squares.

Table 4 | Analysis of Variance for biomass (mg/L)

		Chlorella sp.				Botryococcus sp.			
Source	**DF**	**SS**	**MS**	**F**	***p***	**SS**	**MS**	**F**	***p***
Regression	9	3,190	3,540	8.40	0.001	31,200	3,470	8.96	0.001
Linear	3	1,630	5,430	12.88	0.001	10,700	3,570	9.23	0.003
N	1	1,640	1,640	3.89	0.077	490	4,900	1.26	0.287
P	1	990	990	23.68	0.001	7,070	7,070	18.26	0.002
K	1	4,660	4,660[3]	11.07	0.008	3,160	3,160	8.18	0.017
Square	3	320	1,060	2.54	0.116	1,830	6,130	15.82	0.000
N × N	1	722	880	2.09	0.179	9,160	1,780	4.6	0.058
P × P	1	2,850	1,870	4.44	0.061	1,560	2,240	5.78	0.037
K × K	1	280	280	0.66	0.434	9,070	9,070	23.43	0.001
Interaction	3	1,230	4,120	9.79	0.003	2,110	7,050	1.82	0.207
N × P	1	540	540	1.29	0.282	1,010	1,010	2.61	0.137
N × K	1	4,510	4,510	10.70	0.008	840	840	2.17	0.172
P × K	1	7,320	7,320	17.36	0.002	264	264	0.68	0.428
Residual Error	10	4,210	4,210			3,870	387		
Pure Error	5	390	78			286	57.20		
Total	19	360				35,100			

DF: Degrees of Freedom; SS: Sequential Sum of Squares; MS: Adjusted Mean Squares.

The Wherry adjusted R^2 is a commonly used method to estimate an unbiased, true R^2 for the population. This is the first time that such a method has been applied to the optimization of cultivation conditions in these microalgae having potential significance in the area of biofuel. The only other multifactorial optimization study carried out previously chose to optimize N, Fe, and temperature through a less robust Taguchi procedure that does not provide a model

Table 5 | Response surface regression: lipid (%) versus N, P, K; estimated regression coefficients for lipid (%) using coded units

	Chlorella sp.			Botryococcus sp.		
	Coeff (uncoded)	**Coeff**	**SE Coef**	**Coeff (uncoded)**	**Coeff**	**SE Coef**
Constant	−168.53	41.22	0.46	−109.62	45.54	0.75
N	555.53	0.47	0.42	455.63	2.00	0.69
P	235.43	0.31	0.42	950.12	−3.12	0.69
K	595.93	0.69	0.42	−399.07	1.36	0.69
N × N	−440.00	−1.10	0.80	−461.82	− 1.15	1.32
P × P	−230.55	−0.83	0.80	−209.59	−0.75	1.32
K × K	−156.00	−0.39	0.80	−21.81	−0.05	1.32
N × P	61.25	0.18	0.47	−1,566.67	−4.70	0.77
N × K	−805.50	−2.01	0.47	1,410.00	3.52	0.77
P × K	−83.75	−2.62	0.47	−1,108.33	−3.32	0.77
R^2 (%)		86.72			91.77	
Adj. R^2 (%)		74.76			84.37	

Coef: Coefficients; SE Coef: Standard Error of Coefficients; R: Coefficient of Determination.

Table 6 | Response surface regression: biomass (mg/L) versus N, P, K; estimated regression coefficients for lipid (%) using coded units

	Chlorella sp.			Botryococcus sp.		
	Coeff (uncoded)	Coeff	SE Coef	Coeff (uncoded)	Coeff	SE Coef
Constant	227.75	465.03	7.05	−3,130.35	281.18	6.76
N	−4,786.27	12.80	6.49	10,518.6	−7.00	6.22
P	3,914.92	31.60	6.49	−340.38	26.60	6.22
K	8,136.55	21.60	6.49	11,026.7	−17.80	6.22
N × N	7,163.64	17.90	12.38	−10,181.8	−25.45	11.87
P × P	−7,247.47	−26.09	12.38	7,929.29	28.54	11.87
K × K	−4,036.36	−10.09	12.38	−22,981.8	−57.45	11.87
N × P	2,750.00	8.25	7.26	−3,750.00	−11.25	6.96
N × K	−9,500.00	−23.75	7.26	−4,100.00	−10.25	6.96
P × K	−10,083.3	−30.25	7.26	−1,916.67	−5.75	6.96
R^2 (%)		88.32			88.97	
Adj. R^2 (%)		77.80			79.04	

Coef: Coefficients; SE Coef: Standard Error of Coefficients; R: Coefficient of Determination.

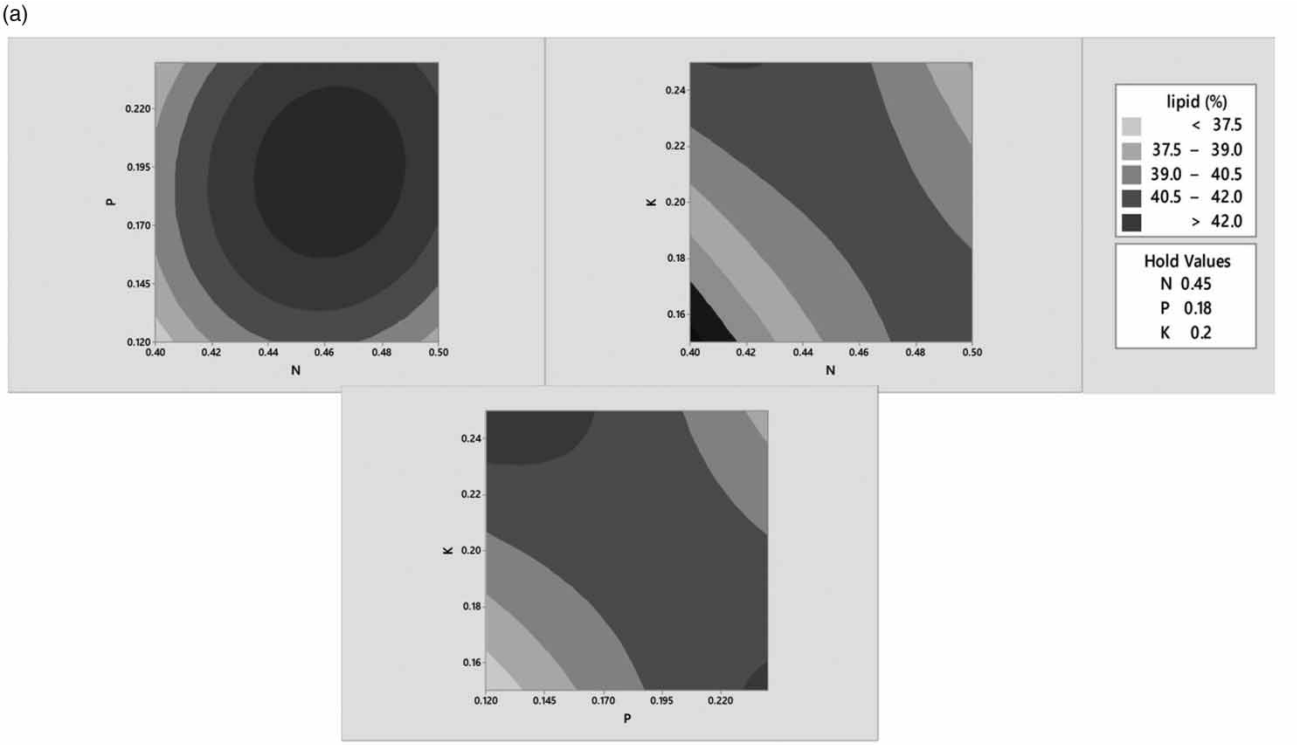
allowing simulation (Wei *et al.* 2013). Moreover, the applicability of those results to large-scale cultivation is doubtful, since obviously it would be impractical and costly to add large amounts of supplemental iron and difficult to finely control the temperatures of outdoor culture facilities.

Interaction among the factors

The response contour plots showed the growth of the *Chlorella* and *Botryococcus* sp. as a function of two factors, while the third was kept at a constant level. Based on the results, three-dimensional plots showed several significant interactions in the center of the range between nitrogen, phosphorus and potassium. The use of this method, allows the assessment of any interaction between these important variables. This also allows the development of a model which predicts both the obtainable biomass and the lipid productivity under any given combination of these variables. While this approach cannot provide details as to the mechanisms involved, it will show which factors drive the largest responses and which ones interact, thus highlighting the areas of focus for other studies involving mechanisms. Plotting the surfaces allows interpretation of these effects, and thus biological meaning and importance. If an interaction term is included in the model, no lack of fit is possible (although it may not be necessary), but when an interaction is not included, lack of fit could occur. Figure 1(a) and 1(b) show the contour/surface plots for the optimization conditions of the variable parameters (nitrogen, phosphorus and potassium concentrations) on lipid yield and biomass productivity in *Chlorella* sp. and Figure 2(a) and 2(b) show the effects on *Botryococcus* sp. The lipid yield increased with an increase in nitrogen concentration, and a further increase resulted in reversal of this trend. A high potassium concentration of 0.20 g/L with nitrogen concentration of 0.45 g/L resulted in improved yields. Studies carried out by other researchers demonstrated that the nitrogen source was an important nutrient in the medium, affecting the growth and lipid accumulation (Li *et al.* 2008) and nitrogen deficiency stimulated lipid accumulation (Mandal & Mallick 2009; Welter *et al.* 2013). With an increase in phosphorus concentration, the conversion ratio increased gradually. With decreasing potassium concentrations from 0.25 g/L to 0.2 g/L, the cellular lipid content in *Chlorella* sp. increased, where the *p*-value was less than 0.001. Furthermore, low phosphate had a positive effect on biomass associated higher lipid content in cells, hence lipid yield was more in low phosphate supplemented medium than with the high phosphate medium.

Maximum lipid yield (62%) at high N concentration of 0.5 g/L, low P concentration of 0.12 g/L and high potassium concentration of 0.25 g/L was observed for *Botryococcus* sp., and the lowest lipid yield (37.8%) was recorded under lowest N concentration. Lipid yield was also affected and showed a low yield when P concentration was maximum (0.24 g/L). The lipid yield was minimum when the lowest concentration of the three nutrient inputs (N, P, K) was used in the cultivation medium.

(a)



(b)

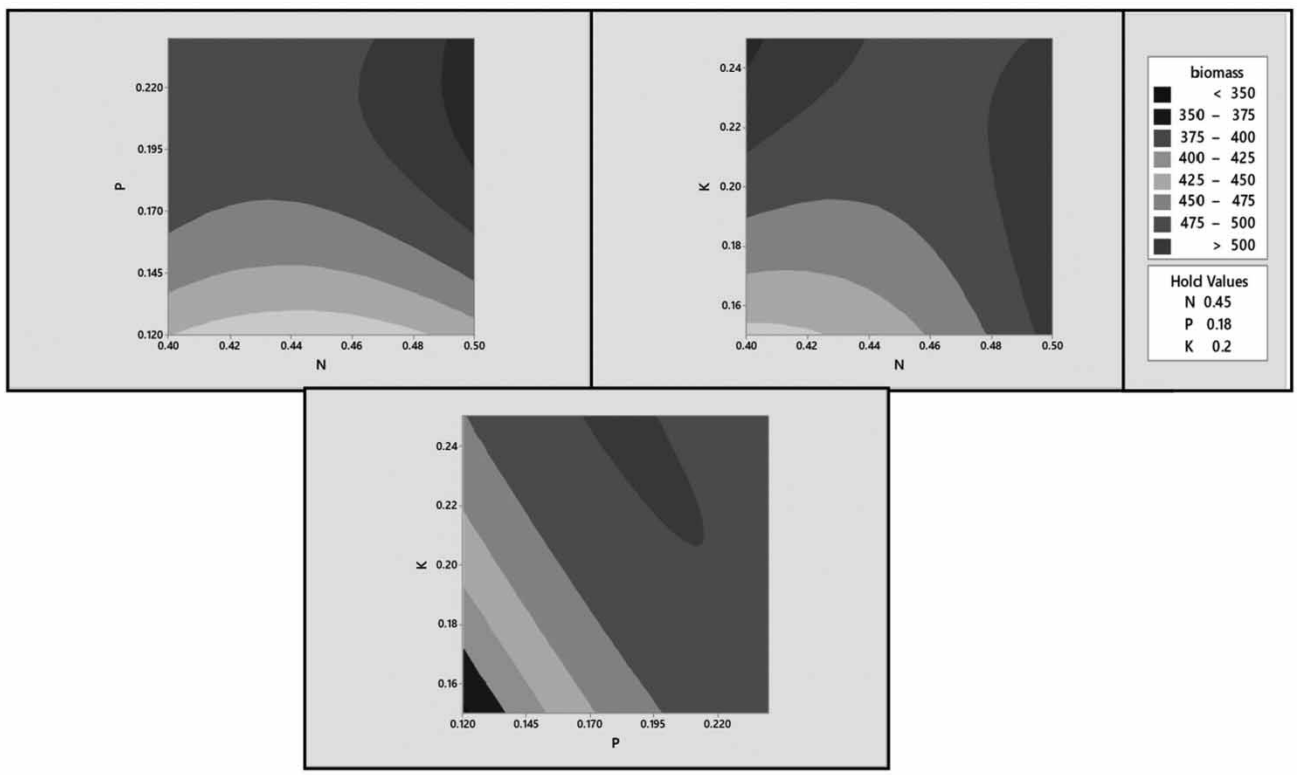
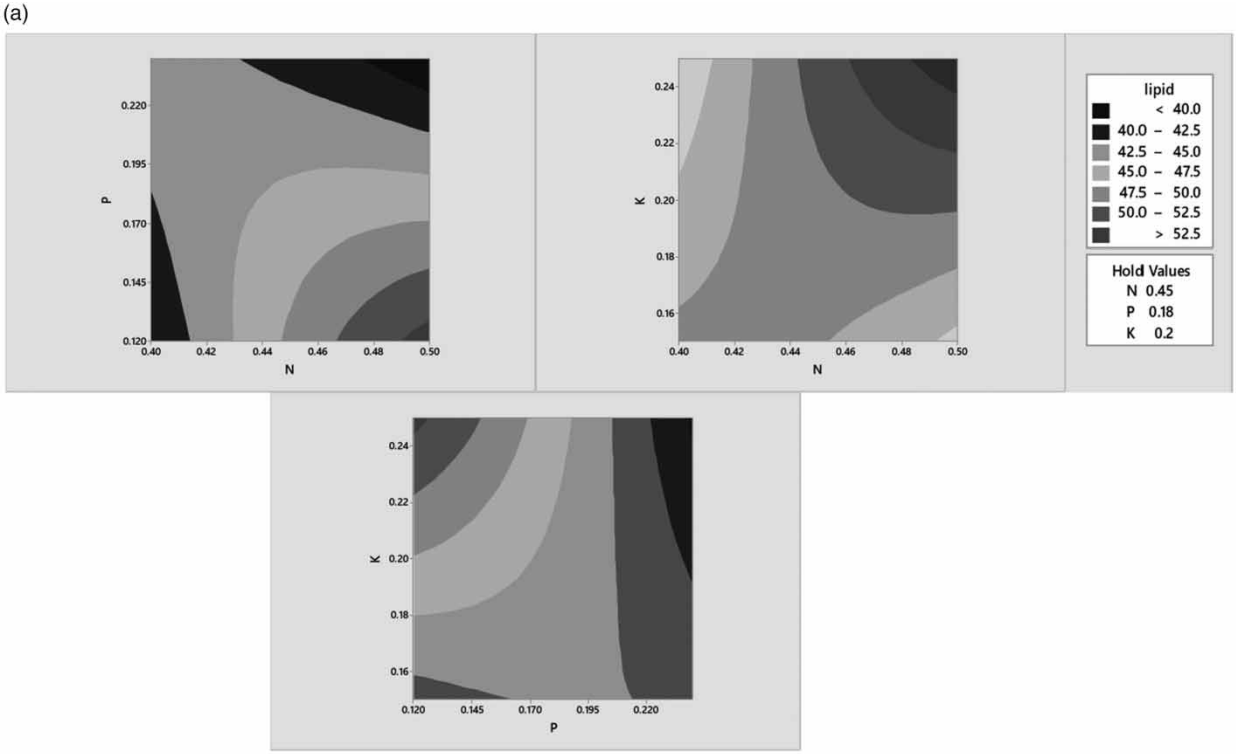


Figure 1 | (a) Contour plots showing the interactive effects of N and P, N and K and P and K for lipid yield (%); and (b) for biomass (mg/L) for *Chlorella* sp.



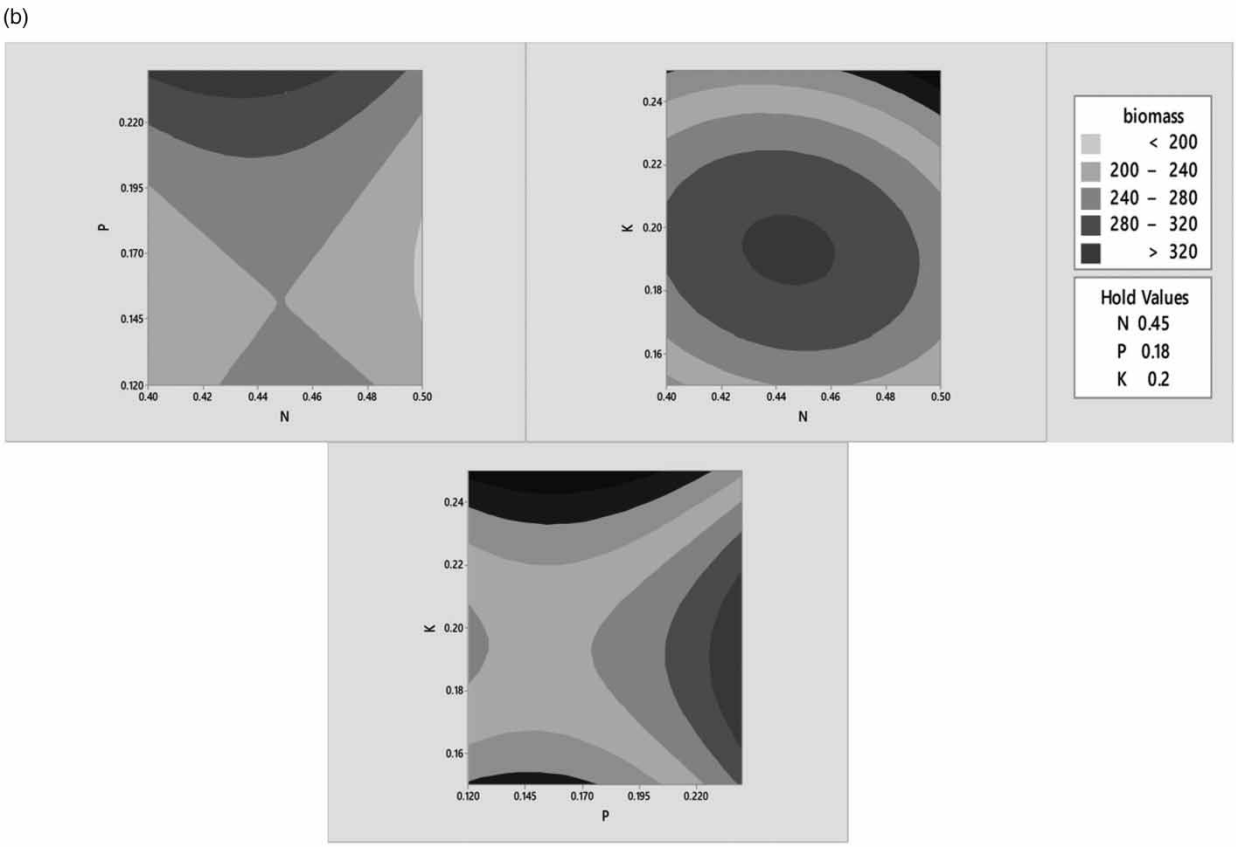


Figure 2 | (a) Contour plots showing the interactive effects of N and P, N and K and P and K for lipid yield (%); and (b) for biomass (mg/L) for *Botryococcus* sp.

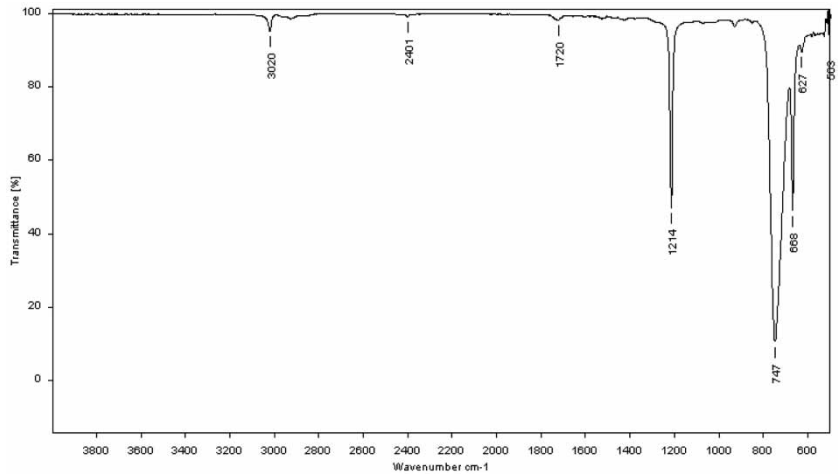


Figure 3 | FTIR spectra of the extracted lipid of *Chlorella* sp.

Validation of the model

The equation demonstrated that the interaction between all the three variables was significant and it could be proven from Figure 3 that these two items showed positive interaction. Only an average nitrogen and potassium and low phosphorus levels were beneficial for enhancement of lipid yield and biomass productivity. In *Chlorella* sp., the optimum concentration for nitrogen, phosphorus and potassium were predicted as 0.42 g/L, 0.14 g/L and 0.22 g/L for maximum lipid yield, whereas for maximum biomass productivity, the optimum concentrations of the three were predicted as 0.4 g/L, 0.15 g/L, and 0.20 g/L. Whereas, in *Botryococcus* sp. the optimum concentrations for nitrogen, phosphorus and potassium were 0.46 g/L, 0.14 g/L and 0.25 g/L for maximum lipid yield, and for maximum biomass productivity, the optimum concentrations of the three were predicted to be 0.45 g/L, 0.18 g/L, and 0.20 g/L.

Although different combinations of nitrogen, phosphorus and potassium concentrations were shown to give the same conversion, from an economic point of view it is desirable to choose the lowest possible concentration. An overall economic process must include high productivity at a minimum concentration, and most of these conditions were achieved in the present study. To validate the optimum concentration, an experiment with the specified conditions, *Chlorella* sp. yielded 440 mg/L biomass and 42% lipid content and *Botryococcus* sp. gave 280 mg/L biomass and 52% lipid content, which showed that the model was useful for predicting the concentration as well as the optimization of the experimental conditions. From the laboratory experiments in a commercial medium containing urea, single super phosphate and muriate of potash in appropriate ratio, the lipid content ranged from 27–30% in both the species. This study shows an increase in the lipid yield of nearly 55% for *Chlorella* sp. and 73% for *Botryococcus* sp. as compared to that obtained in the original medium. Song *et al.* (2012) have used the RSM in *Botryococcus braunii* UTEX 572 and showed micronutrients play a significant role in regulating algal growth and hydrocarbon production. They reported an increase of 34.5% of algal biomass and 27% increase in hydrocarbon using an optimized concentration of trace elements.

Statistical analysis of RSM

The ANOVA results clearly indicated that the model predicted was appropriate. The resulting response surfaces showed the effect of the concentration of various parameters, viz. nitrogen, phosphorus and potassium, on lipid content and biomass productivity (Tables 3 and 4) and the results demonstrated that the response surface had a maximum point. Repeated experiments were performed to verify the predicted optimum, and the results from replications coincided with the predicted values and the model was proven to be adequate.

The statistical analysis of the CCD experimental results, the response surface modeling and the optimization of the culture medium variables carried out using Minitab software showed that the growth of the microalgal sp. were influenced by the nitrogen, phosphorus and potassium of the culture medium and their effects were individual and interactive. When the selected microalgal strains were cultivated in a culture medium having optimized

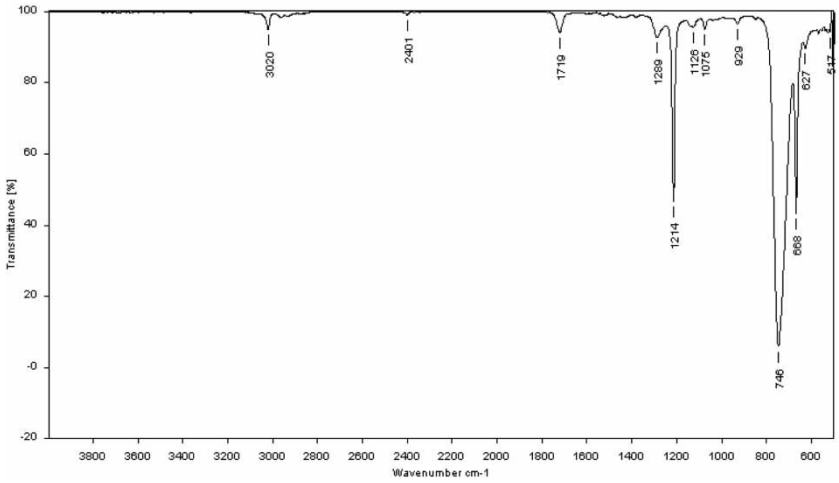


Figure 4 | FTIR spectra of the extracted lipid of *Botryococcus* sp.

concentrations of N, P, K, the cultures grew splendidly and accumulated lipids at moderate levels. Therefore, the present study clearly indicates the utilization of biochemical engineering approaches to selectively trigger lipid synthesis.

FTIR analysis of extracted lipid

The spectrum clearly showed the typical characteristics of absorption bands for common triglycerides as has also been reported in other studies for *Chlorella* sp. (Figure 3). The FTIR spectrum revealed the 3,020 cm^{-1} absorption band occurring in the infrared absorption spectra of methyl esters of unsaturated higher fatty acids was due to the –C – H stretching vibrations of ethylenic double bonds, which is in accordance with the observations reported earlier (Hirabayashi *et al.* 1971). The band present at 1,720 cm^{-1} was attributed to C = O stretching vibrations. The bending vibrations are generally found at lower wave numbers (Renuga Devi & Gayathri 2010). The clear band at 1,214 cm^{-1} was due to C – O stretching. There was a sharp intense band at 747 cm^{-1} due to four adjacent aromatic hydrogen atoms (Premovic *et al.* 2000). Bands at around 627 cm^{-1}, and 668 cm^{-1} were aromatic CH out of plane deformation vibrations and skeletal vibration of straight chain alkanes. The aromatic C-H out of plane deformation bands occurred below 700 cm^{-1}. In addition to the above bands found in *Botryococcus* sp. (Figure 4); the FTIR spectrum also showed bands in the region 1,290–1,130 cm^{-1}. There were weaker C – O bands as well as bands of aliphatic ether (OCH_3) rocking vibration. Bands in the region of 1,000 cm^{-1} were attributed to CCO stretching. Bands observed at 500–670 cm^{-1} were due to aromatic –CH groups and due to alkanes with three or more branches. The high intensity band at 746 cm^{-1} could be attributed to out-of-plane deformation vibration of 3–4 ring aromatic –CH groups with two or more adjacent hydrogen atoms. Small intensity bands were observed at 929 cm^{-1} due to symmetric COC stretch and C = O broad out-of plane bending. Bands at 1,075 cm^{-1} and at 1,126 cm^{-1} were due to C-C-O symmetric stretch of single chain alkane and aromatic C-H deformation and C-O-C asymmetric stretch of single chain alkane.

CONCLUSIONS

A minimal growth formulation comprising N, P and K as urea, single super phosphate and muriate of potash was determined, which enhanced lipid yield and biomass. The optimum values predicted by RSM for *Chlorella* sp. were nitrogen: phosphorus: potassium: 0.42 g/L: 0.14 g/L: 0.22 g/L, and 0.46 g/L: 0.14 g/L: 0.25 g/L for *Botryococcus* sp. FTIR analysis of the extracted lipids revealed the presence of characteristic bands for common triglycerides. An increase in the lipid yield of nearly 55% for *Chlorella* sp. and 73% for *Botryococcus* sp. was obtained as compared to the original culture medium.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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