Chiang Mai J. Sci. 2019; 46(6) : 1055-1068
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Optimization of Culture Conditions for Oil Production by the Double Mutant of *Chlorella sorokiniana* DMKU5202-D223

Mathurin Vichachucherd, Wichien Yongmanitchai and Duenrut Chonudomkul*

Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, 10900, Thailand.

*Author for correspondence; e-mail: fscidrc@ku.ac.th

Received: 10 April 2019
Revised: 21 May 2019
Accepted: 4 July 2019

ABSTRACT

Chlorella sp. is a green microalga and well-known for its ability to accumulate high amount of lipid in the cells. This alga was considered to be a potential energy source for biodiesel production. The objectives of this study were to re-mutate the *Chlorella sorokiniana* DMKU5202-31 which was the UV mutant strain by using Ethyl methanesulfonate (EMS), a chemical mutagen and optimization of culture condition of the double mutant strain for oil production. After treatment with 0.5 M EMS for 1 h 40,323 colonies was obtained and subjected to screening. The double mutant strain *C. sorokiniana* DMKU5202-D223 was selected due to its high lipid production. For optimization of culture condition, the Central Composite Design (CCD) and Response surface methodology (RSM) were employed. The experiment model concluded that glucose concentration of 10 g/L, pH 8, 3% CO_2 and light intensity 3,000 lux were suitable to enhance oil production. The optimization of oil production conditions of *C. sorokiniana* DMKU5202-D223 showed from the RSM and CCD as followed: 6.32 g/L of biomass production, 0.65 g/L of lipid production, and 10.45% of lipid content within 5 days. Comparison of oil production by the double mutant strain under these optimum conditions to the UV mutant strain and the wild type strain revealed that the double mutant strain had oil production higher than UV mutant strain and wild type strain. The UV mutant strain and wild type strain had lipid production 0.54 g/L and 0.22 g/L while lipid content were 8.38% and 4.96%. In conclusion, the double mutant strain showed relatively high oil production and could be used as the raw material for biodiesel production.

Keywords: biodiesel, *Chlorella*, mutation, optimization, microalgae

1. INTRODUCTION

The biodiesel has been used in Thailand over a decade for normal transportation vehicles. Using biodiesel can reduce up to 30% sulfur emissions and 10% carbon monoxide [1]. The number of molecules in the chain of carbon in biodiesel production is about 14-18 carbons. The triglycerides are the main materials for biodiesel production via transesterification [2]. Currently palm oil has been used for this energy substitute. Microalgae are alternative raw materials for lipid production due to their short time of cultivation, high growth rate and

less land requirement. Moreover, CO_2 used during microalgae cultivation is absorbed and release oxygen to the environment [3]. There are certain limitations of cultural conditions to induce lipid accumulation in microalgae [4, 5]. Although, the cost of algal biodiesel is still high, several approaches to solve this problem are improvement of biomass production, high cellular lipid accumulation and extraction technology [6]. In general dissolved carbon dioxide and light intensity are the two major factors to enhance photosynthesis mechanism in microalgae. Several microalgae are able to adapt to a variety of environments and metabolic patterns such as photoautotrophy, heterotrophy, photoheterotrophy and mixotrophy. Photoheterotrophic culture was compared with heterotrophic culture of *Chlorella vulgaris* by light stimulation [7]. The results showed that CO_2 was important for mixotrophic cultivation. *C. vulgaris* used glucose as a carbon source and accumulated high amount of lipid and high biomass productivity without light stimulation [8]. Similarly the lipid yield of *Tetraselmis* sp. was increased by mixotrophic cultivation. The main nutrient of microalgae such as phosphorous, nitrogen and 45% carbon were converted into cell mass [9]. Carbon and nitrogen ration is known to affect lipid and biomass production of microorganisms [10]. Microalgae produced higher lipid and biomass yields when nitrogen sources are limited [11]. In order to minimize the production cost some microalgae can be cultivated in waste water [12]. In addition, several species of microalgae had shown to accumulate high intracellular lipid for biodiesel production [13]. So microalgae have been investigated worldwide for this purpose. *Chlorella* spp. have been reported as a potential lipid producers for biodiesel production [14]. To improve the ability of an alga to accumulate high lipid content in the cells, mutations have been one of the method of choice. As previously report the UV mutant of *C. sorokiniana* improved lipid production significantly [15]. This work aimed to improve *C. sorokiniana* DMKU5202-31, UV-mutant strain by second mutation with mutagenic agents. The selected double mutation strain was then optimized the cultivation condition to improve oil production. The objectives of this study were to improve the oil production of the *C. sorokiniana* DMKU5202-31 mutant by re-mutation using EMS and to optimize the culture condition of the double mutant strain. In this study we employed to statistical methodologies, by using CCD and analyzed by the RSM.

2. MATERIALS AND METHODS

2.1 Microalga

The microalga used in this experiment was *C. sorokiniana* DMKU5202-31. The strain was mutated by UV radiation and shown to improve lipid production compared to wild type [15].

2.2 Culture Medium

The alga was cultivated on NSIII medium [16] consisted of KNO_3 1011 mg/L, KH_2PO_4 240 mg/L, $K_2HPO_4.3H_2O$ 284 mg/L, $MgSO_4.7H_2O$ 124 mg/L, $CaCl_2.2H_2O$ 15 mg/L, NaCl 12 mg/L, Micro A 2 ml/L, Micro B 2 ml/L and Micro C 2 ml/L. The mutant strain was cultivated at 25 °C in a 125 ml flask containing 50 ml NSIII medium. Light was provided at the intensity of 3,000 lux by cool-white fluorescent lamps with 16 h : 8h, light/dark photo-period. The flasks were incubated on an orbital shaker at 140 rpm for 7 days.

2.3 Mutation of the Mutant by EMS Treatment

The suspensions of *C. sorokiniana* DMKU5202-31 mutant at exponential growth phase with cell concentration at 10^6-10^7 cells/ml were treated EMS solution at varying concentration of EMS, i.e. 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 M for 1 h on a shaker at 140 rpm, 25 °C under dark condition. Cell suspensions were

concentrated via centrifugation at 3,300 rpm for 15 min. Cell pellets were washed thrice with 10% (w/v) sodium thiosulfate solution [17]. After that, the cells were re-suspended in NSIII medium at 25 °C in darkness overnight followed by determination of cell survival rate. The optimal cell survival rate was spread on NSIII medium agar plate [18].

2.4 Selection of Double Mutant Strain for High Lipid Production

The mutant strains developed on NSIII broth with 1/4 nitrogen source were incubated as mention in (2.2). Then the cellular lipid contents were analyzed.

2.5 Optimization for Growth and Lipid Production of Double Mutant Strain by Using Statistical Designs

The factors affecting growth and lipid production used for statistical designs experiment were selected from several reports. CO_2, pH, glucose and light intensity were mostly employed culture conditions in microalgal cultivation [19]. Light intensity had impact on lipid contents of *Chlorella kessleri* and *Chlorella protothecoide* [20] and lipid accumulation of *C. sorokiniana* [21]. Optimal glucose concentration could be increased biomass yield, lipid production and lipid content of *C. sorokiniana* [21, 22]. Similarly pH and CO_2 concentration had influenced on biomass, lipid production and lipid content of *C. sorokiniana* [23]. Therefore, these four factors namely CO_2 (coded as X_1), pH (X_2), glucose concentration (X_3) and light intensity (X_4) were chosen to determine their significances on growth and lipid production by CCD. The four-factors produced 27 experiments including sixteen factorial point, eight start point (axial) and three at the center point for optimal data condition were from CCD in a surface response methodology (Table 1). Design Expert software (trail Version 7.0.1.0, Stat-Ease Inc., Minneapolis, MN, USA) were applied with the experiments to analyze the data.

Table 1. There are 4 factors identification of level analyzed by CCD.

Factor	code	Levels				
		-α	-1	0	1	α
CO2 (%)	X1	1	2	3	4	5
pH	X2	6	7	8	9	10
Glucose (g/L)	X3	6	8	10	12	14
Light intensity (lux)	X4	1,000	2,000	3,000	4,000	5,000

2.6 Lipid Extraction and Analysis

The double mutant cells were collected by centrifuged at 3,300 rpm for 15 min. The 0.5 ml of water were added. Lipid extraction was carried out by Bligh and Dyer [24] followed by transmethylation of fatty acids according to to Holub and Skeaff [25]. Fatty acid methylesters were then analyzed by a capillary gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) with flame ionization detector (FID). The column used was the capillary fused silica megabore column (30 m x 0.540 mm x 1 μm film thickness). The temperature of initial column was 190°C. And the temperature of the injector and detector were 250°C.

3. Results and Discussion

3.1 Mutation of *C. sorokiniana* DMKU5202-31 by EMS Treatment

The survival rate of *C. sorokiniana* DMKU5202-31 after treatment with EMS mutagen at the concentrations of 0.1, 0.25,

0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 M for 1 h were in the range of 0 - 82.8%, while 0.5 M was chosen as the optimal concentration. The treated cells after develop on the agar as 40,323 single colonies were exposed to iodine vapor. The light brown to yellowish color were elected as candidates for starchless strains as shown in Figure 1. There were 402 isolates out of colonies were selected.

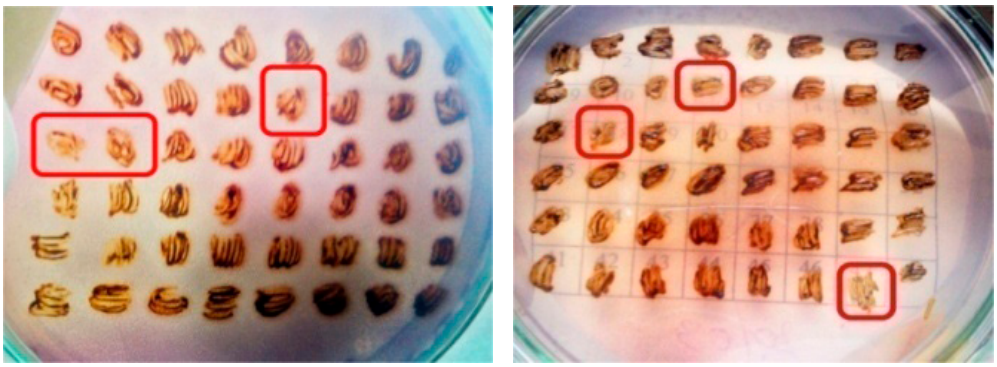

Figure 1. Appearance of *C. sorokiniana* DMKU5202-31 mutant colonies appeared to below starch synthesis after exposure to iodine vapor.

3.2 Selection of Double Mutant Microalgae, *C. sorokiniana* DMKU5202

Primary selection of 402 isolates revealed that the biomass production were in the range of 0.23 - 1.33 g/L while lipid production were 0 - 0.15 g/L and lipid content within the cell were 1.44 - 45.22%. Twenty three isolates were chosen for the secondary stage resulted in 0.06 - 0.14 g/L of biomass, 0.43 - 0.70 g/L of lipid production and 11.71 - 24.44% lipid content. The values were lower than the primary screening because the nitrogen source in the NSIII medium was reduced to 1/4 of original concentration.

In the final selection, 3 isolates were tested, biomass productions were in the range of 3.56 - 4.02 g/L, lipid productions were 0.42 - 0.48 g/L and lipid contents were within the cell 11.93 - 13.13%. Consequently the double mutant microalgae, *C. sorokiniana* DMKU5202-D223 that produced biomass production 4.02 g/L, lipid production 0.48 g/L and lipid content 13.13% was selected for further study.

3.3 Optimization of Growth and Lipid Production by *C. sorokiniana* DMKU5202-D223

3.3.1 Optimization of culture condition for biomass, lipid production and lipid content from *C. sorokiniana* DMKU5202-D223 by CCD

The four factors selected including CO_2 (X_1), pH (X_2), glucose concentration (X_3), light intensity (X_4) were optimized by CCD composed of 27 experiments derived from sixteen factorial points, eight start points (axial) and three at the center points (Table 1). There were five-code levels ($-\alpha$, -1, 0, 1, α) of variables. And the zeroes were central points of code values of all variables. The matrix of predicted values and the experimental data was shown in Table 2. The experimental data were analyzed by Design expert software.

From the CCD experiments, the experiments 25-27 were the center point consisted of 3% carbon dioxide rate, pH 8, glucose concentration 10 g/L, and light intensity 3,000 lux. The results of the experiments revealed that the

Table 2. Biomass, lipid production, and lipid content of experimental data and the results of the CCD.

Run	Factor X_1: CO_2 (%)	Factor X_2: pH	Factor X_3: Glucose (g/L)	Factor X_4: Light intensity (lux)	Biomass (g/L)		Lipid production (g/L)		Lipid content (%)	
					Experiment	Predicted	Experiment	Predicted	Experiment	Predicted
1	-1	-1	-1	-1	5.85	6.32	0.40	0.36	6.85	5.15
2	1	-1	-1	-1	5.80	4.96	0.49	0.48	8.54	7.29
3	-1	1	-1	-1	6.22	5.23	0.65	0.63	10.55	9.49
4	1	1	-1	-1	4.63	5.25	0.56	0.55	12.15	11.45
5	-1	-1	-1	-1	2.93	6.32	0.22	0.36	7.56	5.15
6	1	-1	1	-1	4.49	3.19	0.46	0.45	10.25	9.51
7	-1	1	1	-1	4.84	3.98	0.47	0.47	9.73	8.71
8	1	1	1	-1	3.42	4.15	0.44	0.46	13.07	11.73
9	-1	-1	-1	1	4.56	5.18	0.47	0.42	10.44	8.36
10	1	-1	-1	1	5.52	5.11	0.47	0.45	8.52	6.37
11	-1	1	-1	1	3.18	3.20	0.51	0.50	11.15	8.70
12	1	1	-1	1	4.64	4.51	0.40	0.33	8.68	6.53
13	-1	-1	1	1	3.07	3.93	0.42	0.41	13.83	10.98
14	1	-1	1	1	4.43	4.00	0.53	0.51	12.00	10.07
15	-1	1	1	1	3.18	2.61	0.47	0.44	11.16	9.38
16	1	1	1	1	3.05	4.07	0.30	0.33	10.13	8.28
17	-2	0	0	0	4.58	4.22	0.41	0.45	9.18	9.15
18	2	0	0	0	4.03	4.32	0.45	0.46	11.24	10.19
19	0	-2	0	0	6.40	6.21	0.38	0.41	5.98	6.66
20	0	2	0	0	5.07	5.19	0.47	0.50	9.37	9.22
21	0	0	-2	0	5.08	5.43	0.38	0.47	7.64	8.08
22	0	0	2	0	3.50	3.07	0.39	0.36	10.89	10.98
23	0	0	0	-2	3.52	3.91	0.49	0.47	13.89	7.56
24	0	0	0	2	3.15	2.69	0.34	0.41	10.97	7.32
25	0	0	0	0	6.03	6.30	0.59	0.63	10.02	10.07
26	0	0	0	0	6.43	6.30	0.64	0.63	10.07	10.07
27	0	0	0	0	5.30	6.30	0.64	0.63	15.22	10.07

best experimental sets were experiments 26. The mutant *C. sorokiniana* DMKU5202-D223 produced biomass 6.43 g/L, lipid production 0.64 g/L and lipid content of 10.09%. Based on this experiment, it showed that the experimental planning was appropriate.

ANOVA of the significances of suitable the second-order polynomial equations of the model was shown in Table 3. The *F*-values of 7.68, 6.67 and 7.06 referred to the biomass production, lipid production and lipid content model were significant, respectively. There were only a 0.06%, 0.11% and 0.08% chances that the model could occur to noise. The values of "Prob >*F*" is less than 0.05 implied that the model terms are significant. The significance of variables by the *p*-values indicated the interaction power between independent variables. The less *p*-values had the bigger significance of the related variable [26]. Independent variables influencing the biomass production which has *p*-values less than 0.05 include pH (X_2), glucose

Table 3. Analysis of variance (ANOVA) of multiple regression analysis. To evaluate the results of the studied factors towards biomass production, lipid production and lipid content of *C. sorokiniana* DMKU5202-D223 double mutant strain.

Source	Biomass production (g/L)			Lipid production (g/L)			Lipid content (%)		
	Estimate	F-value	p-value[a]	Estimate	F-value	p-value[b]	Estimate	F-value	p-value[c]
Model		7.6824	0.0006		6.6750	0.0011		7.0678	0.0008
X_1	0.0438	0.1494	0.7058	0.0044	0.1880	0.6723	0.2593	1.8121	0.2031
X_2	-0.2556	5.0991	0.0434	0.0225	4.8246	0.0484	0.6394	11.0191	0.0061
X_3	-0.5893	27.1075	0.0002	-0.0255	6.1852	0.0286	0.7259	14.2027	0.0027
X_4	-0.3039	7.2105	0.0198	-0.0161	2.4518	0.1434	0.0600	0.0970	0.7607
X_1X_2	-0.3457	6.2184	0.0282	-0.0522	17.2576	0.0013	0.0464	0.0387	0.8474
X_1X_3	0.0369	0.0707	0.7948	0.0173	1.9045	0.1927	0.2679	1.2898	0.2783
X_1X_4	0.3208	5.3572	0.0392	-0.0245	3.8174	0.0744	-1.0318	19.1266	0.0009
X_2X_3	0.1647	1.4112	0.2578	-0.0144	1.3198	0.2730	-0.4862	4.2476	0.0617
X_2X_4	-0.2233	2.5955	0.1331	-0.0474	14.2259	0.0027	-1.0013	18.0134	0.0011
X_3X_4	0.1654	1.4229	0.2560	0.0238	3.6018	0.0820	0.3662	2.4093	0.1466
X_1^2	0.0665	17.8450	0.0012	-0.0430	15.6588	0.0019	-1.3731	0.2430	0.6309
X_2^2	0.4243	1.5460	0.2375	-0.0440	16.3878	0.0016	-2.0072	6.8157	0.0228
X_3^2	0.0626	18.1201	0.0011	-0.0530	23.7824	0.0004	-1.6083	0.4337	0.5226
X_4^2	-0.1750	38.8841	0.0001	-0.0468	18.4900	0.0010	-0.8170	10.3350	0.0074

[a] significant at 5% level (p<0.05), R^2 = 0.8996; and R^2_{adj} = 0.7825
[b] significant at 5% level (p<0.05), R^2 = 0.8862; and R^2_{adj} = 0.7534
[c] significant at 5% level (p<0.05), R^2 = 0.8918; and R^2_{adj} = 0.7657

concentration (X_3), light intensity (X_4) and the interaction between them (X_1X_2, X_1X_4, X_1^2, X_3^2, X_4^2). Independent variables that influence lipid production (p-values < 0.05) were pH (X_2), glucose concentration (X_3) and the interactions (X_1X_2, X_2X_4, X_1^2, X_2^2, X_3^2, X_4^2). While the variables influencing the lipid content were pH (X_2), glucose concentration (X_3), X_1X_4, X_2X_4, X_2^2, X_4^2. The coefficient of determination (R^2) were 0.8996, 0.8862 and 0.8918 for biomass production, lipid production and lipid content, respectively implied that 89.96%, 88.62% and 89.18% of the variability in the response could be explained by the good mathematical model was in good agreement with the experimental data and the predicted values.

Table 3 showed the relationships between biomass production, lipid production and lipid content of *C. sorokiniana* DMKU5202-D223 double mutant strain with the factors studied. The multiple regression analysis on the experimental data followed the second order polynomial equation. The regression model equation for biomass production, lipid production and lipid content (P) were as followed:

$$P_{Biomass} = 6.30 + 0.044X_1 - 0.26X_2 - 0.59X_3 - 0.30X_4 - 0.35X_1X_2 + 0.037X_1X_3 + 0.32\,X_1X_4 + 0.16\,X_2X_3 - 0.22X_2X_4 + 0.17X_3X_4 - 0.51X_1^2 - 0.15\,X_2^2 - 0.51\,X_3^2 - 0.75\,X_4^2 \quad (1)$$

$$P_{Lipid\ production} = 0.63 + 4.445X_1 + 0.023X_2 - 0.025X_3 - 0.016X_4 - 0.052X_1X_2 + 0.017X_1X_3 - 0.025X_1X_4 - 0.014X_2X_3 - 0.047X_2X_4 + 0.024\,X_3X_4 - 0.043X_1^2 - 0.044X_2^2 - 0.053X_3^2 - 0.047X_4^2 \quad (2)$$

$$P_{Lipid\ content} = 10.07 + 0.26X_1 + 0.64X_2 + 0.73X_3 + 0.06X_4 + 0.046X_1X_2 + 0.27X_1X_3 - 1.03X_1X_4 - 0.49X_2X_3 - 1X_2X_4 + 0.37X_3X_4 + 0.1X_1^2 - 0.53X_2^2 - 0.13X_3^2 + 0.66X_4^2 \quad (3)$$

P indicated the prediction values of biomass, lipid production and lipid content affected by X_1-CO_2 (%), X_2-pH, X_3-glucose concentration (g/L) and X_4-light intensity (lux).

3.3.2 Analysis of regression equations and model adequacy

The plots of biomass production graph, lipid production and lipid content of the *C. sorokiniana* DMKU5202-D223 mutant were distributed linearly followed the normal probability plot (Figure 2).

The stability of the variances of the error from the scatter plot between the regression standard residual on the Y axis and the regression predicted value on the X axis revealed that the plots were distributed without any particular

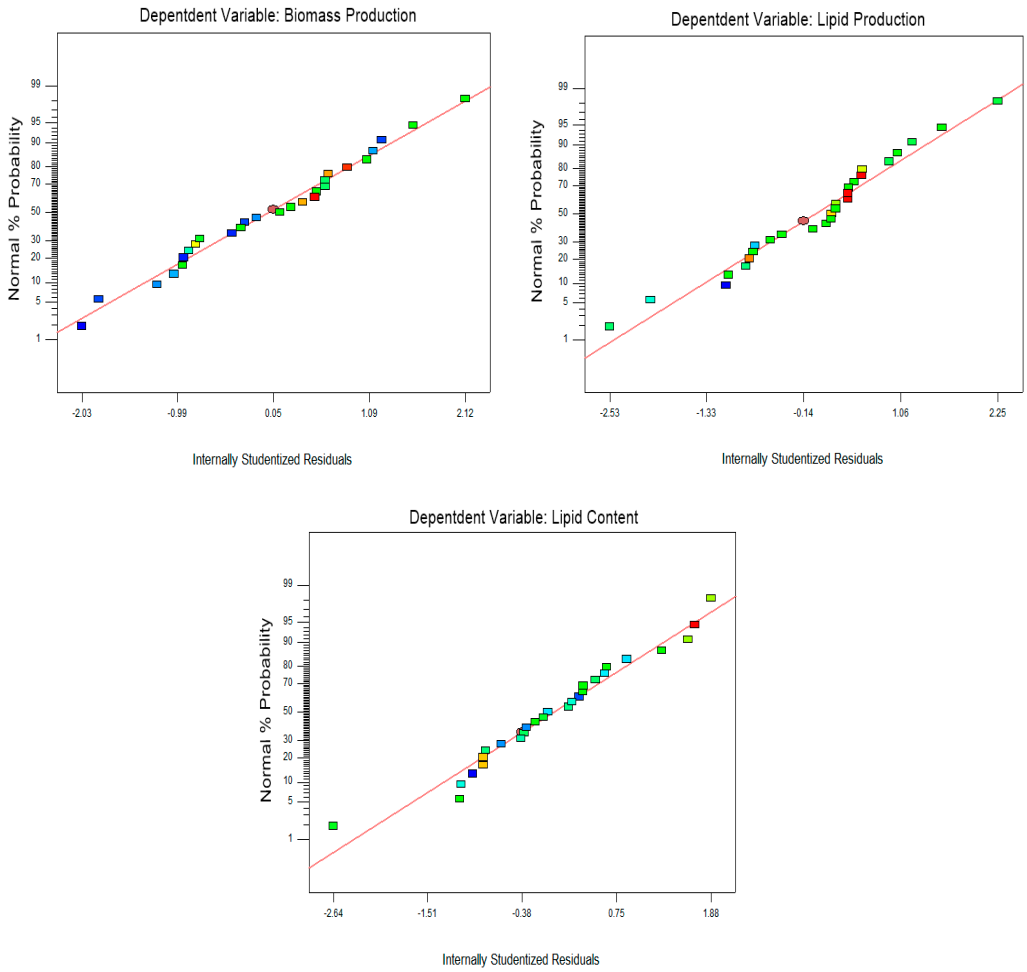

Figure 2. Normal probability plot of biomass production, lipid production and lipid content of the *C. sorokiniana* DMKU5202-D223 mutant.

patterns and scattered around the center line indicated that the variance of the constant error (Figure 3). Therefore, these two plots confirmed the appropriateness of the regression models or the relative equation obtained from the experiment.

Prediction and the actual values of biomass, lipid production and lipid content of the highest *C. sorokiniana* DMKU5202-D223 mutant was shown in Table 2. The experiments 26 composed of 3% carbon dioxide rate, pH 8, 10 g/L glucose concentration and light intensity at 3,000 lux gave the best results. The biomass was 6.43 g/L against the prediction value of 6.30 g/L. The lipid production of the experiment was 0.64 g/L compared to 0.63 g/L of the prediction. In addition the lipid content in the experiment and the prediction were exactly the same at 10.07%. Therefore, the experimental model is consistent according to the examination which was supported by the regression equation of the predict values and the actual experimental data were in good agreement as shown in Figure 4.

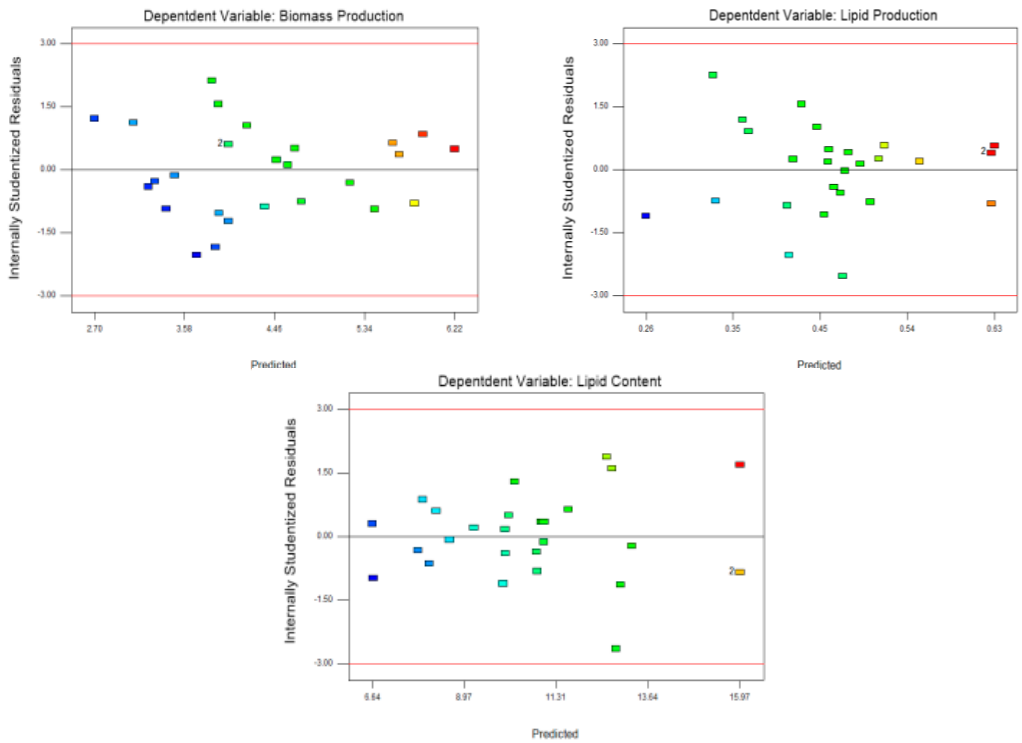

Figure 3. Scatter plot of biomass production, lipid production and lipid content of the *C. sorokiniana* DMKU5202-D223 mutant.

3.3.3 Determination of optimal conditions by using the response surface and contour plot

Figure 5, 6 and 7 showed the response surface plot of selected two factors that had strong significant on the biomass, lipid production and lipid content of *C. sorokiniana* DMKU5202-D223 double mutant strain. In these plots two less important factors were fixed.

The three-dimension response surface plots and contour plots described each independent variable pair of optimize level and their interactions on biomass, lipid production and lipid content could be easily understood [27]. The shape of the compatible contour plots

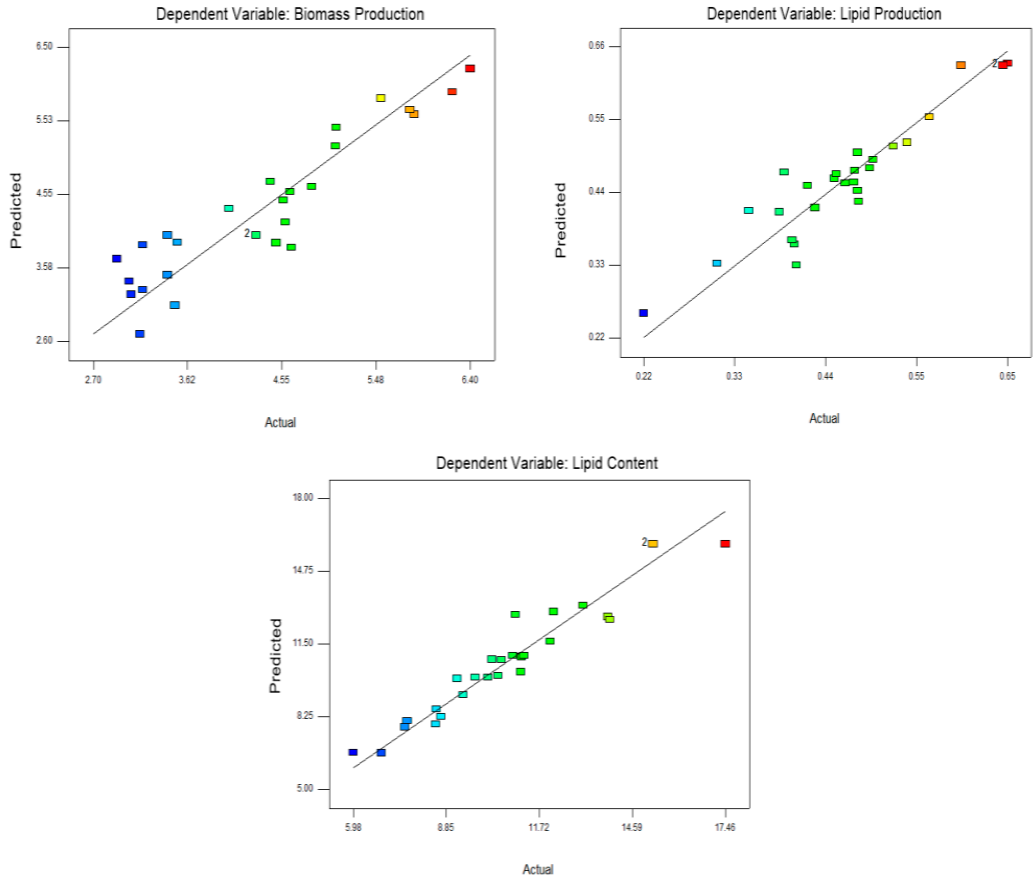

Figure 4. The plot of the predictive values against the actual values from the experiment of biomass, lipid production and lipid content of the *C. sorokiniana* DMKU5202-D223 mutant.

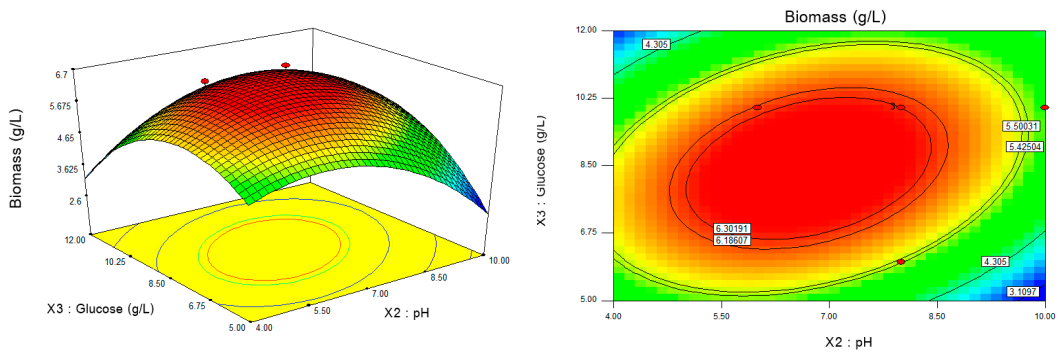

Figure 5. The response surface and contour plot showing the effect of pH (X_2) and glucose concentration(X_3) on biomass by *C. sorokiniana* DMKU5202-D223 with constant CO_2 (3%) and light intensity (3,000 lux).

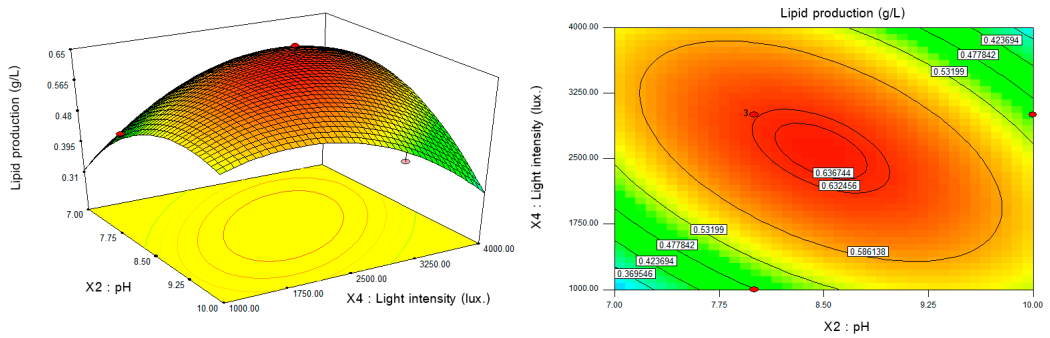

Figure 6. The response surface and contour plot showing the effect of pH (X_2) and light intensity (X_4) on lipid production by *C. sorokiniana* DMKU5202-D223 with constant CO_2 (3%) and glucose concentration (10 g/L).

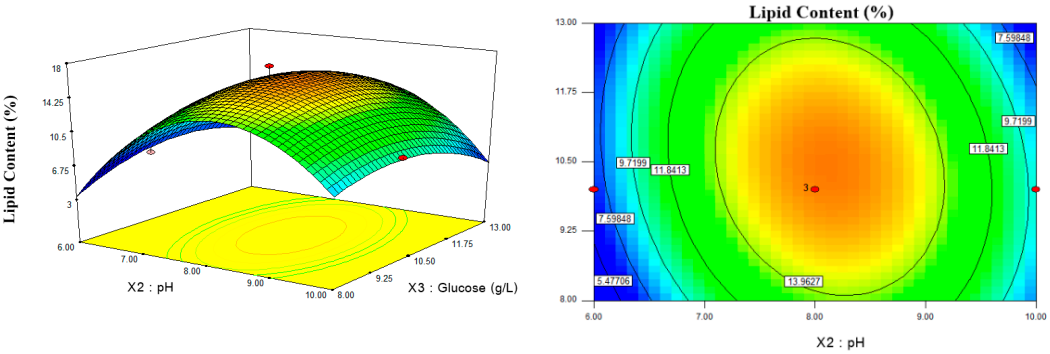

Figure 7. The response surface and contour plot showing the effect of pH (X_2) and glucose concentration (X_3) on lipid content by *C. sorokiniana* DMKU5202-D223 with constant CO_2 (3%) and light intensity (3,000 lux).

implied that the mutual interactions among the independent variables are significant. The independent significant variables were also observed the elliptical figure of contour plots. In this study, the optimal values of CO_2, pH, glucose concentration and light intensity were near center points, respectively.

From the Eq. (1), this model predicted the optimal values of four factors which were most significant variables for biomass were $X_1 = 0$, $X_2 = -1$, $X_3 = -1$ and $X_4 = 0$, corresponding to the actual values of 3% CO_2, pH 7, 8 g/L glucose concentration and light intensity 3,000 lux, respectively. The maximum predicted biomass of *C. sorokiniana* DMKU5202-D223 was 6.32 g/L for 5 days. As for lipid production the optimal levels were $X_1 = 0$, $X_2 = 0$, $X_3 = 0$ and $X_4 = 0$, corresponding to 3% CO_2, pH 8, 10 g/L glucose concentration and light intensity 3,000 lux. respectively. The maximum predicted lipid production was 0.65 g/L for 5 days. On the otherhand the lipid content was maximum at 10.45% under $X_1 = 0$, $X_2 = 1$, $X_3 = 1$ and $X_4 = 0$, corresponding to 3% CO_2, pH 9, 12 g/L glucose concentration and light intensity 3,000 lux. Respectively. Table 4 shown summary of factors that provide the highest response for biomass, lipid production and lipid content of *C. sorokiniana* DMKU5202-D223 mutant.

Table 4. Summary of factors that provide the highest response for biomass, lipid production and lipid content of *C. sorokiniana* DMKU5202-D223 mutant.

Response(Y)	Factors			
	CO_2 (%)	pH	Glucose concentration (g/L)	Light intensity (lux)
Biomass (g/L)	3	7	8	3,000
Lipid production (g/L)	3	8	10	3,000
Lipid content (%)	3	9	12	3,000

Jantasee et al. [15] reported that the mutant of *C. sorokiniana* treated with UV radiation when optimized using KNO_3 0.9 g/L, pH 6.2 and light intensity 4,000 lux yield biomass of 2.58 g/L, lipid production of 1.40 g/L and a lipid content of 54.59% within 5 days under phototrophic cultivation.

The result of this study was not agree with Mayo and Noike [27] who reported that growth rates of *C. vulgaris* was increased by glucose loading rate (25-700 mg/L) but excessive loading rates were effected to the survival of the algae. However Bao Kong et al. [28] confirmed that the growth rate of *C. vulgaris* was not inhibited by higher glucose content which indicated that the tendency of mixotrophic cultivation of *C. vulgaris*.

The statistical design of results showed the optimized culture condition consisted of 3% CO_2, pH 8, 10 g/L glucose concentration and light intensity 3,000 lux. Under these conditions the mutant of *C. sorokiniana* DMKU5202-D223 lipid production was 0.65 g/L for 5 days, equivalent to 0.13 g/L/d, which was much higher than the productivity reported by Rodolfi et al. [29] at 0.044 g/L/d. Similarly the biomass productivity in our experiment at 6.32 g/L in 5 days (equiv. 1.264 g/L/d) was also much better as compared to 0.23 g/L/d from the same report. Although the lipid content in this report was only 10.45% compare to 19.3% of *C. sorokiniana* IAM-212 in BG11 medium under nitrogen deprivation. Table 5 showed the deviation of the experimental data to the predicted values and the errors which was very low and indicated the validity of the model.

Table 6 showed the improvement of lipid production and lipid content of the *C. sorokiniana* DMKU5202-D223 double mutant strain from both UV mutant and the wild type strain. While biomass production was relatively similar. The lipid production of the *C. sorokiniana* DMKU5202-D223 double mutant strain increased as much as 195% as compared to the WT and 20% from original UV mutant. The lipid content of the double

Table 5. The comparison suitable conditions of the predictive value and the actual value from the experiment's biomass production, lipid production and lipid content of the *C. sorokiniana* DMKU5202-D223 double mutant strain.

Response (Y)	Experimental	Predicted	Error (%)
Biomass (g/L)	6.32	6.32	0
Lipid production (g/L)	0.65	0.63	-3.74
Lipid content (%)	10.45	10.28	-1.65

Table 6. The comparison of biomass production, lipid production and lipid content of all 3 *C. sorokiniana* DMKU5202 strains from the study of suitable factor conditions with CCD design.

Isolate	Biomass (g/L)	Lipid production (g/L)	Lipid content (%)
DMKU5202 (WT)	4.59	0.22	4.96
DMKU5202-31 (UV-mutant)	6.48	0.54	8.38
DMKU5202-D223 (double mutant)	6.32	0.65	10.45

mutant strain increase 110% and 24% from WT and UV mutant, respectively. Although the biomass of the double mutant strain increase 38% from WT but decrease 2.4% from the UV mutant. Sarayloo et al. [30] reported that the *C. vulgaris* (UV715-EMS25) mutant strain treated by EMS could increase lipid content 67%, biomass 35% higher than those of the wild type while the lipid production was 91 mg/L/d which was 3.9-fold more than WT.

Therefore, the mutant *C. sorokiniana* DMKU5202-D223 had the lipid production 130 mg/L/d which was much higher than the productivity of *C. vulgaris* (UV715-EMS25) mutant strain.

4. Conclusions

This study improved *C. sorokiniana* DMKU 5202-31UV-mutant strain to *C. sorokiniana* DMKU5202-D223 double mutant strain for high lipid production by using 0.5 M EMS has a survival rate of 41.91%. The selected mutant of *C. sorokiniana* DMKU5202-D223 produced lipid as high as 0.48 g/L, lipid content 11.98% and biomass 4.02 g/L before optimization. The selected four factors namely CO_2, pH, glucose concentration and light intensity were optimized by CCD and further analyzed by RSM. These four factors generated 27 experiments. The experimental data were treated upon multiple regression analysis resulted in second order polynomial equation. The regression model equation for biomass production, lipid production and lipid content (*P*) were:

$$P_{Biomass} = 6.30 + 0.044X_1 - 0.26X_2 - 0.59X_3 - 0.30X_4 - 0.35X_1X_2 + 0.037X_1X_3 + 0.32\,X_1X_4 + 0.16\,X_2X_3 - 0.22X_2X_4 + 0.17X_3X_4 - 0.51X_1^2 - 0.15\,X_2^2 - 0.51\,X_3^2 - 0.75\,X_4^2 \quad (1)$$

$$P_{Lipid\ production} = 0.63 + 4.445X_1 + 0.023X_2 - 0.025X_3 - 0.016X_4 - 0.052X_1X_2 + 0.017X_1X_3 - 0.025X_1X_4 - 0.014X_2X_3 - 0.047X_2X_4 + 0.024\,X_3X_4 - 0.043X_1^2 - 0.044X_2^2 - 0.053X_3^2 - 0.047X_4^2 \quad (2)$$

$$P_{Lipid\ content} = 10.07 + 0.26X_1 + 0.64X_2 + 0.73X_3 + 0.06X_4 + 0.046X_1X_2 + 0.27X_1X_3 - 1.03X_1\,X_4 - 0.49X_2X_3 - 1X_2X_4 + 0.37X_3X_4 + 0.1X_1^2 - 0.53X_2^2 - 0.13X_3^2 + 0.66X_4^2 \quad (3)$$

P indicated the prediction values of biomass, lipid production and lipid content influenced by X_1-CO_2 (%), X_2-pH, X_3-glucose concentration (g/L) and X_4-light intensity (lux).

The equation predicted the biomass, lipid production and lipid content conditions at 6.32 g/L, 0.63 g/L and 10.28% for 5 days, respectively. These experiment models were generated under the culture condition composed

of 3% CO_2, pH 8, glucose concentration 10 g/L and light intensity 3,000 lux after surface response analysis. Under these conditions the mutant of *C. sorokiniana* DMKU5202-D223 the lipid production was 0.65 g/L after 5 days, equivalent to 0.13 g/L/d with lipid content of 10.45 % while the biomass productivity was 6.32 g/L in 5 days (equiv. 1.264 g/L/d). Finally this experiment showed that the lipid production by the mutant of *C. sorokiniana* DMKU5202-D223 could be used for production of biodiesel feedstock.

Acknowledgements

This research was partially supported by Biodiversity-based Economy Development Office Public Organization and National Research Council of Thailand.

References

[1] Antolin G., Tinaut F.V., Briceno Y., Castano V., Perez C. and Ramirez A.I., *Bioresour. Technol.*, 2002; **83**: 111-114. DOI10.1016/s0960-8524-01-00200-0.

[2] Demirbas A., *Energ. Convers. Manage.*, 2003; **44**: 2093-109. DOI 10.1016/s0196-8904-02- 00234-0.

[3] Demirbas A., *Energ. Convers. Manage.*, 2002; **43**: 2349-56. DOI 10.1016/s0196-8904-01-00170-4.

[4] Milne T.A., Evans R.J. and Nagle N., *Biomass*, 1990; **21**: 219-232. DOI 10.1016/0144-4565-90-90066-S.

[5] Minowa T., Yokoyama S.Y., Kishimoto M. and Okakurat T., *Fuel*,1995; **74**: 1735-1738. DOI 10.1016/0016-2361(95)80001-X.

[6] Chisti Y., *Trends Biotechnol.*, 2008; **26**: 126-131. DOI 10.1016/j.tibtech.2007.12.002.

[7] Bhatnagar A., Bhatnagar M., Chinnasamy S. and Das K.C., *Appl. Biochem. Biotechnol.*, 2010; **16**: 523-536. DOI 10.1007/s12010-009-8771-0.

[8] Liang Y., Sarkany N. and Cui Y., *Biotechnol. Lett.*, 2009; **31**: 1043-1049. DOI 10.1007/s10529-009-9975-7.

[9] Singh A., Nigam P.S. and Murphy J.D., *Bioresour. Technol.*, 2011; **102**: 26-34. DOI 10.1016/j.biortech.2010.06.057.

[10] Brennan L. and Owende P., *Sust. Energ. Rev.*, 2010; **14**: 557-577. DOI 10.1016/j.rser.2009.10.009.

[11] Carpenter S.R., *Proc. Nat. Acad. Sci.*, 2008; **105**: 11039-11040. DOI 10.1073/pnas.0806112105.

[12] Pittman J.K., Dean A.P. and Osundeko O., *Bioresour. Technol.*, 2011; **102**: 17-25. DOI 10.1016/j.biortech.2010.06.035.

[13] Chisti Y., *Trends Biotechnol.*, 2008; **26**: 126-131. DOI 0.1016/j.tibtech.2007.12.002.

[14] Xie T., Sun Y., Du K., Liang B., Cheng R. and Zhang Y., *Bioresour. Technol.*, 2014; **118**: 235-242. DOI 10.1016/j.biortech.2012.05.004.

[15] Jantasee W., Yongmanitchai W. and Chonudomkul D., *Kasetsart J. (Nat. Sci.)*., 20015; **49**: 54-66. DOI 2452-316X(0075-5192).

[16] Payer H.D., *Algae Project*, Institute of Food Research and Product Development (IFRPD), Kasetsart University, Thailand, 1971.

[17] Yen Doan T.T. and Obbard J.P., *Algal Res.*, 2012; **1**: 17-21. DOI 10.1016/j.algal.2012.03.001.

[18] Anandarajah K., Mahendraperumal G., Sommerfeld M. and Hu Q., *Appl. Energ.*, 2012; **96**: 371-377. DOI 10.1016/j.apenergy.2012.02.057.

[19] Hu Q., Sommerfeld M., Jarvis E., Ghirardi M., Posewitz M., Seibert M. and Darzins A., *Plant J.,* 2008; **54**: 621-639. DOI 10.1111/j.1365-313X.2008.03492.x

[20] Li Y., Zhou W., Hu B., Min M., Chen P. and Ruan RR., *Biotechnol. Bioeng.*, 2012; **109**: 2222-2229. DOI 10.1002/bit.24491.

[21] Li T.T., Zheng Y.B., Yu L. and Chen S.L., *Biomass Bioenerg.*, 2014; **66**: 204-213. DOI 10.1016/j.biombioe.2014.04.010

[21] Li T.T., Kirchhoff H., Gargouri M., Feng J., Cousins A.B., Pienkos P.T., Gang D.R. and Chen S.L., *Algal Res.*, 2016; **19**: 30-38. DOI 10.1016/j.algal.2016.07.012

[23] Xie M., Qiu Y., Song C., Qi Y., Li Y. and Kitamura Y., *Bioresour. Technol. Rep.*, 2018; **2**: 15-20. DOI 10.1016/j.biteb.2018.03.006

[24] Blight E.G. and Dyer W.J., *Can. J. Biochem. Physiol.*, 1959; **37**: 911-917. DOI 10.1139/059-099

[25] Holub B.J. and Skeaff C.M., *Method. Enzymol.*, 1987; **141**: 234-244. DOI 10.1016/0076-6879(87) 41071-9

[26] Li Y., Cui F., Liu Z., Xu Y. and Zhao H., *Enz. Microb. Technol.*, 2007; **40**: 1381-1388. DOI 10.1016/j.enzmictec.2006.10.015.

[27] Mayo A.W. and Noike T., *Water Res.*, 1994; **28**: 1001-1008. DOI 10.1016/0043-1354(94)90184-8.

[28] Kong W.B., Hua S.F., Cao H., Mu Y.W., Yang H., Song H. and Xia C.G., *J. Taiwan Inst. Chem. E.*, 2012; **43**: 360-367. DOI 10.1016/j.jtice.2011.11.007.

[29] Rodolfi L., Chini Zittelli G., Bassi N., Padovani G., Biondi N., Bonini G. and Tredici R. Mario., *Biotechnol. Bioeng.*, 2009; **102**: 100-112. DOI 10.1002/bit.22033.

[30] Sarayloo E., Simsek S., Unlu Y.S., Cevahir G., Erkey C. and Kavakli I.H., *Bioresour. Technol.*, 2018; **250**: 764-769. DOI 10.1016/j.biortech.2017.11.105.