Optimization of Growth and Hydrocarbon Production from a Green Microalga *Botryococcus braunii* by Plackett-Burman Design and Response Surface Methodology

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ABSTRACT

Statistical experimental designs were used to optimize the culture conditions on growth and hydrocarbon production from a green microalga *Botryococcus braunii* J4-1. In the optimization process, seven independent variables—$NaNO_3$, KH_2PO_4, Fe-citrate, pH, $NaHCO_3$, CO_2 and light intensity—were screened to verify the three most critical variables by the Plackett-Burman design. Fe-citrate, pH and CO_2 were then selected for further optimization by central composite design coupled with response surface methodology. Seventeen experimental tests were run under five levels of the significant variables. The influence of these variables on the responses of biomass, chlorophyll and hydrocarbon was evaluated using a second-order polynomial multiple regression model. Analysis of variance showed a high correlation coefficient of determination value of more than 0.90 and the *P*-values were less than 0.05. These values indicated that the model had a good fit and was acceptable at this level of significance. The optimum values of the variables were Fe-citrate 1.5 mg.L^{-1}, pH 6.8 and CO_2 2.5% (volume per volume) gave maximum yield of biomass at 5.74 g.L^{-1}, 13.51 mg.L^{-1} of chlorophyll and hydrocarbon 1.44 g.L^{-1}. Validation of the experimental values using the optimal conditions showed that the experimental values were quite close to the predicted values. Furthermore, the corresponding results of the deviations for the production of biomass, chlorophyll and hydrocarbon were 10.17, 11.19 and 1.41%, respectively, suggesting that the experimental designs used in this work were effective for the optimization of the process parameters on biomass, chlorophyll and hydrocarbon production.

Keywords: optimization, *Botryococcus braunii*, Plackett-Burman design, response surface methodology, central composite design

INTRODUCTION

The continued use of fossil fuels is now widely recognized as an unsustainable energy source because of depleting supplies and the contribution of these fuels to the accumulation of carbon dioxide in the environment (Khan *et al*., 2009). One of the promising alternative energy sources is biodiesel (Hill *et al*., 2006).

Microalgae are currently considered to be one of the most promising alternative sources of feedstock for biodiesel production (Sheehan *et al*., 1998). *Botryococcus braunii* is a green colonial microalga that is widespread in freshwater, brackish lakes, reservoirs and ponds (Wake and Hillen, 1980, 1981). *B. braunii* is regarded as a potential renewable resource because of its ability to synthesize and accumulate an unusually

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high level of hydrocarbons in the range 15–75% dry weight (Metzger *et al*., 1985; Sawayama *et al*., 1994; Kalacheva *et al*., 2002; Metzger and Largeau, 2005). *B. braunii* is classified into three races—A, B and L—depending on the type of hydrocarbon synthesis (Metzger and Largeau, 2005). Race A produces C_{23} to C_{33} odd-numbered n-alkadienes, mono-, tri-, tetra-, and penta-enes, which are derived from fatty acids (Metzger *et al*., 1990). Race B produces C_{30} to C_{37} unsaturated hydrocarbons known as botryococcenes as major hydrocarbons and small amounts of methyl-branched squalenes (Metzger and Largeau, 2005). Race L produces a single C_{40} tetraterpenoid hydrocarbon known as lycopadiene (Metzger *et al*., 1990). Media and culture conditions were observed to have an influence on the growth and hydrocarbon production of microalgae. Optimization of these parameters requires many experiments, but the total number of experiments can be reduced depending on the experimental design technique (Kincl *et al*., 2005).

A statistical method is a technique for investigating multiple process variables because it allows for the process to be easily optimized with fewer experiments (Bajaj *et al*., 2009). Several experimental design models can be employed to reduce the number of experiments under different conditions. The Plackett-Burman design and central composite design (CCD) are a statistical methodology widely used to determine the effects of several variables. The Plackett-Burman design is a screening design used for identifying important factors among a large number of variables; thus, this screening technique is quite useful in preliminary studies to select variables that can be fixed or eliminated in further optimization processes (Stanbury *et al*., 2003). The response surface methodology (RSM) coupled with CCD was used to optimize important nutritional factors that have been screened by Plackett-Burman design. This combination is an efficient strategic experimental tool for the development, improvement and optimization of complex processes (Manohar and Divakar, 2004). The objective of the current study was to determine the significant variables using the Plackett-Burman design and to further optimize the levels of the screened variables using RSM coupled with CCD for biomass, chlorophyll and hydrocarbon production from a newly isolated *B. braunii*.

MATERIALS AND METHODS

Microorganism and cultivation conditions

In this study, a green microalga *B. braunii* J4-1 was isolated from freshwater samples collected from the Huai Saphan Reservoir in Chon Buri province, Thailand. The stock culture was maintained in AF-6 medium (Kato, 1982) at 25 °C under a light intensity of 55 $\mu mol \cdot m^{-2} . s^{-1}$ with a 16:8 hr light-dark cycle.

The experiments were carried out in 200 mL conical culture tubes each containing 150 mL of AF-6 medium. Light from fluorescent lamps was provided laterally. The microalgal culture 15% (volume per volume; v/v) was inoculated into the medium and incubated at 25 °C with a 16:8 hr light-dark cycle for 32 d. All the experiments were carried out in triplicate.

Analytical procedures
Biomass analysis

A known volume of microalgal culture was filtrated using a Whatman GF/C filter and then dried to constant weight at 80 °C. The dry weight of the algal biomass was determined gravimetrically and expressed in grams per liter (Frenz *et al*., 1989).

Chlorophyll analysis

A known volume of microalgal culture was centrifuged at 3,300 rpm for 15 min. Cells were suspended in a known volume of methanol and kept in a water bath at 60 °C for 30 min. This extraction step was repeated till the microalgal cells turn colorless. The chlorophyll content in the pooled extract was estimated for it absorbance at 652 and 665 nm using spectrophotometry

(UV-visible Spectrophotometer, UV-Pharma Spec 1700; Shimadzu Corp.; Kyoto, Japan) and then evaluated using Lichtenthaler equations (Lichtenthaler, 1987).

Hydrocarbon analysis

Hydrocarbon was extracted from the dry biomass using hexane in a sonicator bath for 30 min. The supernatant was collected and evaporated to complete dryness under a stream of nitrogen. The hydrocarbon content was measured gravimetrically and expressed as a dry weight percentage (Maxwell *et al.,* 1968; Sawayama *et al.,* 1992). The hydrocarbon was analyzed using gas-liquid chromatography (GC-14B; Shimadzu Corp.; Kyoto, Japan) equipped with a flame ionization detector and split injector, using a capillary column (30 m × 0.54 mm × 1 µm). Identification of the hydrocarbon profile was obtained by comparison of fragmentation patterns with standards (kindly provided by K. Kaya, the University of Tsukuba, Tsukuba, Japan).

Experimental and data analysis
Plackett-Burman design

The Plackett-Burman design was used to screen the important significant variables that influenced biomass, chlorophyll and hydrocarbon production. The factors or independent variables considered in this study were seven factors (X_1 to X_7) representing $NaNO_3$, KH_2PO_4, Fe-citrate, pH, $NaHCO_3$, CO_2 and light intensity, respectively. The seven independent variables were organized in eight experiments according to the Plackett-Burman design matrix. All variables were investigated at two levels of +1 (high) and –1 (low). Table 1 shows the factors under investigation as well as the levels of each factor used in the experimental design. The Plackett-Burman design matrix and response values are listed in Table 2. The effect of each variable was determined according to Equation 1:

$$E_{(xi)} = (\Sigma M_{i+} - M_{i-}) / N \qquad (1)$$

where $E_{(xi)}$ is the concentration effect of the tested

Table 1 Experimental variables at different levels used in the Plackett-Burman design.

Variable	Unit	Code	Low level (-)	High level (+)
$NaNO_3$	$mg.L^{-1}$	X_1	120	160
KH_2PO_4	$mg.L^{-1}$	X_2	5	15
Fe-citrate	$mg.L^{-1}$	X_3	1	3
pH		X_4	6	8
$NaHCO_3$	% (w/v)	X_5	0	0.5
CO_2	% (v/v)	X_6	0	3
Light intensity	$µmol.m^{-2}.s^{-1}$	X_7	37	74

w/v = Weight per volume; v/v = Volume per volume.

Table 2 Plackett-Burman experimental design matrix for screening of variables for biomass, chlorophyll and hydrocarbon production from *B. braunii* J4-1.

Treatment	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Biomass $(g.L^{-1})$	Chlorophyll production $(mg.L^{-1})$	Hydrocarbon production $(g.L^{-1})$
1	+1	+1	+1	-1	+1	-1	-1	0.055	0.002	0.0020
2	-1	+1	+1	+1	-1	+1	-1	1.402	0.443	0.2750
3	-1	-1	+1	+1	+1	-1	+1	0.043	0.002	0.0012
4	+1	-1	-1	+1	+1	+1	-1	0.083	0.002	0.0039
5	-1	+1	-1	-1	+1	+1	+1	0.370	0.038	0.0255
6	+1	-1	+1	-1	-1	+1	+1	3.000	0.563	0.5949
7	+1	+1	-1	+1	-1	-1	+1	0.392	0.044	0.0398
8	-1	-1	-1	-1	-1	-1	-1	0.453	0.095	0.0427

variable, M_{i+} and M_{i-} are the biomass, chlorophyll and hydrocarbon production from the trials where the variable (xi) measured was present at high and low levels, respectively and N is the number of trials.

The experimental design and statistical analysis of the data were done using the SPSS for Windows software package (version 11.5, 2002; SPSS Inc.; Chicago, IL, USA). Factors significant at the 95% level ($P < 0.05$) were considered reliable.

Central composite design coupled with response surface methodology

The factors identified by the Plackett-Burman design to significantly influence biomass, chlorophyll and hydrocarbon production were further optimized using the central composite design. The three significant variables selected assigned as X_1, X_2 and X_3 were employed within 17 experiments. These variable was investigated at five levels (-1.68, -1, 0, +1, and +1.68), as shown in Table 4.

The regression equation is described in Equation 2:

$$Y = \beta_0 + \Sigma\beta_iX_i + \Sigma\beta_{ii}X^2_i + \Sigma\beta_{ij}X_iX_j \quad (2)$$

where, Y is the predicted response, X_i and X_j are independent variables, β_0 is the offset term, β_i is the linear coefficient, β_{ii} is the squared coefficient and β_{ij} is the interaction coefficient.

The effects of each variable were determined using the statistical software package.

Design-Expert 7.0 (Stat-Ease, Inc.; Minneapolis, MN, USA) which carried out the regression analysis of the experimental data and also plotted the response surface graphs. The quality of fit of the model equation was expressed via the coefficient of determination (R^2). The statistical significance of the model equation and model term was evaluated using the P-value ($P < 0.05$).

RESULTS AND DISCUSSION

Screening of significant variables using Plackett-Burman design

The seven independent variables ($NaNO_3$, KH_2PO_4, Fe-citrate, pH, $NaHCO_3$, CO_2 and light intensity) were screened with regard to their effects on biomass, chlorophyll and hydrocarbon production using a Plackett–Burman design as shown in Table 1. The results revealed that treatment 6 in the design matrix presented the highest production of biomass (3.00 g.L^{-1}), chlorophyll (0.563 mg.L^{-1}) and hydrocarbon (0.595 g.L^{-1}). The regression analysis data for the Plackett-Burman design are shown in Table 3. Factors with P-values less than 0.05 were considered to have significant effects on the response; consequently, these factors were selected for further optimization. The analysis of variance (ANOVA) of the three responses showed that Fe-citrate, pH, $NaHCO_3$ and CO_2 were significant variables. The effects of Fe-citrate and CO_2 were positive on the biomass, chlorophyll and

Table 3 Effects of variables and statistical analysis of the biomass, chlorophyll and hydrocarbon production from *B. braunii* J4-1 using the Plackett-Burman design.

Term	Biomass			Chlorophyll production			Hydrocarbon production		
	Effect	T-value	P-value	Effect	T-value	P-value	Effect	T-value	P-value
X_1	0.162	3.678	0.002	0.020	0.499	0.624	0.162	3.678	0.002
X_2	-0.177	-4.023	0.001	-0.080	-2.002	0.062	-0.177	-4.023	0.001
X_3	0.415	9.431	0.000	0.490	12.306	0.000	0.415	9.431	0.000
X_4	-0.252	-5.728	0.000	-0.122	-3.060	0.007	-0.252	-5.728	0.000
X_5	-0.605	-13.766	0.000	-0.650	-16.321	0.000	-0.605	-13.766	0.000
X_6	0.504	11.462	0.000	0.534	13.404	0.000	0.504	11.462	0.000
X_7	0.235	5.348	0.000	0.062	1.557	0.139	0.235	5.348	0.000

hydrocarbon production which were greater at the high levels, whereas the pH and $NaHCO_3$ had negative effects and the influences of these variables were greater at the low levels. Among the significant variables identified, $NaHCO_3$ was excluded due to its negative coefficient which was indicated by improvement of the responses at decreased concentrations.

Terry and Abadia (1986) reported that Fe was essential for chlorophyll biosynthesis, inorganic nitrogen assimilation and as a redox catalyst in electron transport reactions in green plants and cyanobacteria. Therefore, Fe could be an important factor affecting the thylakoid membrane structure and function which are essential in photochemical energy conversion. Fe limitation also affected the reduction of pigment concentration and decreased the maximum chlorophyll specific rate of photosynthesis (Terry and Abadia, 1986; Greene *et al*., 1991; Morales *et al*., 1991). Algae grown in low Fe concentrations decreased P700, the reaction center chlorophyll of photosystem I and c-type cytochromes (Oquisr, 1974; Bohme *et al*., 1978) The effect of Fe-citrate agreed with Behrenfeld *et al*. (2006) who reported that iron had a key function in regulating microalgal biomass. Increased concentrations of iron also affected oil accumulation in four stains of *Botryococcus—*TRG, KB, SK and PSU (Yeesang and Cheirsilp, 2010). The results also corresponded with Tanoi *et al*. (2014) who reported that iron concentration strongly influences the size, shape, growth and oil production of *B. braunii* BOT-22.

Considering the influence on algal growth, it was apparent that the pH affected the distribution of carbon dioxide species dissolved in the culture medium. The availability of inorganic carbon directly affected algal photosynthesis (Azov, 1982; Celia and Edward, 1994), especially in the form of carbon dioxide. Lehman (1978) supported that free CO_2 is the only direct substrate for the Calvin cycle. On the other hand, the pH and CO_2 concentration were the main factors influencing the growth and chemical composition of *Botryococcus* sp. (Rousch *et al*., 2003; Metzger and Largeau, 2005). It also conformed with Chirac *et al.* (1985) who compared non-CO_2 enriched air and air enriched with 1% (v/v) CO_2, the latter abruptly enhanced growth with a mean doubling time of the biomass in the exponential phase of approximately 2 d against 7 d and the hydrocarbon production increased five-fold.

Therefore, in the screening tests using the Plackett-Burman design, Fe-citrate, the pH and CO_2 impacted on the biomass, chlorophyll and hydrocarbon production of *B. braunii* J4-1. These factors were further optimized using central composite design.

Optimization of significant variables using central composite design coupled with response surface methodology

The three selected variables were investigated at five levels (-1.68, -1, 0, +1, and +1.68), as shown in Table 4. The design matrix, with the corresponding results of the 17 experiments, as well as the predicted results, are presented in Table 5.

Table 4 Experimental code, range and levels of significant independent variables used in central composite design.

Independent Variable	Unit	Code	Level				
			-1.68	-1	0	+1	+1.68
Fe-citrate	mg.L^{-1}	X_1	0.32	1	2	3	3.68
pH		X_2	5.32	6	7	8	8.68
CO_2	% (v/v)	X_3	0	0.8	2	3.2	4.0

v/v = Volume per volume.

Table 5 Central composite design matrix of experimental design and predicted responses of significant variables on biomass, chlorophyll and hydrocarbon production.

Treatment	X_1	X_2	X_3	Biomass ($g.L^{-1}$)		Chlorophyll production ($mg.L^{-1}$)		Hydrocarbon production ($g.L^{-1}$)	
				Predicted	Experimental	Predicted	Experimental	Predicted	Experimental
1	-1	-1	-1	3.25	3.16	5.99	5.91	0.52	0.55
2	-1	-1	+1	3.89	3.46	7.46	6.66	0.82	0.69
3	-1	+1	-1	2.86	3.74	6.05	5.80	0.68	0.64
4	-1	+1	+1	2.26	2.84	7.34	6.67	0.85	0.50
5	+1	-1	-1	1.66	2.01	6.31	5.97	0.43	0.29
6	+1	-1	+1	3.58	3.61	7.30	6.52	0.98	0.64
7	+1	+1	-1	3.03	2.79	6.37	6.15	0.35	0.34
8	+1	+1	+1	3.71	3.15	7.18	6.22	0.63	0.51
9	-1.68	0	0	3.54	3.08	7.72	8.30	0.73	0.77
10	0	-1.68	0	3.99	4.09	7.48	8.15	0.66	0.68
11	0	0	-1.68	1.57	1.14	4.77	4.85	0.30	0.23
12	+1.68	0	0	3.42	3.75	7.85	8.72	0.49	0.54
13	0	+1.68	0	3.71	3.40	7.42	8.15	0.66	0.75
14	0	0	+1.68	2.68	3.01	6.69	8.09	0.74	0.96
15	0	0	0	4.89	4.97	10.78	11.77	1.20	1.27
16	0	0	0	4.89	4.98	10.78	10.50	1.20	1.25
17	0	0	0	4.89	4.77	10.78	9.81	1.20	1.08

Multiple regression analysis was used in the analysis and the data were fitted to a second-order polynomial equation. The response of biomass production ($Y_{biomass}$), chlorophyll production ($Y_{chlorophyll}$), and hydrocarbon production ($Y_{hydrocarbon}$) from *B. braunii* J4-1 could be expressed in terms of the regression Equations 3–5:

$$Y_{biomass} = -20.2839 + 1.1044X_1 + 5.66321X_2 + 4.23862X_3 + 0.044105X_1X_2 + 0.26654X_1X_3 - 0.25474X_2X_3 - 0.4958X_1^2 - 0.37917X_2^2 - 0.67734X_3^2 \tag{3}$$

$$Y_{chlorophyll} = -58.32149 + 4.49111X_1 + 16.63383X_2 + 5.93025X_3 - 2.80E-03X_1X_2 - 0.10361X_1X_3 - 0.037124 X_2X_3 - 1.05642X_1^2 - 1.18366X_2^2 - 1.24607 X_3^2 \tag{4}$$

$$Y_{hydrocarbon} = -10.23013 + 0.67081X_1 + 2.78796X_2 + 0.97705X_3 + 5.20E-04X_1X_2 + 0.054772X_1X_3 - 0.04667X_2X_3 - 0.21397X_1^2 - 0.19274X_2^2 - 0.16338X_3^2 \tag{5}$$

where X_1 is Fe-citrate, X_2 is the pH and X_3 is the CO_2 concentration.

The results of the second-order response surface model fitted in the form of ANOVA are given in Table 6. The model presented coefficient of determination (R^2) values for the production of biomass, chlorophyll and hydrocarbon of 0.9133, 0.8427 and 0.9050, respectively, which showed response variability of 91.33, 84.27 and 90.50%, respectively. From these values, it was clear that a good correlation was obtained, indicating a good fit by the model. Hu (1999) concluded that a model could be accepted when $R^2 \geq 0.75$. The probability P-values ($P < 0.05$) were used to determine the significance of the model equations and model terms. In this study, the P-values of the models for the production of biomass, chlorophyll and hydrocarbon were 0.0056, 0.0365 and 0.0075, respectively, indicating that the three models were significant. In these models, X_3, X_1^2, X_2^2 and X_3^2 were significant variables for biomass and hydrocarbon production, while X_1^2, X_2^2 and X_3^2 were significant variables for chlorophyll production.

Table 6 Analysis of variance for variables of the quadratic model of biomass, chlorophyll and hydrocarbon production from *B. braunii* J4-1.

Source	Biomass			Chlorophyll production			Hydrocarbon production		
	Estimate	*F*-value	*P*-level	Estimate	*F*-value	*P*-level	Estimate	*F*-value	*P*-level
Model	4.8900	8.1900	0.0056	10.7800	4.1700	0.0365	1.2000	7.4100	0.0075
X_1	-0.0370	0.0960	0.7654	0.0390	0.0160	0.9027	-0.0720	3.5400	0.1021
X_2	-0.0660	0.3100	0.5944	-0.0170	0.0032	0.9563	-0.0027	0.0050	0.9454
X_3	0.3300	7.8800	0.0262	0.5700	3.5400	0.1018	0.1300	11.1100	0.0125
X_1X_2	0.0440	0.0800	0.7850	-0.0028	0.0000	0.9946	0.0005	0.0001	0.9920
X_2X_3	0.3200	4.2300	0.0788	-0.1200	0.0980	0.7639	0.0660	1.7300	0.2296
X_3X_1	-0.3100	3.8600	0.0902	-0.0450	0.0130	0.9140	-0.0560	1.2600	0.2991
X_1^2	-0.5000	14.2900	0.0069	-1.0600	9.9100	0.0162	-0.2100	25.8300	0.0014
X_2^2	-0.3800	8.3600	0.0233	-1.1800	12.4400	0.0096	-0.1900	20.9600	0.0026
X_3^2	-0.9800	54.5700	0.0002	-1.7900	28.2200	0.0011	-0.2400	30.8100	0.0009

The optimal value of each variable was clearly represented in the three dimension surface plots and contour plots as shown in Figures 1, 2 and 3 for the production of biomass, chlorophyll and hydrocarbon, respectively. Each response surface plot represented the effect of two independent variables, while maintaining other variables at the central point level.

Figure 1 represents the model and Equation 3 for biomass production shows the relative effect of variable interactions. The effect of Fe-citrate and pH on biomass production is shown in Figure 1a. The effect of Fe-citrate and CO_2 is shown in Figure 1b. The effect of pH and CO_2 is shown in Figure 1c. A similar profile is presented in Figure 2 for chlorophyll production and in Figure 3 for hydrocarbon production.

The experimental data were fitted into the aforementioned equation, and the optimum levels of Fe-citrate, pH and CO_2 were 1.5 mg.L^{-1}, 6.8 and 2.5% (v/v), respectively. For this combination of conditions, the predicted responses of biomass, chlorophyll and hydrocarbon production were 5.21 g.L^{-1}, 12.15 mg.L^{-1} and 1.42 g.L^{-1}, respectively. However, under these optimal conditions, the observed experimental values of biomass, chlorophyll and hydrocarbon production were 5.74 g.L^{-1}, 13.51 mg.L^{-1} and 1.44 g.L^{-1}, respectively.

The statistical results for the production of biomass, chlorophyll and hydrocarbon in the modified AF-6 medium supplemented with 1.5 mg.L^{-1} of Fe-citrate, a pH of 6.8 and 2.5% (v/v) of CO_2 were compared with the original AF-6 medium. The results showed that maximal algal biomass, chlorophyll and hydrocarbon production levels were 5.74 g.L^{-1}, 13.51 mg.L^{-1} and 1.44 g.L^{-1}, respectively, which were higher than those produced in the original AF-6 medium (2.32 g.L^{-1}, 0.38 mg.L^{-1} and 0.73 g.L^{-1}, respectively). It was clear that these concentrations of Fe-citrate, pH level and CO_2 + air mixture were suitable for cultivation of *B. braunii* J4-1 and resulted in improved levels of biomass, chlorophyll and hydrocarbon production.

The biomass production of *B. braunii* J4-1 in this study was 5.74 g.L^{-1} which was much higher than reported by Dayananda *et al.* (2005). They investigated the effect of the medium composition on growth and hydrocarbon production by *B. braunii* SAG30.81 using RSM, and they found that potassium nitrate and ferric citrate exhibited effects on the levels of biomass and hydrocarbon production. However, the maximum biomass yield was only 0.65 g.L^{-1}. Similarly, the biomass production under optimal conditions in the current study was almost 20 times higher than reported

by Tran *et al*. (2010) who studied the effect of the media composition on the growth and lipid production of *B. braunii* LB572 using CCD. The results indicated that potassium phosphate and magnesium sulfate were major impact factors with the optimal concentrations at 0.058 g.L^{-1}and 0.09 g.L^{-1}, respectively. The biomass production increased to 4.57 g.L^{-1}. On the other hand, the value of hydrocarbon production was comparable to the work of Song *et al*. (2012) who used RSM to study the micronutrient requirements in *B. braunii* UTEX 572. Their model showed that the concentrations of iron, manganese, molybdenum and nickel played significant roles in the regulation of algal growth and hydrocarbon production. The optimal levels of micronutrients for hydrocarbon production were 10.43 µM of iron, 6.53 µM of manganese, 0.012 µM of molybdenum and 1.73 µM of nickel which maximized hydrocarbon production at 1.32 g.L^{-1}.

Validation of optimal conditions

          Validation was undertaken of the optimal conditions for the predicted conditions derived from analysis of CCD. The validation experiments were performed comparing the experimental values with the predicted values as shown in Table 7. The optimal conditions were conducted by running test experiments in AF-6 medium using Fe-citrate at 1.5 mg.L^{-1}, the pH at 6.8 and CO_2 at 2.5 % (v/v). Under these optimum conditions, the observed experimental values were 5.74 g.L^{-1} of biomass production, 13.51 mg.L^{-1}of chlorophyll production and 1.44 g.L^{-1} of hydrocarbon production. These values compared with the modeled predicted levels of production for biomass, chlorophyll and hydrocarbon of 5.21 g.L^{-1}, 12.15 mg.L^{-1} and 1.42 g.L^{-1}, respectively, which indicated that the experimental values were quite close to the predicted values. Furthermore, the corresponding results of the deviations of the experimental values and predicted values for the production of biomass, chlorophyll and hydrocarbon were 10.17, 11.19

and 1.41%, respectively. These results suggested that the experimental designs used in this study were effective in predicting the optimized culture conditions.

CONCLUSION

          The Plackett-Burman design and central composite design coupled with response surface methodology were employed to screen and optimize the media constituents and culture conditions for biomass, chlorophyll and hydrocarbon production from *B. braunii* J4-1. The seven variables—$NaNO_3$, KH_2PO_4, Fe-citrate, pH, $NaHCO_3$, CO_2 and light intensity—were examined using the Plackett-Burman design. Three variables—Fe-citrate, pH and CO_2—were found to exert significant effects. These significant variables were used for further optimization of the biomass, chlorophyll and hydrocarbon production using CCD. The optimum levels of each variable were: Fe-citrate, 1.5 mg.L^{-1}; pH, 6.8; and CO_2, 2.5 % (v/v). Under these optimal culture conditions, the maximum levels of biomass, chlorophyll and hydrocarbon production were 5.74 g.L^{-1}, 13.51 mg.L^{-1} and 1.44 g.L^{-1}, respectively. Therefore, these results suggested that a statistical optimum strategy was an effective tool for the optimization of process parameters on the growth and chlorophyll and hydrocarbon production from *B. braunii* J4-1. Consequently, this optimization strategy would be useful in the development of technology for biodiesel production.
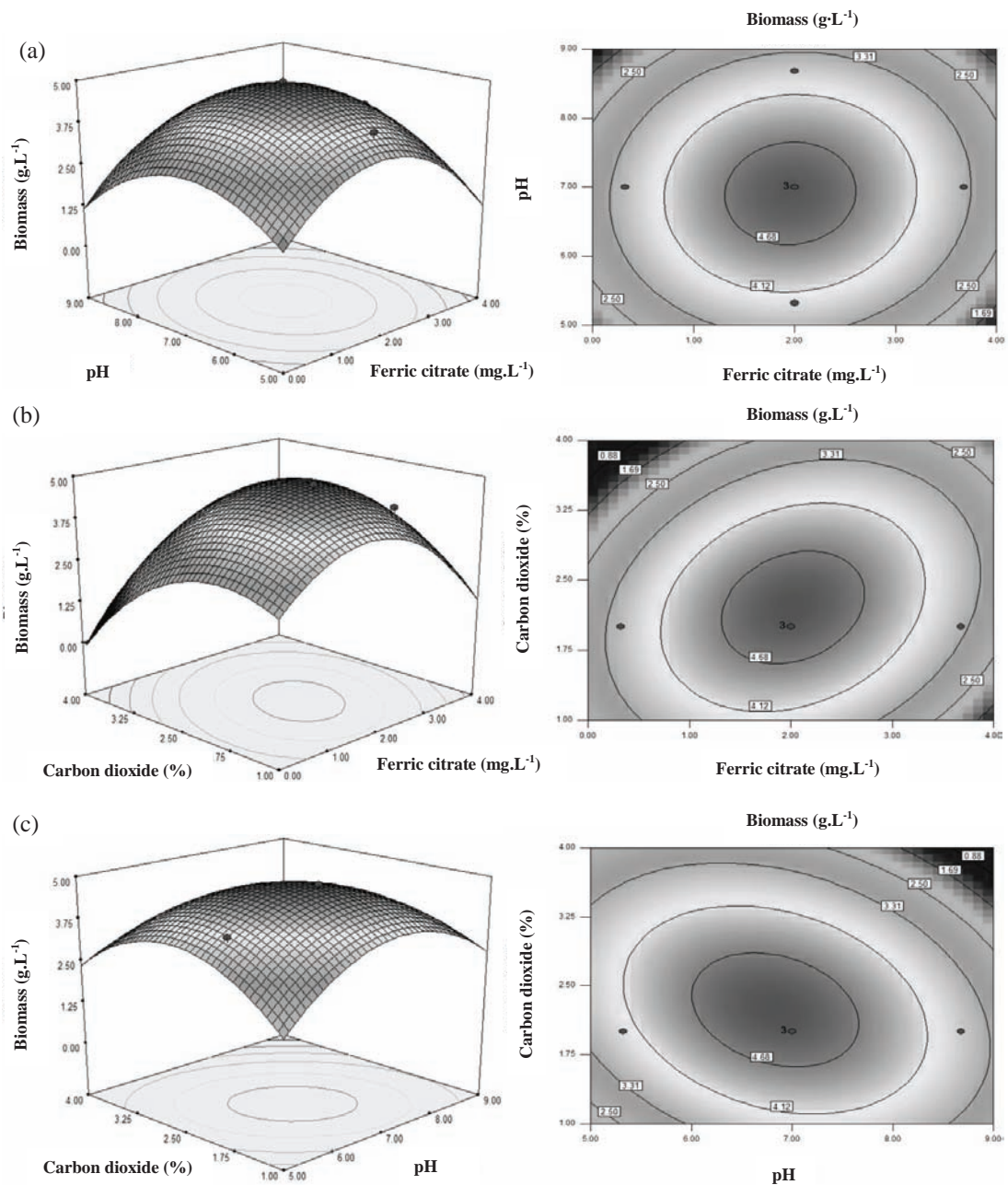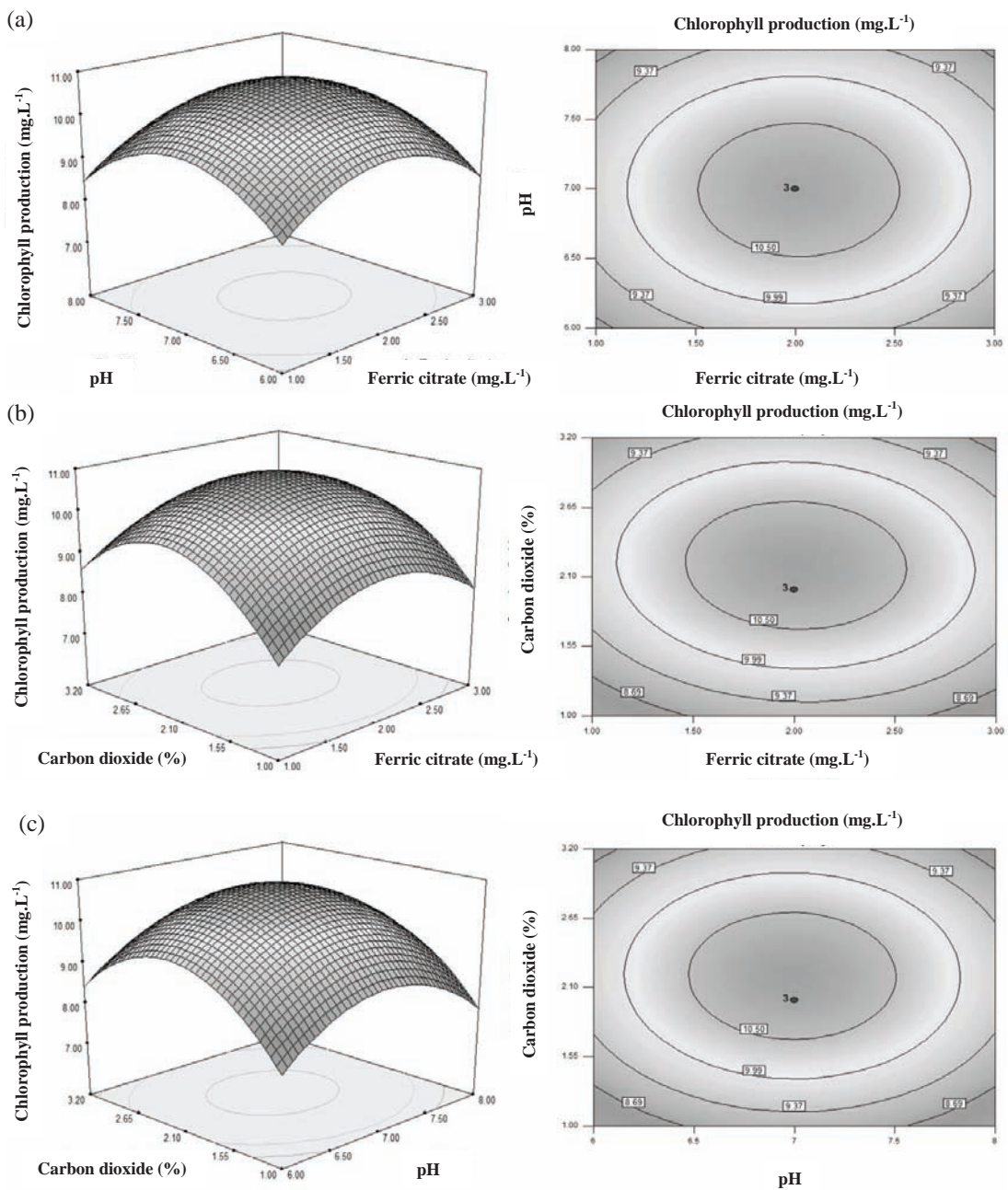
Figure 1 Three-dimensional response surface plots and two-dimensional contour plots of biomass production by *B. braunii* J4-1 showing independent variable interactions of: (a) Fe-citrate and pH; (b) Fe-citrate and CO_2; (c) pH and CO_2.

(a)

(b)

(c)

Figure 2 Three-dimensional response surface plots and two-dimensional contour plots of chlorophyll production by *B. braunii* J4-1 showing independent variable interactions of: (a) Fe-citrate and pH; (b) Fe-citrate and CO_2; (c) pH and CO_2.
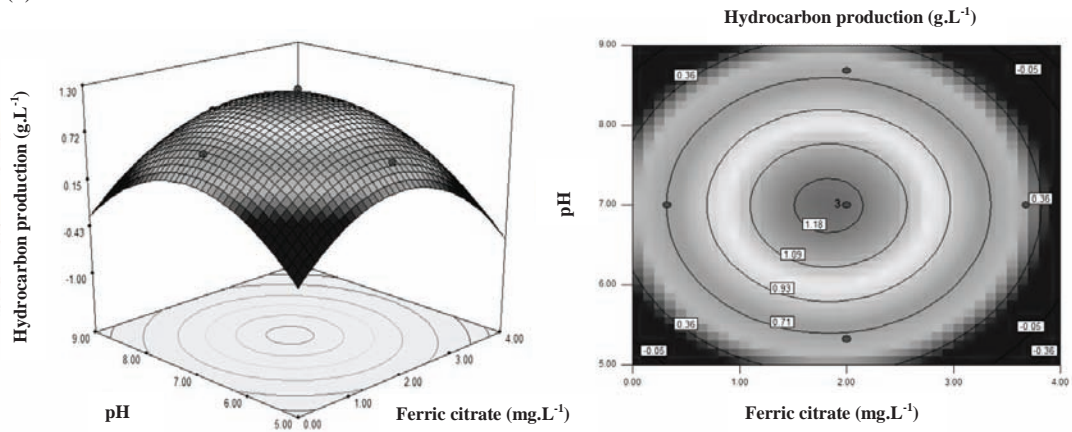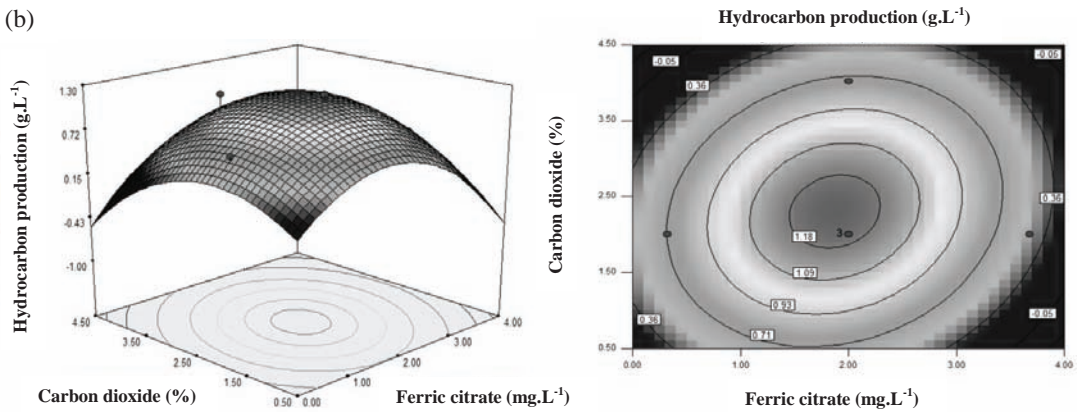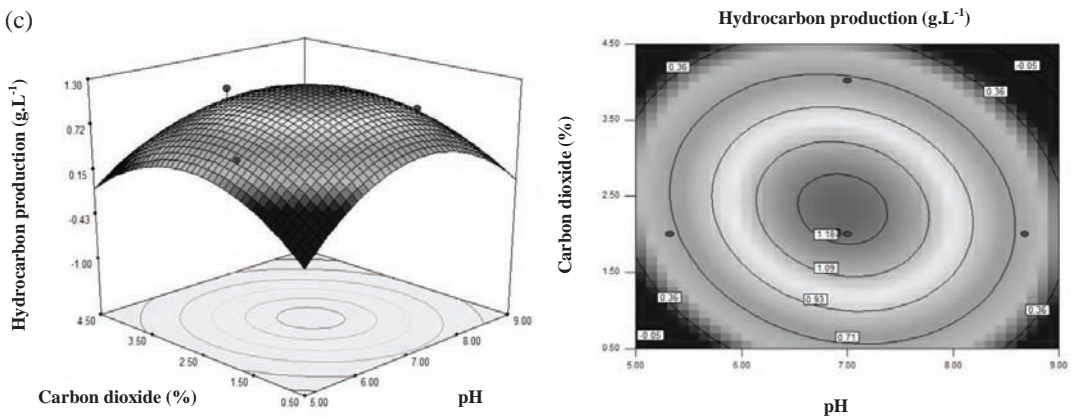
(a)

(b)

(c)

Figure 3 Three-dimensional response surface plots and two-dimensional contour plots of hydrocarbon production by *B. braunii* J4-1 showing independent variable interactions of: (a) Fe-citrate and pH; (b) Fe-citrate and CO_2; (c) pH and CO_2.

Table 7   Validation of central composite design for optimization of biomass, chlorophyll and hydrocarbon production from *B. braunii* J4-1.

Response	Predicted	Experimental	Error (%)
Biomass (g.L^{-1})	5.21	5.74	10.17
Chlorophyll production (mg.L^{-1})	12.15	13.51	11.19
Hydrocarbon production (g.L^{-1})	1.42	1.44	1.41

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