Effect of glucose concentration and cultivation days on *Chlorella vulgaris* growth via immobilization technique for biodiesel production

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Abstract. Immobilization technique had been utilized to simplify the separation process of microalgae biomass for biodiesel production in the present study. The optimization using response surface methodology (RSM) through central composite design (CCD) approach had been applied to maximize the number of cells growth and minimized the cells loss of *Chlorella vulgaris* cells via immobilization technique. Two effects were optimized by CCD consisting of glucose concentration and cultivation days. The glucose concentration at 23.99 g/L and 7.96 days of cultivation time were found to be the optimum conditions for the maximum number of cells growth (3.30×10^9 cells/mL) and a minimum number of cells lost (1.07×10^4 cells/mL). The optimization using CCD had increased the lipid to 51.6 % and the result of fatty acid methyl ester (FAME) profile is similar to non-bearing oil crop. The findings had revealed the potential of immobilized microalgae biomass as an alternative feedstock for biodiesel production. Moreover, this study had reported optimum conditions for an efficient recovery process via immobilization technique using mixed matrices.

1. Introduction

Recently, microalgae have emerged as a new and alternative bio-energy source for biodiesel production. The potential of microalgae for large scale cultivation had triggered interest for researchers compared to other sources such as soybean, oil palm, coconut oil and sunflower [1]. Nevertheless, harvesting of microalgae is the major challenges in large scale cultivation because of the low density and small size of algal cells (3-30 μm) making it difficult in separation process. Hence, immobilization method using natural or synthetic matrix have been executed to separate microalgae since it is easier in handling the process and consume low energy [2]. Unlike oleaginous crops, microalgae are the least possibility to prompt any food-security problem because of the flexibility of microalgae that can grow on degraded land and salt water [3]. However, having these advantages are insufficient to produce a maximum lipid yield for biodiesel production. Hence, it is crucial to identify and discover the factors that promote the growth of microalgae cells.

Supplementation of nutrients such as glucose, citric acid, vitamin and industrial effluent is one of the important factors in influencing microalgae growth. The addition of these nutrients had resulted in variety responses in lipid accumulation and also served as a carbon sources for the growth of microalgae [2,4]. Glucose had widely been used to increase lipid productivity of microalgae, however, excessive supply of glucose might disturb and limit the microalgae growth [5]. Another important factor in enhancing microalgae growth is the cultivation days. Cultivation or harvesting days play an important role in affecting the growth of microalgae cells. Each microalga species have their specific cultivation time where this period may produce the highest growth rate, biomass and also the lipid content [6]. However, the primary hurdle of microalgae cultivation is the cultivation period which usually takes 10 - 14 days to reach the maximum growth rate which resulted in high electricity and energy consumption. Thus, optimization involving factors to improve the growth and production of lipid is needed to reduce the time and concurrently connecting all factors for an economical biodiesel production.

Up till now, response surface methodology (RSM) have been used and reported to be fast, can avoid the deficiencies brought by one-factor at a time method and complete many sets of experiment simultaneously. The one-factor-at-a-time approach is typically being used even though it is time and energy consuming and regularly leads to results misunderstanding in the optimization process [4]. RSM via central composite design (CCD) approach is an efficient, created better models and generated smaller number of experiments compared to other methods such as Box-behnken and full factorial design. This statistical method had been utilized in variety of applications including the chemical industry, engineering and biological science [7,8]. Several reports had been utilized the application of CCD in optimization of microalgae cultivation process for biodiesel production [6,9]. Nevertheless, most previous study employed the biomass of free cells of *C. vulgaris* for lipid and biodiesel production, but less and rarely found any research focusing on optimizing factors that increased immobilized microalgae growth using CCD until now. Thus, this study was performed to fill the knowledge gap by optimizing two significant factors consists of glucose concentration and cultivation time of immobilized *C. vulgaris* to achieve maximum cell growth and minimum cell lost using CCD. Consequently, the lipid extraction and transesterification of dried immobilized biomass from the validation experiment was exhibited to analyze the fatty acid methyl ester profile of immobilized microalgae.

2. Materials and methods

2.1 Strain, culture media, and stock culture

The *Chlorella vulgaris* 211/11B strain was purchased from the Culture Collection of Algae and Protozoa (CCAP), United Kingdom. The microalgae was cultured in modified Bold's Basal Medium (BBM) with 3-fold nitrogen and vitamins [11]. The microalgae were cultivated in a 250 mL Erlenmeyer flask containing 250 mL sterilized distilled water at room temperature (25–28 °C) with aeration supply at constant pressure. The flask was incubated under a fluorescent lamp (Philip TL-D 36W/865, light output 3050 lm) for 12 days.

2.2 Preparation of immobilized beads

The chemicals including sodium carboxymethyl cellulose (C_{MC}), sodium alginate (S_A) and alginic acid calcium salt (calcium alginate, C_A) were purchased from R&M chemicals and Sigma-Aldrich. 10 mL of microalgae stock culture (approximately $8 \times 10_8$ cells/mL) was mixed with 2% (w/v) of mixed matrices (S_A, C_A and C_{MC}) solution which had been prepared following to ratios 2:1:1 for S_A, C_A and C_{MC} in 5.03 g/L $NaNO_3$ solution. The solution was stirred until it completely dissolved and dropped using a 1 mL micropipette into 1 % (w/v) of $CaCl_2$ solution. After all the beads stabilized for 1 h in $CaCl_2$ solution, it were filtered and rinsed two times with sterilized distilled water. The cultivation of immobilized beads was carried out in 250 mL of modified BBM medium with addition of glucose solution (2 mL) with different concentration in 250 mL Erlenmeyer flask. The flask was supplied with aeration at constant pressure in room temperature (25–28 °C) for 24 hours of photoperiod under a fluorescent lamp (Philip TL-D 36W/865, light output 3050 lm) at different cultivation days. The detail values of coded levels for glucose concentration and cultivation days are presented in Table 1. The pore size image of the immobilized bead was examined using a Scanning Electron Microscope (SEM) (FEI Quanta 450, USA). The immobilized beads were attached on an aluminium plate in order to scan at 2500x magnification in low vacuum mode. The measurement of the beads was repeated three times.

2.3 Optimization using Central Composite Design (CCD)

The RSM experiment was performed through central composite design (CCD) to determine the optimum condition that maximized the number of cells growth and minimized the cells lost to the medium. The Design Expert software, Version 7.1.6 (State-Ease, Inc.) was used in this study and the optimization was executed using two independent variables which were identified as the significant factors for the responses. The independent variables were different glucose concentration added to the medium (22.97 – 24.97 g/L) and cultivation days (6 – 10 days) (Table 1). The experimental results were fitted by regression to a quadratic model as presented in Eq. 1:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \cdot \sum_{j=1}^{n} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y represents the value of the responses, β_o, β_i, β_{ii}, and β_{ij} represent the constant, linear, quadratic and interaction coefficients, respectively. n was denoted as the number of variables, X_i and X_j represents the levels of the independent variables, related to A and B which shown in Table 1. As shown in Table 2, thirteen experiments were performed and the responses were analyzed using Analysis of Variance (ANOVA). The significance level at 95 % with $P < 0.05$ were considered to have significant effect to the number of cells growth and minimum effect to the cells lost of the bead. The experiments were conducted in triplicate for the data analysis.

Table 1 Experimental and levels values of central composite design (CCD)

No	Variables	Coded	Actual values of coded levels					Units
			-2	-1	0	1	2	
1	Glucose concentration	A	22.97	23.47	23.97	24.47	24.97	g/L
2	Cultivation days	B	6	7	8	9	10	Days

2.4 Determination of immobilized microalgae number of cells growth and cells lost

The cells growth of microalgae in immobilized beads were harvested at different days (refer to Table 1 and 2) and dissolved in 1 w/v % of sodium carbonate anhydrous. The absorbance reading of the dissolved beads was measured at 600 nm by using UV-vis spectroscopy (Varian Cary 50 Probe). Concurrently, the absorbance reading of the culture medium was also determined to calculate the cells lost of the microalgae cells into the medium. The number of cells growth and lost were calculated using Neubauer-Improved Haemocytometer (Hirschmann®) cell counting chambers through light microscope (Axiostar plus, Germany) at 5x magnification supported with eye piece camera view (Dino-Eye AM4023X). The absorbance (optical density) versus number of cells growth and cells lost were constructed to determine the calibration curve. All the experiments were carried out in three replication to determine the mean and standard deviation for all the samplings.

2.5 Validation models experiment

The recommended optimum conditions of glucose concentration and cultivation days for maximum cell growth and minimum cell lost obtained from the CCD result were validated through experiments. The predicted and experimental values were compared to confirm the validity of the model. The experiments were performed in triplicate. Subsequently, the dried biomass of immobilized beads was seal in closed vial to further use for lipid extraction.

2.6 Lipid extraction of immobilized microalgae biomass

Approximately 0.03 g of the immobilized microalgae dried biomass was extracted in a screw capped test tubes. In order to lyse the cells, the dried biomass was undergo the pre-treatment method using an ultrasonic (Fisher brand, FB15051) for 15 min at 40 °C in a 5.5 mL distilled water added to the test tubes [12,13]. After that, 12 mL of methanol and chloroform mixture (2:1) was mixed with the biomass and extracted for 24 h at 60–65 °C [14]. Then, the mixture solution was centrifuged at 3000 rpm for 10 min to collect the lipid at the bottom layer of the solution. The solvent of the samples was evaporated using the vacuum oven at 50 °C and the lipid content was measured gravimetrically.

Table 2 Experimental values for optimization of cell growth and cell lost of immobilized *C. vulgaris*

Standard Order	Coded values of variables		No. of cells growth ($\times 10^9$) cells/mL	No. of cells lost ($\times 10^4$) cells/mL
	A	B		
1	-1	-1	2.855	1.156
2	1	-1	2.942	1.092

3	-1	1	2.775	1.039
4	1	1	2.855	1.281
5	-2	0	2.432	1.519
6	2	0	2.444	1.372
7	0	-2	2.440	1.436
8	0	2	2.426	1.479
9	0	0	3.681	0.910
10	0	0	3.545	0.913
11	0	0	3.681	0.982
12	0	0	3.654	0.991
13	0	0	3.545	0.999

2.7 Transesterification process

Transesterification process of lipid obtained from section 2.7 was performed using 4.25 mL of methanol, 5 mL of hexane and 215 µL of HCl (37% vol) added to the lipid. The mixture solution was mixed at 750 rpm and heated to the temperature of 80 – 85 °C for 2 hours. Then, the mixture was cooled down before it centrifuged at 3000 rpm for 10 minutes which resulted into two layers [12]. The upper layer contained the desired product which is the fatty acid methyl ester and the lower layer consisted of the remaining HCl, excess methanol and glycerol. The lipid extraction and transesterification process were repeated three times.

2.8 Fatty acid methyl ester (FAME) composition analysis

The FAME composition of the samples was analyzed using gas chromatograph (Agilent Technologies 7890A) equipped with a flame ionization detector and HP-Innowax column (30 m × 0.25 mm × 0.25 µm). The oven temperature was started at 150 °C for 1 minute and raised to 230 °C at ramping rate 2.9 °C/min and remained constant at this temperature for 30 minutes. The injection temperature was set to 260 °C. The fatty acid compounds consisted of C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 were identified by comparing the peak areas and retention time with standard chemicals. All the analysis was replicated three times.

3. Results and discussion

3.1 Analysis of variance (ANOVA) of optimization using CCD

The successes of immobilized cells are depending on the stability and viability of the cells to increase in size and undergo physiological activities in the matrix [15,16]. These indicators are very important in immobilized cells research to ensure the survival of the microorganisms due to some limitations involving the movement of the cells, diffusion of nutrients towards the inner matrix and bead ruptured problem [17]. Thus, in the present study, two responses were analyzed to solve these impediments which are cell growth in the immobilized bead and cell lost of *C. vulgaris* into the medium.

Through CCD approach, these two main responses can be analyzed simultaneously and rapidly while optimizing the selected independent variables and showing interaction effects between them [3,18]. According to Rakić et al. (2014), CCD was generally chosen for optimization purpose due to the number of required experiments and the quality of the data obtained was better compared to other experimental design (Rakić et al., 2014). Table 2 presents the number of cells growth ranged from 2.426×10^9 to 3.681×10^9 cells/mL and cells lost from 0.910×10^4 to 1.519×10^4 cells/mL of immobilized *C. vulgaris*. The maximum number of cells growth and minimum number of cell lost were achieved at centre points (standard 9 -13) which suggested from the software were at 23.99 g/L of glucose concentration and 7.96 days of cultivation.

The multiple regression analysis was performed to study the relationships between the responses (number of cell growth and cell lost) and the two factors (glucose concentration and cultivation days).

The second order polynomial equations (2) and (3) in terms of coded form was obtained after the software regression analysis of the experimental data as follows:

$$N_{cell\ growth} = 3.57 - 0.016\ A - 0.016\ B - 1.813 \times 10^{-3}\ AB - 0.30\ A_2 - 0.30\ B_2 \quad (2)$$

$$N_{cell\ lost} = 0.94 - 9.624 \times 10^{-3}\ A + 0.013\ B + 0.076\ AB + 0.12A_2 + 0.12B_2 \quad (3)$$

where the $N_{cell\ growth}$ and $N_{cell\ lost}$ were denoted as number of cells growth and number of cells lost, and A and B are represent the glucose concentration and cultivation days, respectively. The A and B are the coded terms of main effects, while AB is referred as the interaction between the factors. The quadratic terms (A_2 and B_2) are shown to demonstrate the existence of curvature in the model.

In Table 3 and 4, the analysis of variance (ANOVA) was shown for the number of cells growth and cells lost. The Model F-values of 41.12 and 21.48 indicated that the model is significant for the number of cells growth and cells lost, respectively. A higher calculated F-value implied that the model is good [8]. Values of "*p*-value" less than 0.05 shows that the model terms were significant. In this study, both of the models show A_2 and B_2 are significant model terms. Meanwhile, the "lack of fit" is not significant with *p*-value 0.059 and 0.0752 for both of the models, signified that the model adequately explained the data in the region of experimentation. The lack of fit test is used to check the adequacy of the model. If the model is not significant, it implies that the parameters had considerable effect on the responses and the proposed model fit the experimental data [20]. By referring to the results, the coefficient of determination, R_2 values obtained were 0.9671 and 0.9953 in which it demonstrated that the model is well fitted for the predicted and experimental data. According to Shamsuddin et al. (2015), a good model was verified through the R_2 value which should be above 80% [21].

Table 3. ANOVA analysis for number of cell growth of immobilized *C. vulgaris*

Source	Sum of Squares	Mean Square	F Value	p-value Prob> F	
Model	3.2040	0.6408	41.1212	< 0.0001	Significant
A-Glucose concentration	0.0030	0.0030	0.1956	0.6716	
B-Cultivation days	0.0032	0.0032	0.2042	0.6650	
AB	< 0.0001	< 0.0001	0.0008	0.9776	
A_2	2.0436	2.0436	131.1447	< 0.0001	
B_2	2.0621	2.0621	132.3275	< 0.0001	
Residual	0.1091	0.0156			
Lack of Fit	0.0891	0.0297	5.9411	0.0590	not significant
Pure Error	0.0200	0.0050			
Cor Total	3.3131				
R-Squared	0.9671				
Adj R-Squared	0.9436				

3.2 Effects of glucose concentration and cultivation days to immobilized C. vulgaris cell growth and cell lost

Figure 1 illustrated the effect of glucose concentration on the number of cells growth and number of cells lost of immobilized *C. vulgaris*. By referring to Figure 1(a), the number of cells was increased as well as the glucose concentration increased due to availability of many carbon sources to support the growth. However, raising the concentration beyond 23.99 g/L had resulted in reduction of number of cells. This result was in line with Kong et al. (2012) that further increased the glucose concentration > 25 g/L will not give significant effect to the biomass content of *C. vulgaris*. Kong et al. (2012) verified that from the experimental data, the *C. vulgaris* cells were not fully consumed the glucose concentration above 25 g/L [4].

Table 4. ANOVA analysis for number of cell lost into the medium of immobilized *C. vulgaris*

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F	
Model	0.5578	0.1116	21.4830	0.0004	Significant

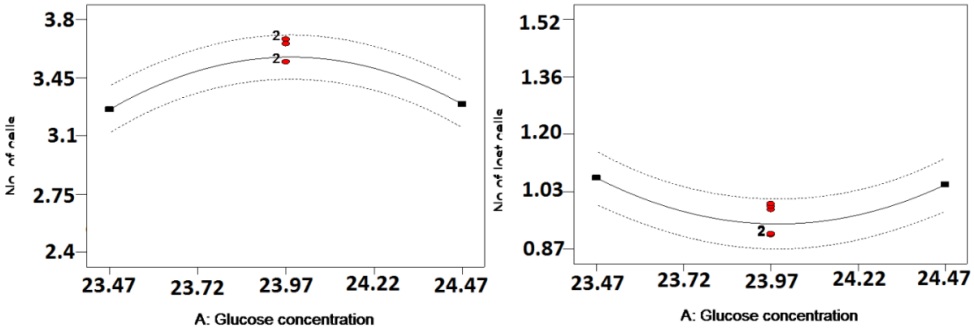
A-Glucose concentration	0.0011	0.0011	0.2140	0.6577	
B-Cultivation days	0.0021	0.0021	0.3985	0.5479	
AB	0.0232	0.0232	4.4694	0.0723	
A_2	0.3325	0.3325	64.0206	< 0.0001	
B_2	0.3498	0.3498	67.3531	< 0.0001	
Residual	0.0364	0.0052			
Lack of Fit	0.0288	0.0096	5.0786	0.0752	not significant
Pure Error	0.0076	0.0019			
Cor Total	0.5942				
R-Squared	0.9388				
Adj R-Squared	0.8951				

Furthermore, excessive glucose supplied might contribute to the bead ruptured problem and increase the cells lost due to the confined space of an immobilized bead which had limited the growth of microalgae cells in the matrix to some extent [13]. This was proven in Figure 1(b) that the cells lost were increased as the glucose concentration exceeded 23.99 g/L. Based on previous report by Lam and Lee (2012), a high concentration of nutrient supply will promote undesirable free cells biomass because of over saturated of microalgae cells in the immobilized bead. The leakage of immobilized cells into the medium may lead to uncontrollable of free cells culture growth [2]. Therefore, to avoid this consequence, the glucose concentration should be considered for optimization of immobilized microalgae cells growth and lost.

Figure 2 shows the effect of cultivation days to the number of cells growth and number of cell lost of immobilized *C. vulgaris*, respectively. By comparing both Figure 1 and 2, a similar trend of curved shapes were observed for the two responses. The number of cells growth were increased as the cultivation days increased and started to decrease after 7.96 days of cultivation period (Figure 2(a)). This phenomenon is related with Figure 2(b) that the cells lost initially increased during cultivation period of more than 7.96 days. The result was comparable to the cultivation days reported in literature, which the maximum cell growth lies between 5 to 10 days of cultivation period before it begin to reduce after day 8 [2,22]. These problems could occur because of medium contamination and instability of the bead due to the over saturated of microalgae cells. The medium started to be cloudy at 8 days of cultivation and this observation proven that the medium was contaminated based on the increasing number of cells lost in Figure 2 (b). Although the microalgae cells entrapped between the matrices bead were protected from the surrounding medium, the probability of the bead to be ruptured is still high because of the pores structure within the matrices as shown in Figure 3. The pores were irregularly distributed on the bead surface with pores size range between 10.8 ± 0.05 and 32.78 ± 0.05 µm. Hence, longer cultivation days would not be suitable for immobilized *C. vulgaris* and the optimum day is equally essential to obtain maximum cell growth without neglecting the cells lost of the immobilized bead.

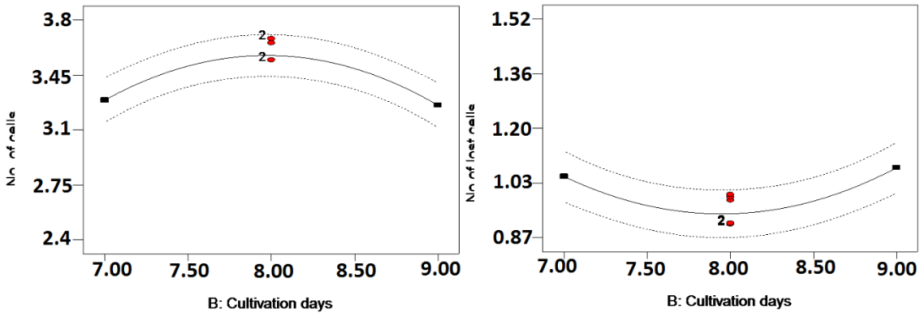
3.3 Effect of interaction factors on the cells growth and cells lost of immobilized C. vulgaris

The response surface plots of interaction between glucose concentration and cultivation days are shown in Figure 4. Figure 4(a) shows the 2D contour plot while 4(b) illustrated 3D view of dome shaped curve which present the relationship between the glucose concentration and cultivation days to the immobilized cells growth. From the figure, the cells growth seems to be increased and achieved the maximum number at 23.99 g/L glucose concentration and 7.96 days of cultivation time, and begins to decrease beyond that number. Figure 5(a) shows 2D contour plot and Figure 5(b) presents 3D view of capsize dome shaped curve with both figures demonstrated the correlation of two main factors to the cells lost of immobilized *C. vulgaris* cells. The same value of optimum points were also obtained for the cells lost response surface plot. Through supplement of glucose to the BBM medium, the growth of the immobilized cells had increased faster compared to the medium without the addition of glucose. This statement had been justified by Abu Sepian et al. (2019) that the same immobilized bead cultured in BBM medium had reached maximum number of cells at 10 days of cultivation time [13]. Thus, by providing certain amount of glucose, the cultivation days had been shortened and this clearly explained the interaction of glucose concentration with cultivation days.



(a) (b)

Figure 1 The effect of glucose concentration (a) to cells growth (b) to cells lost of immobilized *C. vulgaris*



(a) (b)

Figure 2 The effect of cultivation days (a) to cells growth (b) to cells lost of immobilized *C. vulgaris*

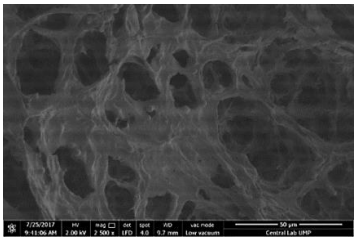


Figure 3 SEM membrane surface images of immobilized microalgae bead.

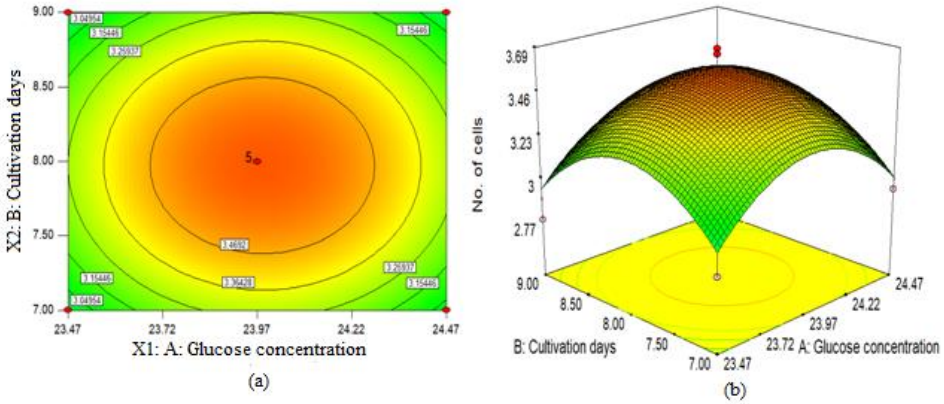


Figure 4 Response surface plot (a) 2D contour (b) 3D view for the effect of interaction between glucose concentration and cultivation days on number of cells growth

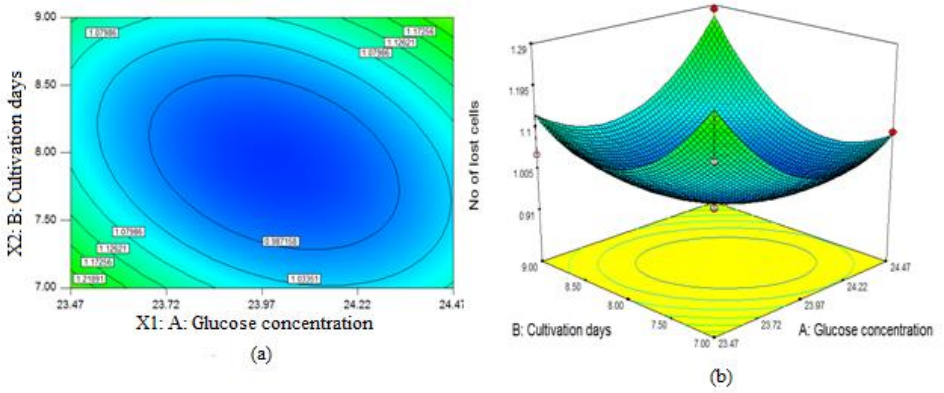


Figure 5 Response surface plot (a) 2D contour (b) 3D view for the effect of interaction between glucose concentration and cultivation days on number of cells lost

3.4 Validation of the models

In order to confirm the adequacy of the model equations on maximum number of cells growth and minimum of cells lost, a validation experiment was carried out in triplicates using the suggested optimized conditions (23.99 g/L glucose concentration and 7.96 cultivation days) obtained from the CCD result. Based on the results, the discrepancies of experimental values were found to be slightly close to the predicted values (number of cells growth 3.57 $\times 10^9$ cells/mL, number of cells lost 0.94 $\times 10^4$) cells/mL) for number of cells growth ranging and number of cells lost from 5.04 – 9.24 % and 5.32 – 8.51 %, respectively. The optimization using CCD had increased 40.8 % of cells growth and decreased 33.6 % of cells lost from the previous experiment using fractional factorial design (FFD) (data not shown). The results from the validation experiment show a good agreement between the experimental and predicted values and this verified the model equations (Eq. 2 and 3).

3.5 Lipid extraction and fatty acid methyl esters (FAME) profile of immobilized C. vulgaris biomass

The main purpose of cultivation parameters optimization using CCD is to obtain a maximum cells growth with minimum cells lost which produced a high lipid percentage for biodiesel production. The dried immobilized biomass from the validation experiment (Section 3.4) was used and higher percentage of lipid, 51.56 % was achieved compared to 47.7 % from previous extraction result (data not shown). Thus, it is verified that using CCD as an optimization tool can improved the production of lipid of

immobilized microalgae biomass.

The fatty acid methyl ester composition of immobilized *C. vulgaris* is shown in Table 5. There are six main FAME compounds existed after transesterification process of immobilized *C. vulgaris* which consisted of C16:0 (palmitic acid methyl ester), C16:1 (palmitoleic acid methyl ester), C18:0 (stearic acid methyl ester), C18:1 (oleic acid methyl ester), C18:2 (linoleic acid methyl ester) and small amounts of C18:3 (linolenic acid methyl ester). As shown in Table 5, the extracted FAME contained greater percentage of unsaturated fatty acid (UFA) which 14.03 % higher than saturated fatty acid (SFA). This result was contradicted with Lam and Lee (2012) and Abu Sepian et al. (2019) which obtained higher SFA (> 60 %) than UFA (< 40 %) for extraction of immobilized *C. vulgaris*. However, the result was similar with most previous studies that the fatty acid profile of *C. vulgaris* consists of more UFA (> 60 %) than SFA (< 40 %) [6,10,20,23]. Thus, it revealed the efficient of optimization through CCD to increase the FAME percentage specifically the SFA of immobilized *C. vulgaris* biomass. It is noted that high content of unsaturated FAME can decreased the pour point of biodiesel which allowed it to be utilized in cold weather countries [23]. A high proportion of saturated and monounsaturated fatty acids (C16:1 and C18:1) are minimal point for a high quality biodiesel. Meanwhile, polyunsaturated fatty acids (C18:2 and C18:3) are suitable sources for health products and it have an excellent cold-flow properties but more exposed to oxidation [20]. According to European regulation (EN 14213 and EN14214), a good quality of biodiesel depends on the concentration of linolenic acid (not exceed 12%) due to the two bis-allylic locations at C-11 and C-14 of the carbon chains are more susceptible to oxidation [6]. In the present study, the percentage of C18:3 is less than 12 % and the C18:1 is slightly higher compared to other FAME which indicate a realistic balance of fuel properties [23]. Hence, the biofuel derived from the immobilized *C. vulgaris* able to meet the requirement of European regulation for vehicle use; thus have the potential as a good candidate for biodiesel production.

Table 5 FAME compositions of immobilized *C. vulgaris*

FAME	Amount of fatty acid (mg/g dw)
C 16:0	7.57
C 16:1	8.08
C 18:0	8.38
C 18:1	9.08
C 18:2	8.63
C 18:3	4.21
SFA (%)	34.72
UFA (%)	65.26

4. Conclusion

The optimization through central composite design (CCD) approach had been performed in this study to increase the number of cells growth and minimized the cells lost of immobilized *Chlorella vulgaris* for lipid and biodiesel production. Two factors including glucose concentration and cultivation days were examined. The glucose concentration at 23.99 g/L and 7.96 days of cultivation time were found to be the optimum conditions. The number of cells growth approximately 3.30×10^9 cells/mL and the number of cells lost (1.07×10^4 cells/mL) were obtained from the optimization experiment. Interestingly, the lipid percentage was increased to 51.56 % compared from previous study (47.7 %) and the fatty acid profile of transesterification process revealed the potential of immobilized *C. vulgaris* as a feedstock for biodiesel production. Furthermore, this study had exposed the benefit of CCD in optimization of microalgae cultivation system for biodiesel production.

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