Kasetsart J. (Nat. Sci.) 49 : 54 - 66 (2015)

Optimization of Lipid Accumulation by Starchless Mutant *Chlorella sorokiniana* for Biodiesel Production

Watchara Jantasee, Wichien Yongmanitchai and Duenrut Chonudomkul*

ABSTRACT

Improvement of oil accumulation in *Chlorella sorokiniana* was conducted by ultraviolet mutagenesis. In total, 63 starchless mutants from over 40,000 colonies were screened using an iodine fumigation technique. Eight starchless mutants had relatively high levels of oil accumulation compared to wild type strains. The lipid content of starchless *C. sorokiniana* DMKU5202-31 increased from 15% to 21.16%. Therefore, this strain was selected for further optimization using a statistical experimental design. Seven factors were screened using a Plackett-Burman design and further optimized using a central composite design (CCD). The Plackett-Burman design presented three significant factors—KNO_3, pH and light intensity—that affected biomass and lipid accumulation significantly. CCD was used to optimize the significant factors and indicated that the optimal values for KNO_3, pH and light intensity were 0.9 $g.L^{-1}$, 6.2 and 4,000 lux, respectively. The response surface plots revealed that the maxima for biomass, lipid production and lipid content were 2.58 $g.L^{-1}$, 1.40 $g.L^{-1}$ and 54.49%, respectively. Thus, it could be concluded that ultraviolet mutation and the statistical experimental design can be used to improve oil accumulation in *C. sorokiniana*. After mutation and optimization, strain DMKU5202 had a lipid content increase from 15% to 54.49%. Therefore, these techniques were very efficient for the development of an upstream process for biodiesel production.

Keywords: *Chlorella sorokiniana*, starchless mutant, oil content, Plackett-Burman design, response surface methodology, central composite design

INTRODUCTION

Currently, the increased consumption of fossil fuels has caused the rapid depletion of this resource and there is an anticipated shortage in the near future (Amaro *et al.*, 2012). In addition, massive use of petroleum is the major cause of global warming caused by greenhouse gases (Amaro *et al.*, 2012). Therefore, biodiesel production from renewable sources is becoming more crucial and microalgae are a promising alternative source of energy with several advantages over other sources as they are photosynthetic, have higher productivity and high growth rates, and do not require agricultural land for cultivation (Chisti, 2007). There are some species that can accumulate very large amounts of triacylglycerols (TAG) and they are environmentally friendly due to their removal of carbon dioxide instead of its emission (Chisti, 2007; Mata *et al.*, 2010). However, biodiesel production from microalgae has not materialized due to its high cost of production caused by factors such as relative low lipid contents, expensive

Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fscidrc@ku.ac.th

Received date : 27/05/14 Accepted date : 04/08/15

harvesting technology and the large amount of water needed for microalgae cultivation (Amaro *et al*., 2012). Thus, the reduction of the cost of upstream and downstream biodiesel processing from microalgae is necessary.

In order to increase lipid accumulation in the cell, organic carbon compounds derived from photosynthesis should be converted to the biosynthesis of fatty acids instead of starch. Improvement of cellular lipids in microalgae by mutation has been suggested as a method of choice (Anandarajah *et al*., 2012). Mutation was induced by several mutagens and ultraviolet (UV) rays are a common method that has been used to induce mutation for over-production of starch and lipid in microalgae (Ramazanov and Ramazanov, 2006). Starch and lipid synthesis in microalgae share common carbon precursors (Li *et al*., 2010). However, regulation of starch and lipid synthesis is less understood, especially the possible interaction between the two pathways (Weselake *et al*., 2009).

A Plackett-Burman design is one of the statistical designs for screening key parameters among a large number of independent factors and can be quite useful in preliminary studies that then apply further optimization tools such as response surface methodology (RSM). RSM is based on a central composite design to study the interaction between each determined factor that affects the responses of dependent factors (Song *et al*., 2007).

The purposes of this study were to improve oil accumulation of *C. sorokiniana* by UV mutagenesis and screening for starchless mutants using iodine vapor. The culture conditions of the selected mutant were optimized for growth and lipid accumulation using statistical experimental design.

MATERIALS AND METHODS

Mutagenesis by ultraviolet radiation

Two strains of microalgae—*C. sorokiniana* DMKU5201 and DMKU5202—which are relatively high in lipid contents were mutated using UV radiation. Suspensions of wild type algal strains in NSIII medium (Ramazanov and Ramazanov, 2006) with a cell density of approximately 1×10^6 to 1×10^7 cells.mL^{-1} were treated under UV irradiation at 254 nm for 40 min resulting in a 2–5% survival rate (Ramazanov and Ramazanov, 2006). After treatment, cell suspensions were spread on NSIII agar medium and incubated at 25°C under dark conditions for 7 d. The surviving colonies were then picked up for further studies.

Primary screening of starchless mutants by iodine vapor staining

The mutant colonies were replica-plated on NSIII and N-free NSIII (nitrogen starvation) media and incubated in a growth chamber for 7 d. The agar plates with developed algal colonies were exposed to iodine vapor on the NSIII (nitrogen starvation) medium. The starchless mutant colony appeared yellow-orange in color, whereas the wild type colony was a dark-blue color (Ramazanov and Ramazanov, 2006).

Secondary screening of starchless mutants

Starchless mutants and the wild type strain were cultivated in N-limited NSIII medium (containing one-fourth strength of the original nitrogen concentration) under a 16:8 hr light:dark cycle at 25°C for 7 d. Cells were collected and extracted for lipid analysis according to the method described by Bligh and Dyer (1959). Pentadecanoic acid (C15:0) (Sigma Co., Ltd., St. Louis, MO. USA) was used as an internal standard. The total lipids were measured as fatty acid methyl esters (FAMEs) after transmethylation by the method described by Holub and Skeaff (1987). The FAME was analyzed using capillary gas chromatography (GC-14B; Shimadzu Corp.; Kyoto, Japan) with a flame ionization detector equipped with a capillary column (DB-225, J&W Scientific; Folsom, CA, USA). FAMEs were identified by comparing to

authentic standards (Sigma Co., Ltd., St. Louis, MO, USA) and quantified by internal standard quantitation.

Optimization of starchless mutant for biomass and lipid accumulation statistical analyses

Plackett and Burman experimental design

A Plackett and Burman design was used for screening the prime factors affecting the biomass, lipid production and lipid content of the starchless mutant. The experiment was designed with eight runs (treatments) from seven determined factors—KNO_3, KH_2PO_4+K_2HPO_4, $MgSO_4.7H_2O$, NaCl, pH, CO_2 and light intensity. Each factor was tested at two levels—a high level (+) and a low level (-)—as shown in Table 1. The code levels, real values and matrix for the Plackett and Burman experimental design are shown in Table 2. The effect of each variable was determined using Equation 1:

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

where $E_{(xi)}$ is the effect of variable test, M_{i+} and M_{i-} are the biomass, lipid production and lipid content where the variables were at high and low levels, respectively, and N is the total number of experiments.

The software package SPSS (version 18; SPSS (Hong Kong) Ltd, Westlands Centre, Quarry Bay, Hong Kong) was used to analyze the significance levels. Factors significant at the 90% level ($P < 0.1$) were considered reliable.

Central composite design

Response surface methodology (RSM) is an optimization tool for determining the relationships between the response (dependent factors) against variables (independent factors). RSM was used to optimize the significant factors obtained from the Plackett-Burman design using the CCD. Three significant factors were used in

Table 1 Code levels and actual values in Plackett and Burman design.

Variable code	Factor	Low level (-)	High level (+)
X_1	KNO_3 ($g.L^{-1}$)	0.2	2.0
X_2	$KH_2PO_4 + K_2HPO_4$ ($g.L^{-1}$)	0.1	1.0
X_3	$MgSO_4.7H_2O$ ($g.L^{-1}$)	0.01	1.0
X_4	NaCl ($g.L^{-1}$)	0.01	1.0
X_5	pH	5.5	7.0
X_6	CO_2 (%, volume per volume)	0	5.0
X_7	Light intensity (lux)	1,000	5,000

Table 2 Matrix for Plackett and Burman experimental design.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Biomass ($g.L^{-1}$)	Lipid production ($g.L^{-1}$)	Lipid content (%)
1	+	+	+	-	+	-	-	0.2131	0.0206	9.65
2	-	+	+	+	-	+	-	0.1405	0.005	3.57
3	-	-	+	+	+	-	+	1.8862	0.1387	7.35
4	+	-	-	+	+	+	-	0.1091	0.0057	5.22
5	-	+	-	-	+	+	+	2.2727	0.1804	7.93
6	+	-	+	-	-	+	+	1.4718	0.1073	7.28
7	+	+	-	+	-	-	+	1.8185	0.1249	6.87
8	-	-	-	-	-	-	-	0.1707	0.0107	6.28

this model. These factors were tested at five levels as shown in Table 3, and employed within 17 experiments, as shown in Table 4. The responses were then calculated based on Equation 2:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X^2_i + \Sigma\beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, X_i and X_j are independent factors that influence the response Y, β_0 is the intercept, β_i is the linear effect, β_{ii} is the squared effect and β_{ij} is the interaction effect.

The significance of the model was determined using an *F*-test. Response was measured in the terms of the biomass, lipid production and lipid content using the software Design-Expert (version 7.0; Stat-Ease, Inc., Minneapolis, MN, USA).

RESULTS AND DISCUSSION

Screening of starchless mutants by ultraviolet mutagenesis

Over 40,000 colonies of the UV-treated algae were picked up for screening of the starchless mutant on nitrogen-free medium using iodine vapor staining. After fumigation, the starchless

Table 3 Experimental codes, ranges and levels of the independent variables in the central composite design.

Factor	Unit	Code	Level				
			-1.68	-1	0	1	1.68
KNO_3	$g.L^{-1}$	X_1	0	0.2	1.1	2.0	2.6
pH		X_2	5.0	5.5	6.2	7.0	7.5
Light intensity	lux	X_3	0	1,000	3,000	5,000	6,360

Table 4 Code and real value of the experimental in central composite design.

Treat-ment	Variable			Biomass ($g.L^{-1}$)		Lipid production ($g.L^{-1}$)		Lipid content (%)	
	KNO_3 ($g.L^{-1}$)	pH	Light intensity (lux)	Experi-mental	Predicted	Experi-mental	Predicted	Experi-mental	Predicted
1	0.2	5.5	1,000	1.174	0.953	0.117	0.042	9.99	13.36
2	0.2	5.5	5,000	2.228	1.970	0.661	0.663	29.66	35.24
3	0.2	7.0	1,000	1.072	0.596	0.125	0.041	11.66	11.89
4	0.2	7.0	5,000	2.079	2.021	0.402	0.484	19.32	26.51
5	2.0	5.5	1,000	0.604	0.828	0.111	0.063	18.37	14.80
6	2.0	5.5	5,000	1.436	2.076	0.099	0.295	6.92	10.33
7	2.0	7.0	1,000	0.374	0.797	0.077	0.108	20.60	18.64
8	2.0	7.0	5,000	2.066	2.452	0.137	0.244	6.65	6.91
9	0	6.2	3,000	0.412	1.725	0.151	0.809	36.66	44.19
10	1.1	5.0	3,000	2.133	1.975	0.289	0.265	13.54	12.07
11	1.1	6.2	0	0.129	0.600	0.021	0.398	16.16	27.70
12	2.6	6.2	3,000	2.269	1.335	0.239	0.085	10.53	15.24
13	1.1	7.5	3,000	2.086	1.992	0.159	0.153	7.62	7.96
14	1.1	6.2	6,360	2.858	2.477	1.033	0.831	36.16	29.97
15	1.1	6.2	3,000	2.939	2.483	1.616	1.368	54.97	55.99
16	1.1	6.2	3,000	2.072	2.483	1.100	1.368	53.07	55.99
17	1.1	6.2	3,000	2.411	2.483	1.374	1.368	56.99	55.99

mutant colony appeared yellow-to-orange in color, while the non-starchless mutant colonies were a dark-to-blue color. The starchless mutants had good potential to accumulate higher lipid contents because starch and lipid synthesis share common carbon precursors (Weselake *et al*., 2009). Sixty-three starchless mutants from over 40,000 colonies were selected after iodine vapor staining. The cellular lipid contents of these strains revealed that eight isolates of the mutants accumulated relatively high levels of lipid compared to the wild type strain (Figure 1). The lipid contents increased from 10.27% in the wild type DMKU5201-WT to 17.89% in the mutant DMKU5201-05, while they increased from 15% in the wild type DMKU5202-WT to 21.16% in the mutant DMKU5202-31. Therefore, the mutant *C. sorokiniana* DMKU5202-31 was selected for further optimization. However, the lipid content of this mutant was lower than for *C. pyrenoidosa* mutants (25.5% to 38% of cell dry weight) reported by Ramazanov and Ramazanov, (2006). Li *et al*. (2010) reported that disruption of ADP-glucose pyrophosphorylase in the *Chlamydomonas* starchless mutant could produced 10-fold as much triacylglycerol (TAG) under stress (high-light and nitrogen-starved) conditions, suggesting that shunting the carbon precursor from starch to TAG synthesis may represent a more effective strategy than direct manipulation of the lipid synthesis pathway to over produce TAG. Yu *et al*. (2013) reported that *Chlamydomonas* BafJ5 (a starchless mutant) increased the lipid content to 42% with input acetate concentration under a nitrogen-limited and mixotrophic state.

Optimization of mutants for growth and lipid accumulation by experimental design and data analysis

Plackett and Burman experimental design

The seven factors tested in the Plackett and Burman design for biomass (shown in Table 5) revealed that the mutant strain DMKU5202-

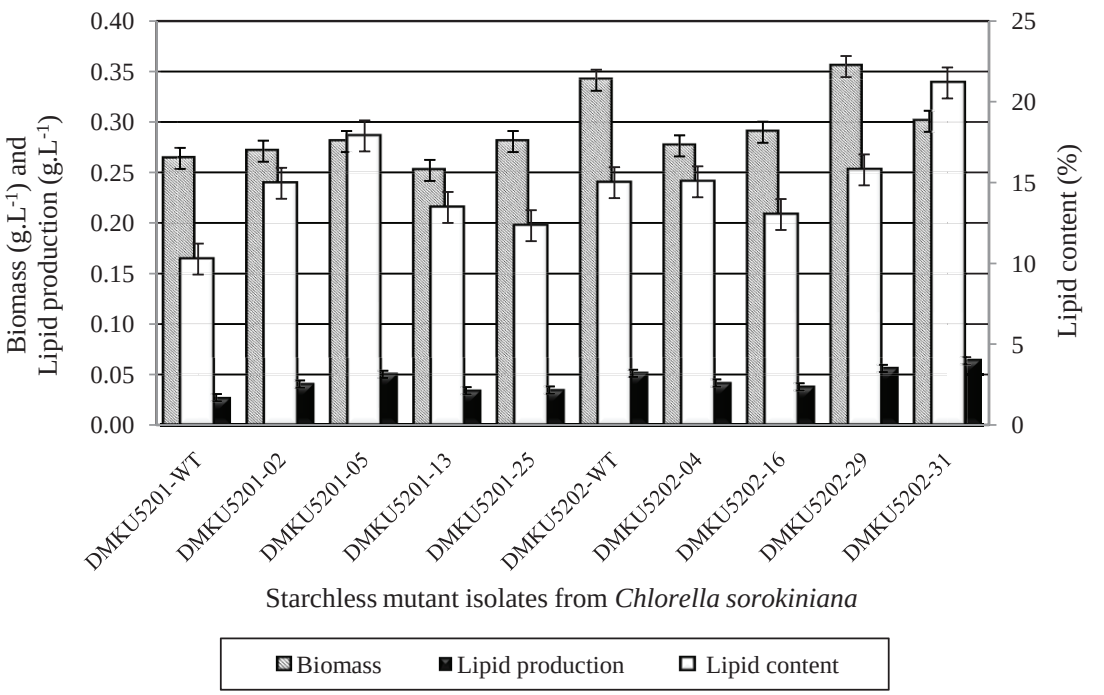

Figure 1 Biomass, lipid production and lipid content of wild type and starchless mutants of *C. sorokiniana* DMKU5201 and DMKU5202.

31 had the highest combined biomass and lipid accumulation in Run 5 which was composed of 0.2 $g.L^{-1}$ KNO_3, 1 $g.L^{-1}$ KH_2PO_4+K_2HPO_4, 0.01 $g.L^{-1}$ $MgSO_4.7H_20$, 0.01 $g.L^{-1}$ NaCl, pH 7.0, 5% v/v CO_2 and 5,000 lux light intensity. Three factors—KNO_3, pH and light intensity—were significant at $P < 0.1$. While the pH and light intensity had a positive effect on the biomass, lipid production and lipid content, KNO_3 had a negative effect on the growth and lipid production, but had a significant effect on the lipid content. The results coincided with those of Hu *et al*. (2008); Widjaja *et al*. (2009) reported that nitrogen starvation was the most important factor regulating lipid accumulation in *C. vulgaris*. Therefore, these factors were selected for further optimization using RSM.

Central Composite Design

The optimal levels of the selected variables; KNO_3, pH and light intensity were determined for the interactions between each factor on the biomass, lipid production and lipid content from the starchless mutant *C. sorokiniana* DMKU5202-31. The experiments were performed using the statistically design combinations. The results of the experimental and predicted responses from each individual experiment are shown in Table 4.

Multiple regression analysis from the experimental data derived the second-order polynomial equation for the biomass production shown in Equation 3:

$$Y_{biomass} = -10.332 + 0.455641X_1+3.614022X_2-0.000469X_3 + 0.120593\ X_1X_2+0.0000321\ X_1X_3 +0.0000678\ X_2X_3-0.55472\ X_1^2-0.31548X_2^2 - 0.000000099\ X_3^2 \quad (3)$$

where $Y_{biomass}$ is the predicted response, X_1 is KNO_3, X_2 is pH and X_3 is light intensity.

The results of the analysis of variance (ANOVA) are shown in Table 6. The model resulted in an *F*-value of 2.47 and a *P*-value of 0.12. Although the *P*-value was greater than 0.05 which indicated that the model terms were not significant, the coefficient of determination (R^2) was close to 1. The R^2 value for biomass production was 0.7602 indicating that this model provided a moderate representation of biomass production. Djekrif-Dakhmouche *et al*. (2006) and Li *et al*. (2011) suggested that *P*-values less than 0.30 were considered to have significant influence on a response. Hu (1999) also reported that a model with a confidence level of 75% or higher could be accepted.

The second-order polynomial equation for the lipid production is shown in Equation 4:

$$Y_{\text{lipid production}} = -28.5536+0.948434X_1+9.077213X_2+0.00071X_3-0.047333\ X_1X_2-0.000054\ X_1X_3+0.000016\ X_2X_3-0.51962\ X_1^2-0.73008\ X_2^2 -0.000000076X_3^2 \quad (4)$$

where $Y_{\text{lipid production}}$ is the predicted response, X_1 is KNO_3, X_2 is pH and X_3 is light intensity.

Table 5 Analysis of variance of Plackett and Burman design for biomass, lipid production and lipid content of starchless mutant *C. sorokiniana* DMKU5202-31.

Variable	Biomass		Lipid production		Lipid content	
	Effect	*P*-level	Effect	*P*-level	Effect	*P*-level
KNO_3 ($g.L^{-1}$)	-0.016	0.186	-0.172	0.119	2.466	0.057 [a]
KH_2PO_4 + K_2HPO_4 ($g.L^{-1}$)	0.020	0.114	0.244	0.032 [a]	1.891	0.137
$MgSO_4.7H_2O$ ($g.L^{-1}$)	-0.010	0.418	-0.122	0.259	1.718	0.174
NaCl ($g.L^{-1}$)	-0.008	0.486	-0.001	0.994	-0.332	0.787
pH	0.027	0.036 [a]	0.263	0.023 [a]	2.926	0.027 [a]
CO_2 (%, v/v)	0.004	0.769	0.019	0.858	0.226	0.854
Light intensity (lux)	0.130	0.000[a]	1.747	0.000 [a]	2.848	0.031 [a]

[a] = Significant ($P<0.1$).

The results of the ANOVA in Table 6 show an *F*-value and a *P*-value for the model of 8.8 and 0.0045, respectively. The *P*-value was less than 0.05 which indicated that the model terms are significant. The R^2value was 0.9189 for lipid production.

The lipid content is predicted as shown in Equation 5:

$$Y_{\text{lipid content}} = -1126.85+25.37506X_1+361.7263 X_2+0.029057 X_3- 1.967412 X_1X_2-0.00366 X_1X_3-0.00121 X_2X_3-14.4246 X_1^2-28.9511 X_2^2-0.0000027 X_3^2 \quad (5)$$

where $Y_{\text{lipid content}}$ is the predicted response, X_1 is KNO_3, X_2 is pH and X_3 is light intensity.

Table 6 shows the results of the ANOVA of the factors giving an F-value of 14.24 and a P-value of 0.0010. The values indicated that the model terms are significant with an R^2 value of 0.9482.

The response surface and corresponding contour plots are shown in Figures 2, 3 and 4 with the interactions between two factors, such as KNO_3 and pH, KNO_3 and light intensity, pH and light intensity, while keeping another factor constant at the central point level. The maximum points on the contour plots of each pair of variables while fixing the remaining variable are shown in Table 7. The maximum lipid production yield of 1.39 $g.L^{-1}$ was obtained when using KNO_3 at 1 $g.L^{-1}$, a pH of 6.2 and a light intensity of 3,700 lux while the maximum lipid content was substantially improved to 55.98% when using KNO_3 at 0.9 $g.L^{-1}$, pH at 6.2 and light intensity at 3,500 lux. The optimal conditions of KNO_3 at 0.9 $g.L^{-1}$, pH at 6.2 and light intensity at 4,000 lux were then selected from the regression equations 3–5 for the predicted values of maximum biomass, lipid production and lipid content. Kong *et al*. (2012) used a similar Plackett and Burman design technique to evaluate the significances of various factors and then optimized using RSM to maximize the biomass production of *C. vulgaris*. The results revealed that the biomass increased 2.24-fold with glucose at 25 $g.L^{-1}$, $MgSO_4.7H_2O$ at 1.33 $g.L^{-1}$, KNO_3 at 1.3 $g.L^{-1}$ and NaCl at 3.02 $g.L^{-1}$ under mixotrophic conditions. Cheng *et al*. (2013) used RSM optimizing culture media of *C. protothecoides* UTEX 250 for growth and lipid production and found that the biomass and lipid production were higher than in the original medium. Likewise, Cheng *et al*. (2013) successfully optimized the culture conditions for biomass and lipid production of *C. protothecoides* UTEX 250 using RSM with a Box-Behnken design. The optimal concentration of medium for maximum biomass concentration of 1.19 $g.L^{-1}$ was obtained with 12.9% lipid content.

Table 6 Analysis of variance for response surface methodology factors.

Source	Biomass			Lipid production			Lipid content		
	Estimate	*F*-value	*P*-level	Estimate	*F*-value	*P*-level	Estimate	*F*-value	*P*-level
Model	2.4881	2.4652	0.1237	1.3654	8.8122	0.0045	55.1795	14.2376	0.0010
X_1	0.0767	0.1871	0.6783	-0.0536	0.8188	0.3956	-4.5424	7.8358	0.0266
X_2	0.0050	0.0008	0.9783	-0.0341	0.3315	0.5828	-1.2210	0.5661	0.4763
X_3	0.6717	14.3374	0.0068	0.1883	10.0938	0.0156	2.6037	2.5746	0.1526
X_1X_2	0.0814	0.1233	0.7358	0.0320	0.1702	0.6923	1.3280	0.3923	0.5509
X_1X_3	0.0578	0.0621	0.8103	-0.0964	1.5502	0.2532	-6.5906	9.6629	0.0171
X_2X_3	0.1017	0.1926	0.6740	-0.0244	0.0991	0.7621	-1.8148	0.7327	0.4203
X_1^2	-0.4493	5.2960	0.0549	-0.4209	41.6215	0.0003	-11.6839	42.7958	0.0003
X_2^2	-0.1775	0.8261	0.3936	-0.4107	39.6252	0.0004	-16.2850	83.1378	0.0001
X_3^2	-0.3952	4.0972	0.0826	-0.3035	21.6384	0.0023	-10.7770	36.4098	0.0005

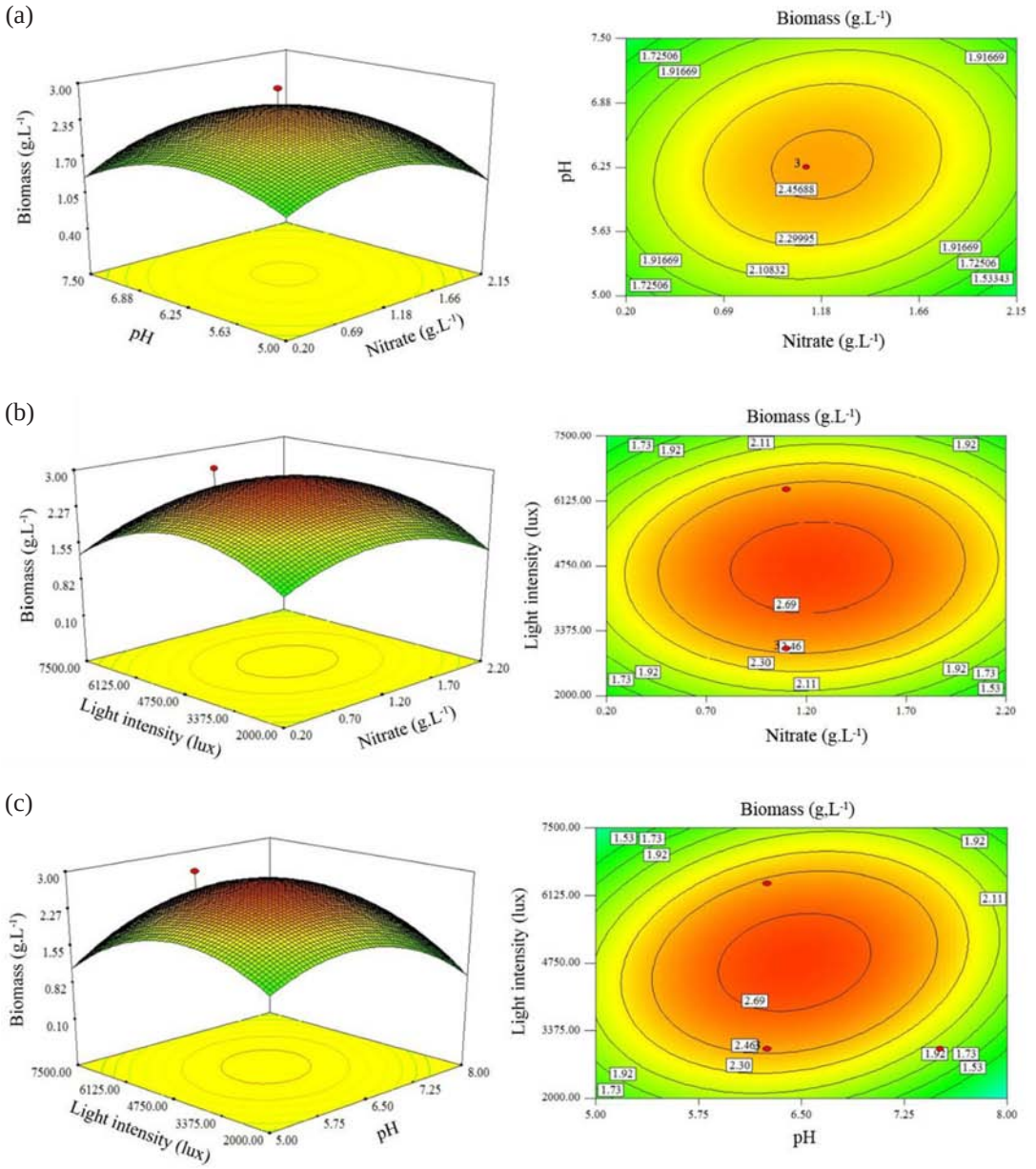

Figure 2 Three-dimensional response surface plots and two-dimensional contour plots of biomass production by *C. sorokiniana* DMKU5202-31 showing interaction of two variables while the remaining factor is held constant: (a) KNO_3 and pH; (b) KNO_3 and light intensity; (c) pH and light intensity.

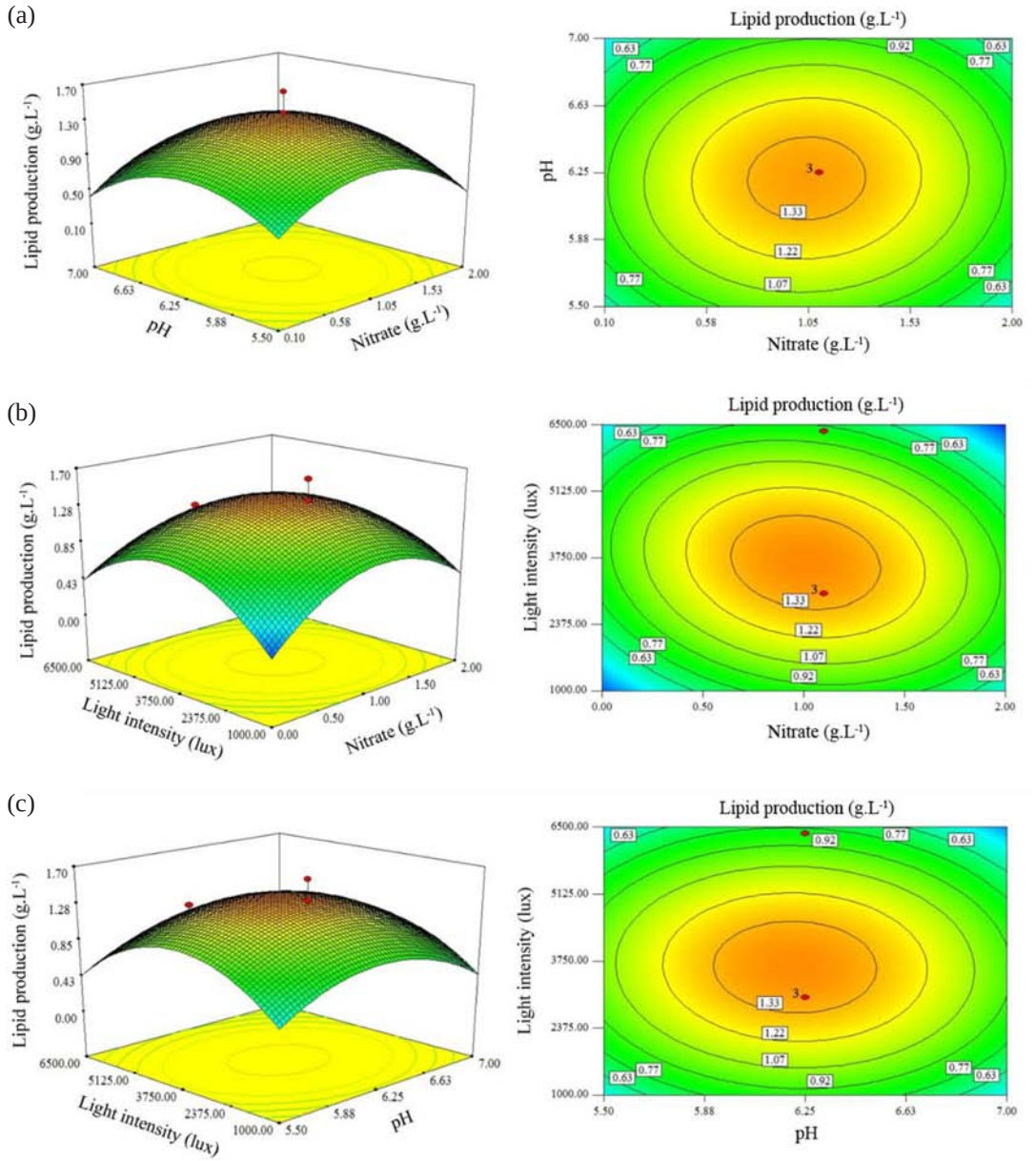

Figure 3 Three-dimensional response surface plots and two-dimensional contour plots of lipid production by *C. sorokiniana* DMKU5202-31 showing interaction of two variables while the remaining factor is held constant: (a) KNO_3 and pH; (b) KNO_3 and light intensity; (c) pH and light intensity.

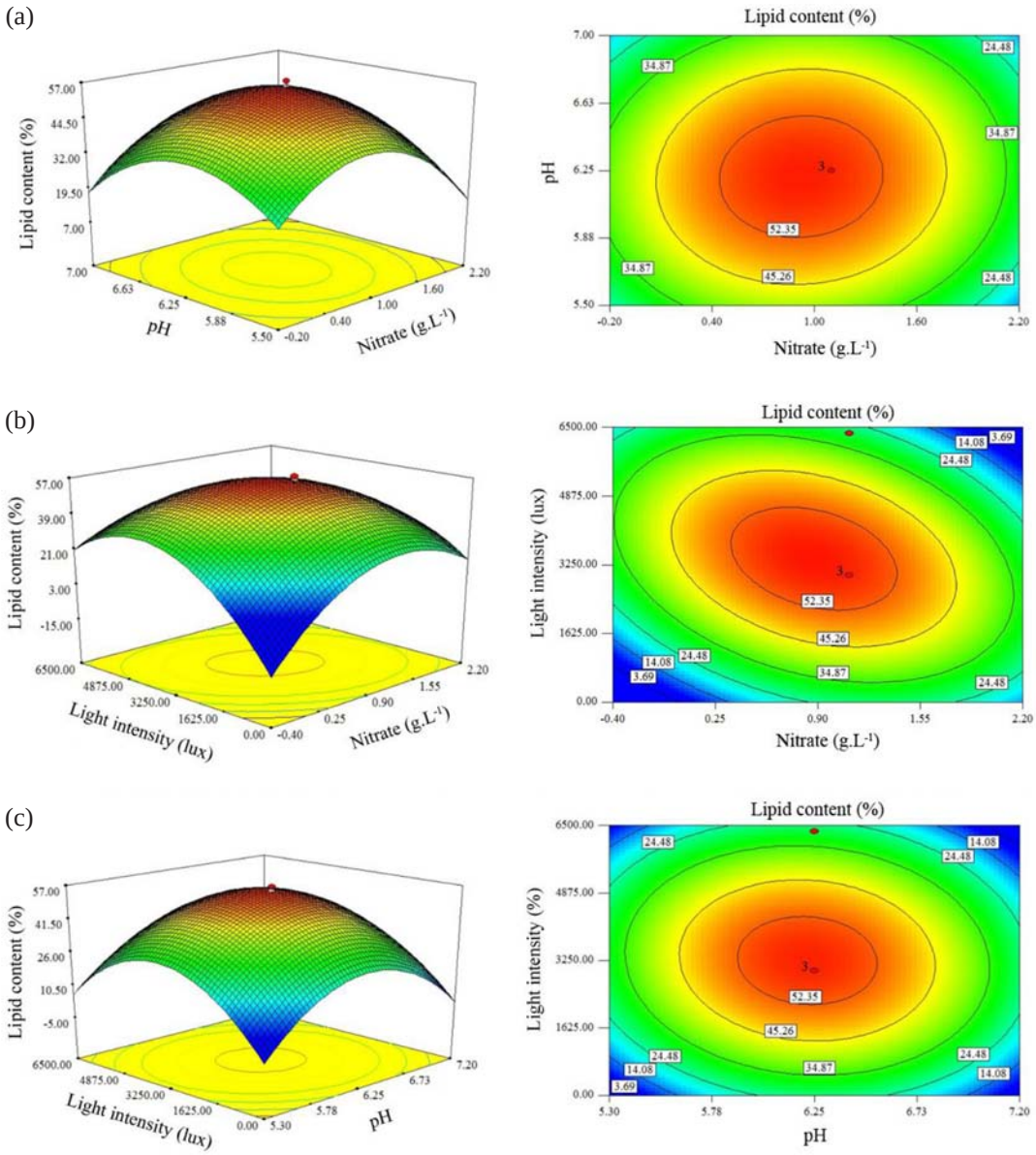

Figure 4 Three-dimensional response surface plots and two-dimensional contour plots of lipid content by *C. sorokiniana* DMKU5202-31 showing interaction of two variables while the remaining factor is held constant: (a) KNO_3 and pH; (b) KNO_3 and light intensity; (c) pH and light intensity.

Table 7 Maximum response values on contour plots of each pair of variables by fixing the remaining variable (in parentheses).

Response	Variable			Yield
	KNO_3 (g.L^{-1})	pH	Light intensity (lux)	
Biomass production (g.L^{-1})	1.2	6.3	(3,000)	2.49
	1.25	(6.2)	4,700	2.78
	(1.1)	6.4	4,800	2.79
Lipid production (g.L^{-1})	1	6.2	(3,000)	1.37
	1	(6.2)	3,700	1.39
	(1.1)	6.3	3,700	1.39
Lipid content (%)	0.9	6.2	(3,000)	55.65
	0.9	(6.2)	3,500	55.98
	(1.1)	6.2	3,300	55.36

Model validation

In order to demonstrate the optimal values of the three variables for biomass, lipid production and lipid content of starchless mutant *C. sorokiniana* DMKU5202-31, the model was validated using the selected conditions of 0.9 g.L^{-1} KNO_3, pH 6.2 and light intensity at 4,000 lux. The results from this experiment showed that the maxima produced were: production of biomass, 2.58 g.L^{-1}; lipid production, 1.40 g.L^{-1}; and lipid content, 54.49% while the predicted values were 2.67 g.L^{-1}, 1.39 g.L^{-1} and 55.80%, respectively. The deviations from the predicted values for the biomass production, lipid production and lipid content were -3.47, 1.01 and -2.35%, respectively (Table 8). The results of the validation suggested that there was good agreement on the optima between the experimental and the predicted values.

CONCLUSION

The starchless mutant *C. sorokiniana* DMKU 5202-31 obtained from UV mutagenesis improved its lipid content from 15% in the wild type strain to 21.16%. The relatively high lipid level of this microalgal mutant together with its ability to grow rapidly under autotrophic conditions increased the opportunity for utilization of the strain for biodiesel production. Statistical experimental designs were used to screen and optimize the culture conditions for biomass, lipid production and lipid content of the mutant strain. The significance of the seven selected factors (KNO_3, KH_2PO_4+K_2HPO_4, $MgSO_4.7H_2O$, NaCl, pH, CO_2 and light intensity) evaluated by Plackett and Burman design indicated that three factors (KNO_3, pH and light intensity) were significant and consequently, they were selected for further optimization using RSM. The model showed that the optimal conditions for the three significant factors were 0.9 g.L^{-1} KNO_3, pH 6.2 and 4,000 lux of light intensity. Under the optimum conditions, biomass production of 2.58 g.L^{-1}, lipid production of 1.40 g.L^{-1} and a lipid content of 54.59% were obtained within 5 d under phototrophic cultivation.

Table 8 Comparison of biomass, lipid production and lipid content for model validation.

Response (Y)	Experimental	Predicted	Error (%)
Biomass (g.L^{-1})	2.58	2.67	-3.47
Lipid production (g.L^{-1})	1.40	1.39	1.01
Lipid content (%)	54.49	55.80	-2.35

The validation of the biomass, lipid production and lipid content comparing the experimental and predicted values showed -3.47, 1.01 and -2.35% deviations from the predicted values, respectively. This study indicated that UV mutation techniques could dramatically improve the lipid accumulation in *C. sorokiniana*. A Plackett-Burman design and CCD were suitable for screening and optimization of the culture conditions for growth and lipid production of microalgae. After optimization, the lipid content of the starchless mutant *C. sorokiniana* DMKU5202-31 increased from 21.16% to 54.59%.

ACKNOWLEDGEMENTS

The authors would like to thank the Biodiversity-Based Economy Development Office (Public Organization) and the National Research Council of Thailand for financial support and the Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand for providing the research facilities.

LITERATURE CITED

Amaro, H.M., A.C. Macedo and F.X. Malcata. 2012. Microalgae: An alternative as sustainable source of biofuels. **Energy.** 44: 158–166.

Anandarajah, K., G. Mahendraperumal, M. Sommerfeld and Q. Hu. 2012. Characterization of microalga *Nannochloropsis* sp. mutants for improved production of biofuels. **Appl. Energy.** 96: 371–377.

Bligh, E.G. and W.J. Dyer. 1959. A rapid method for total lipid extraction and purification. **Can. J. Biochem. Physiol.** 37: 911–917.

Cheng, K.C., M. Ren and L. Kimberly Ogden. 2013. Statistical optimization of culture media for growth and lipid production of *Chlorella protothecoides* UTEX250. **Bioresour. Technol.** 128: 44–48.

Chisti, Y. 2007. Biodiesel from microalgae. **Biotechnol. Adv.** 25: 294–306.

Djekrif-Dakhmouche, S., B. Gheribi-Aoulmi, Z. Meraihi and L. Bennamoun. 2006. Application of a statistical design to the optimization of culture medium for alpha-amylase production by *Aspergillus niger* ATCC16404 grown on orange waste power. **J. Food Eng.** 73: 190–197.

Holub, B.J. and C.M. Skeaff. 1987. Nutritional regulation of cellular phosphatidyl inositol. **Methods Enzymol.** 141: 234–244.

Hu, R. 1999. **Food Product Design: A Computer-Aided Statistical Approach.** Technomic Publishing Co. Ltd. Lancaster, PA, USA.

Hu, Q., M. Sommerfield, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert and A. Darzins. 2008. Microalgae triacylglycerols as feedstocks for biofuel production: perspectives and advances. **Plant J.** 54: 621–639.

Kong, W.B., S.F. Hua, H. Cao, Y.W. Mu, H. Yang, H. Song and C.G. Xia. 2012. Optimization of mixotrophic medium components for biomass production and biochemical composition biosynthesis by **Chlorella vulgaris** using response surface methodology. **J. Taiwan Inst. Chem. E.** 43: 360–367.

Li, Y., D. Han, G. Hu, D. Dauvillee, M. Sommerfeld, S. Ball and Q. Hu. 2010. **Chlamydomonas** starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. **Metab. Eng.** 12: 387–391.

Li, Y., W. Zhou, B. Hu, M. Min, P. Chen and R.R. Ruan. 2011. Integration of algae cultivation as biodiesel production feedstock with municipal wastewater treatment: Strains screening and significance evaluation of environmental factors. **Bioresour. Technol.** 102: 10861–10867.

Mata, T.M., A.A. Martins and N.S. Caetano. 2010. Microalgae for biodiesel production and other applications: A review. **Renew Sustain. Energy Rev.** 14: 217–232.

Ramazanov, A. and Z. Ramazanov. 2006. Isolation and characterization of a starchless mutant of *Chlorella pyrenoidosa* STL-PL with

a high growth rate, and high protein and polyunsaturated fatty acid content. **Phycol. Res.** 54: 255–259.

Song, X., X. Zhang, C. Kuang, L. Zhu and N. Guo. 2007. Optimization of fermentation parameters for the biomass and DHA production of *Schizochytrium limacinum* OUC88 using response surface methodology. **Proc. Biochem.** 42: 1391–1397.

Weselake, R.J, D.C. Taylor, M.H. Rahman, S. Shah, A. Laroche, P.B.E. McVetty and J.L. Harwood. 2009. Increasing the flow of carbon into seed oil. **Biotechnol Adv.** 27(6): 866–878.

Widjaja, A., C.C. Chien and Y.H. Ju. 2009. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J. Taiwan Inst. **Chem. Eng.** 40: 13–20.

Yu, S., Q. Zhaoa, X. Miao and J. Shi. 2013. Enhancement of lipid production in low-starch mutants *Chlamydomonas reinhardtii* by adaptive laboratory evolution. **Bioresour. Technol.** 147: 499–507.