Korean J. Chem. Eng., 33(9), 2575-2581 (2016)
DOI: 10.1007/s11814-016-0101-y

pISSN: 0256-1115
eISSN: 1975-7220

Optimization of algal lipid extraction by mixture of ethyl acetate and ethanol via response surface methodology for biodiesel production

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(*Received 11 January 2016 • accepted 14 April 2016*)

Abstract−The effects of extraction time, extraction temperature, solvent to biomass ratio and solvent composition on lipid yield from lyophilized *Chlorococcum* sp. biomass using a mixture of ethyl acetate and ethanol (EAE), a new proposed solvent, were studied. Subsequently, the process conditions of extraction by EAE were optimized using Box-Behnken design (BBD). The results revealed that the extraction temperature had the greatest effect on lipid extraction efficiency, followed by volume ratio of ethyl acetate to ethanol (EA/E) and extraction time. The largest lipid extraction yield of 15.74% was obtained under the following extraction conditions: 40 mL solvents per gram of biomass for 270 min with gentle stirring at 80 °C by EAE with an EA/E of 1.0. Furthermore, palmitic acid, stearic acid, oleic acid, and linoleic acid were the most abundant fatty acids in the lipids extracted, indicating the great potential of the proposed lipid extraction procedure for microalgae-based biodiesel production.

Keywords: Biomass, Lipids, Microalgae, Biodiesel

INTRODUCTION

The escalating prices of the irreversible fast depleting fossil energy and the impact of global warming generated by the combustion of fossil fuel have become issues of worldwide concern and lead to the search the alternative fuels for engines [1,2]. Compared with other renewable energy, such as wind, solar and tidal, biofuels, microalgal biodiesel are generally considered to be one of the most promising renewable energy for the substitutes of conventional diesel for industry and transportation, as the biodiesel has the advantage of being stored easily and can be used after blending with petroleum diesel without any alteration of the engine [2,3].

Currently, vegetable oil, animal fats, and waste cooking oil are the major feedstocks for biodiesel production. However, conventional feedstocks of biodiesel are not sustainable, and thus difficult to meet the growing consumption demand. Microalgae can be produced year-round and therefore have much higher lipid productivity than land plants; some species can accumulate up to 20-50% triacylglycerol [4]. Some species can even grow in contaminated and wastewater, saline water, non-arable land and use flue gas as a carbon source, providing a further advantage compared to traditional biodiesel feedstocks [5-8]. Therefore, microalgae-based biodiesel has become a hot research subject in recent years [9,10].

The general steps for microalgae based biodiesel production consist of algae cultivation and harvesting, cell disruption, lipid extraction and transesterification [11,12]. Among which, lipid extraction is one of the most energy intensive processes and the chemicals involved may cause health and safety issues. Lipid extraction with chloroform and methanol is the most efficient method, and thus these are the most widely utilized solvents for algal lipid extraction. However, chloroform is a type of carcinogenic chemical and methanol causes harmful effects to the retina, which is contrary to the demand for bio-compatible and less toxic solvents. To overcome the disadvantages of chloroform and methanol, Halim [13,14] and Cheng [15] examined the performance of supercritical carbon dioxide ($SCCO_2$) in the extraction of lipids from marine *Chlorococcum* sp. and *Pavlova* sp. respectively. Hideki Kanda [16] extracted lipids from several species of natural blue-green microalgae using dimethyl ether as a solvent, achieving lipid yield comparable to the Bligh and Dyer's method. However, the above-mentioned methods usually operate at high pressure, which is expensive and has its own safety issues. Another traditional lipid extraction process with high volume of hydrophobic organic solvent, such as hexane, resulted in low lipid extraction yield; the water molecule could hinder the contact between solvent and lipid [13]. The routine uses of lab-scale extraction process to determine microalgal lipid content are not well understood, and the variables affecting lipid extraction from microalgae are not well studied. An ideal lipid extraction process for microalgal biodiesel production in commercial scale needs to be lipid specific towards desirable lipid fractions, be directed to

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reduce the co-extraction of non-lipid contaminants and increase operational safety.

In a previous study, we proposed a mixture of ethyl acetate and ethanol (EAE) as the extraction solvent to extract lipids from *Chlorella* sp. followed by a primary study on the effect of extraction process parameters on lipid extraction efficiency [17]. However, the correlation of different variables and optimum extraction conditions has not been determined, which is crucial for cost-effective scaling up of the proposed process. Furthermore, evaluation of the feasibility of lipid extraction from other species has not been fully confirmed.

Therefore, the objective of this work was to identify an optimal range of EAE extraction conditions, namely extraction time, extraction temperature, ethyl acetate to ethanol (EA/E) and solvent to biomass ratio through single-factor experiments. The optimization of lipid extraction by the mixed solvent using response surface methodology (RSM) from lyophilised biomass of *Chlorococcum* sp. was conducted.

MATERIALS AND METHODS

1. Microalgae Cultivation

The *Chlorococcum* sp. strain obtained from the Guangzhou Institute of Energy Conversion (GIEC), Chinese Academy of Sciences (CAS) was first inoculated in 100 mL Erlenmeyer flasks containing modified BG-11 culture medium. The culture medium was composed of $NaNO_3$ (1.5 g·L^{-1}), $K_2HPO_4{\cdot}3H_2O$ (40 mg·L^{-1}), Na_2CO_3 (20 mg·L^{-1}), $CaCl_2{\cdot}2H_2O$ (36 mg·L^{-1}), $MgSO_4{\cdot}7H_2O$ (75 mg·L^{-1}), EDTA (1 mg·L^{-1}) and $(NH_4)5Fe(C_6H_4O_7)_2$ (4 mg·L^{-1}), citric acid (6 mg·L^{-1}) and 1 mL·L^{-1} of trace elements solution consisting of $MnCl_2{\cdot}4H_2O$ (1.81 mg·L^{-1}), H_3BO_3 (2.86 mg·L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.39 mg·L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.08 mg·L^{-1}), $Co(NO_3)_2{\cdot}6H_2O$ (0.05 mg·L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.22 mg·L^{-1}). The seeds were inoculated in photobioreators (PBRs, 1.3 L glass photobioreactor, Φ6×70 cm) containing 1,000 mL BG-11 medium. After inoculation, the cultures were sparged with mixed gas of air and CO_2 (3-5%, vol./vol.) through the bottom of the PBRs, and maintained at temperature of 23±1 °C as well as a continuous illuminating intensity of 3,000±10 lux. The total cultivation time was 15 days.

2. Lipid Extraction by EAE

For lipid extraction, approximately 20 mg of vacuum dried (–80 °C) algal biomass and a predefined volume of EAE were taken and placed in a 10 ml glass centrifuge tube inside a temperature-controlled shaker (Yuhua instrument, China) and stirred by a magnetic bar. Then the mixture was cooled, and 1 mL hexane and 2 mL distilled water were added to form two phases and to separate lipids fraction. Finally, the liquid and solid phases were separated by centrifugation (4,000 rpm, 10 min, Eppendorf, Germany). The upper phase rich in lipids was transferred to a pre-weighted flask and subjected to N_2 stream to remove solvents. The lipid yield (%, w/w) was recorded by percentage of lipid based on dry weight of algal biomass [18].

3. Experimental Design

First, the effect of extraction process variables, including extraction time, extraction temperature, solvent to biomass ratio and EA/E was studied in order to obtain an optimum range of each variable. Then, the extraction process variables were optimized using RSM. For the RSM experiment, a total of 17 experimental runs of three independent variables was designed with Box-Behnken design (Box and Behnken, 1960) using the Design-Expert software (Version 8.0.6.1, Stat-Ease, Inc., USA). The lipid yield was taken as the response of the design experiment. Regression analysis was performed for the experimental data, and the following equation was expressed by an empirical second order polynomial model (Eq. (1)):

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response; β_0 is a constant; β_i is the linear coefficient; β_{ii} is the quadratic coefficient; β_{ij} is the interaction coefficient of the variables i and j; X_i and X_j are independent variables.

Finally, lipid extraction experiments were carried out under the optimal extraction condition obtained by the RSM predictive equation. The validity of the mathematical model was verified by comparing the experimental data and predicted values.

4. Analysis of Fatty Acid Composition

To evaluate the potential as feedstock for biodiesel production, extracted lipids were methylated as described in the literature with minor modifications [19]. In summary, approximately 20 mg of freeze-dried biomass sample was weighed and introduced into a glass tube, 2.5 mL of methanol with 2 wt% of concentrated sulfuric acid were added as the catalyst. The screw capped glass tubes were incubated at 80 °C in a water bath for 2.5 h, using magnetic bars for mixing. The mixtures then were cooled to room temperature. After that, 2 mL of hexane and 1 mL of saturated NaCl aqueous solution was added to extract and purify the obtained fatty acid methyl esters (FAMEs). Finally, the mixture was subjected to a vortex oscillator (Dragon Lab, Dragon Laboratory Instruments Limited, Beijing) for 30 s to ensure thorough mixing followed by centrifugation (3,000 rpm, 17 min, Shanghai Centrifuge Institute Co. LTD.) to form two phases. The upper phase rich in FAMEs was then filtered to a clean bottle and dried under N_2 stream. The filtered FAMEs samples were used for composition and content analysis, which was carried out by gas chromatography equipped with an FID detector (Shimadzu GC-2010, Japan) with a DB-Wax column of length 30 m, ID 0.25 mm, film 0.25 μm (Agilent Technologies, Inc.). The injector and detector temperature were 300 and 250 °C, respectively. The time-temperature was programmed at 190 °C for 5 min, and then elevated by 10 °C/min to 250 °C for 7 min. Methyl hexadecanoate (C17:0) was used as internal standard and Ar as the carrier gas. FAMEs were identified and quantified by comparing their retention time with those of the authentic standards (Sigma) and the internal standard concentration, respectively.

5. Statistical Analysis

All the experiments were conducted in duplicate and the average values as well as standard deviations were reported as the final results. Data analysis was performed with EXCEL (Microsoft Office Enterprise, 2003) and Origin Pro 8.6 (Origin Lab, USA) and analysis of variance (ANOVA) was determined wherever applicable.

RESULTS AND DISCUSSION

1. Single-factor Experiments

It is well known that lipid extraction from microalgae can be

considered to be a solid-liquid extraction process. According to previous studies, it is affected by several typical variables, such as extraction time, extraction temperature, solvent to biomass ratio and characteristics of extraction solvent. Therefore, a single-factor experiment was carried out to evaluate various process variables on the lipid extraction efficacy in order to screen the parameters of significance and obtain their optimal range to achieve the highest yield of lipid. The three variables of interest were extraction time, extraction temperature, and EA/E. The results of 17 runs using RSM are shown in Table 2, which include the design, observed responses and the predicted values. A close agreement between experimental and predicted values was found. In addition, it was observed that the highest lipid extraction yield of 15.74% was obtained under the optimum extraction conditions.

1-1. Effect of Extraction Time on Lipid Yield

Microalgal lipid extractions were carried out at different times (30, 60, 90, 150, 210 and 270 min) while other conditions were set as follows: extraction temperature 80 °C, EA/E 1.0 (v/v) and solvent to biomass ratio 10 : 1 (v/m). The effect of extraction time on lipid yield is plotted in Fig. 1(a). From Fig. 1(a), it can be observed that lipid yield increased rapidly with extended extraction time from 30 min to 90 min. After 30 min lipid yield was calculated 6.98%, while extraction time was extended to 90 min, the amount of lipid was recorded at 13.87%. The highest amount of lipid was measured to be 16.79% at 210 min. This might be due to the time requirement of the exposure of the solute to the release medium where the liquid entered into the dried microalgae cells, dissolved the solute and subsequently diffused out from the cells. Another possible reason for the increase in microalgae lipid yield with an extraction time would possibly due to longer contact time between lipids and solvent, which had a positive effect on lipid yield. However, after 210 min extending the period of extraction could lead to slight lipid yield reduction. According to Gan [20], this occurrence might be due to the applied extraction temperature, which degrades the antioxidant of microalgae cells at the extended extraction period, thus loses the phenolic activity for releasing of lipid compound. Longer incubation time in high temperature, however, can cause the degradation of sensitive bioactive compounds in the microalgal cell [21].

1-2. Effect of EA/E on Lipid Yield

It is widely recognized that a combination of a polar and a nonpolar solvent could result in an increase in lipids' extraction efficiency than that of single solvent [22]. Our previous work compared the lipid yield of ethyl acetate, ethanol, and a mixture of ethyl acetate and ethanol at 1 : 1 (v/v) [17], which were in accordance with the traditional extraction method proposed by Bligh and Dyer,

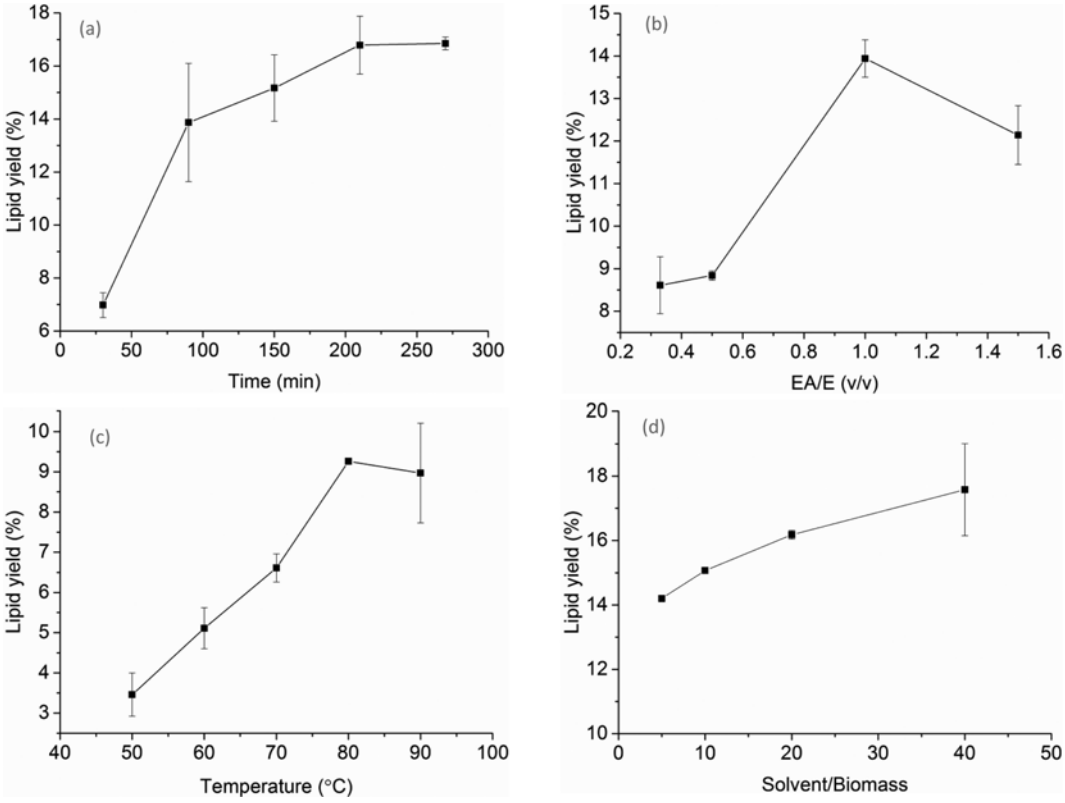

Fig. 1. Effect of process variables on the lipid extraction yield (a) extraction time. The experiments were performed under the following conditions: algal biomass, approximately 20 mg; The extraction temperature, 80 °C; EA/E, 1.0; Solvent to biomass ratio, 10 : 1; (b) EA/E. The experiments were performed under the following conditions: algal biomass, approximately 20 mg; Extraction time, 210 min; Extraction temperature, 80 °C; Solvent to biomass ratio, 40 : 1; (c) Extraction temperature. The experiments were carried out in the following conditions: algal biomass, approximately 20 mg; The extraction time, 30 min; EA/E, 1.0; Solvent to biomass ratio, 10 : 1; and (d) Solvent to biomass ratio. The experiments were performed under the following conditions: algal biomass, approximately 20 mg; The extraction time, 210 min; The extraction temperature, 80 °C; EA/E, 1.0.

in which a mixture of chloroform, methanol and water resulted in a much higher lipid extraction than that of single components. Extractions by different compositions of mixed solvents, i.e. 0.33 (v/v), 0.5 (v/v), 1.0 (v/v), 1.5 (v/v) in terms of volume ratio of ethyl acetate and ethanol were conducted and results were illustrated in Fig. 1(b), while other conditions were set as follows: extraction temperature 80 °C, extraction time 210 min and solvent to biomass ratio 40 : 1 (v/m). As shown in Fig. 1(b), no obvious variation of lipid yield was recorded as EA/E increased from 0.33 (v/v) to 0.5 (v/v). The highest lipid yield (13.94%) was found when the EA/E value was increased to 1.0. However, the lipid yield decreased to 12% as the EA/E raised to 1.5. This phenomenon can be attributed to the higher penetration capacity of the mixed solvent with a volume ratio of 1 : 1 (v/v) through the cell membrane into the cytoplasm, where it can interact with the lipid complex [10].

1-3. Effect of Extraction Temperature on Lipid Yield

To determine the optimal range of extraction temperature, temperatures of 50, 60, 70, 80, and 90 °C were employed in the extraction experiment. Other conditions were set as follows: extraction time 30 min, EA/E 1.0 (v/v), and solvent to biomass ratio 10 : 1 (v/m). As shown in Fig. 1(c), lipid yield increased steadily with increasing extraction temperature and reached maximal value of 9.5% at 80 °C, while lipid yield was recorded at 3.45%, 5.11% and 6.61% at 50 °C, 60 °C and 70 °C, respectively. However, lipid yield decreased when the extraction temperature exceeded 80 °C. According to Shi [23], higher temperature would cause softening of the plant tissue, disrupting the interactions between phenolic compounds and lipid or protein or polysaccharides and increasing the rate of diffusion, thus giving a higher rate of extraction. While the reduction in lipid yield when the extraction temperature exceeded 80 °C for long incubation time (270 min) could be attributed to the degradation of pigments such as chlorophyll, carotenoid and xanthophyll dissolved in the mixed solvent.

1-4. Effect of Solvent to Biomass on Lipid Yield

Fig. 1(d) presents the effect of solvent to biomass on lipid extraction yield. Extraction experiments were carried out at the solvent to biomass at a range of 5 (v/m) to 40 (v/m); while extraction time, EA/E and extraction temperature were set at 210 min, 1.0 (v/v) and 80 °C, respectively. It can be clearly observed that lipid yield increased proportionally with the addition of solvent quantity. Lowest lipid yield (14.2%) was recorded at low solvent 5 (v/v) treatment and highest lipid (17.58%) was recorded at high solvent 40 (v/v) treatment. This is likely due to an increase in concentration gradient between the biomass and solvent and thus an increase in mass transfer efficiency. Although an increase in solvent to biomass ratio resulted in higher lipid extraction yield, excess amount of solvent consumed is not cost-effective for lipid extraction, as the separation of lipid and solvent is an energy intensive process.

Consequently, an extraction time between 150-270 min, extraction temperature 70-90 °C, and EA/E 0.5-1.5 were selected for RSM experiments based on results from the single-factor experiment.

2. Optimization of Extraction Process by RSM

2-1. Model Development

Table 1 illustrates the independent variables and the corresponding levels in actual and coded factor for the RSM experiment. The RSM experimentally-obtained results and statistically-predicted values are presented in Table 2. Each experimentally-obtained value is the average of duplicate independent runs. It can be observed the response varied greatly from 5.41% to 15.33%. Moreover, the response obtained allows us to determine a typical quadratic model (Eq. (2)) expressed in terms of code factors using multiple regression analysis.

$$Y=15.04X_1+1.47X_2+1.54X_1X_2+0.14X_1X_3+0.08X_2X_3+0.83X_1^2-1.97X_2^2-5.35X_3^2 \quad (2)$$

where Y is the lipid yield, X_1, X_2 and X_3 are the coded variables for extraction time, extraction temperature and EA/E, respectively.

The maximum extraction yield of 16.01% was found from the fitted model under the experimental conditions: extraction time 270 min, extraction temperature 80 °C, and EA/E 1.0 (v/v) on the basis of the fitted model.

The quality of the models developed can be evaluated based on the correlation coefficient R^2 and also on the lack-of fit value [24].

Table 1. Factors and levels for response surface methodology, BBD matrix (in actual and coded levels of three variables)

Process variables	Coded symbols	Levels		
		−1	0	+1
Extraction time (min)	X_1	150	210	270
Extraction temperature (°C)	X_2	70	80	90
EA/E	X_3	0.5	1.0	1.5

Table 2. Experimental runs and corresponding results regarding BBD

Run	X_1/min	X_2/°C	X_3	Lipid yield, %	
				Experimental value	Predicted value
1	150	80	0.5	8.98	8.16
2	210	80	1	15.54	15.04
3	150	80	1.5	9.93	9.72
4	210	80	1	16.65	15.04
5	210	70	1.5	7.20	7.09
6	150	90	1	14.62	15.33
7	270	90	1	14.25	13.93
8	270	70	1	14.77	14.02
9	210	80	1	14.06	15.04
10	270	80	1.5	10.84	11.66
11	210	90	0.5	8.09	8.20
12	210	70	0.5	4.91	5.41
13	210	80	1	14.86	15.04
14	210	80	1	14.10	15.04
15	210	90	1.5	10.70	10.20
16	150	70	1	8.99	9.31
17	270	80	0.5	9.35	9.56

Note: The total lipid in the microalgae biomass was determined to be 16.64±1.27% according to the Bligh and Dyer method [26], all experiment data presented in the table were average value of duplicate experiments

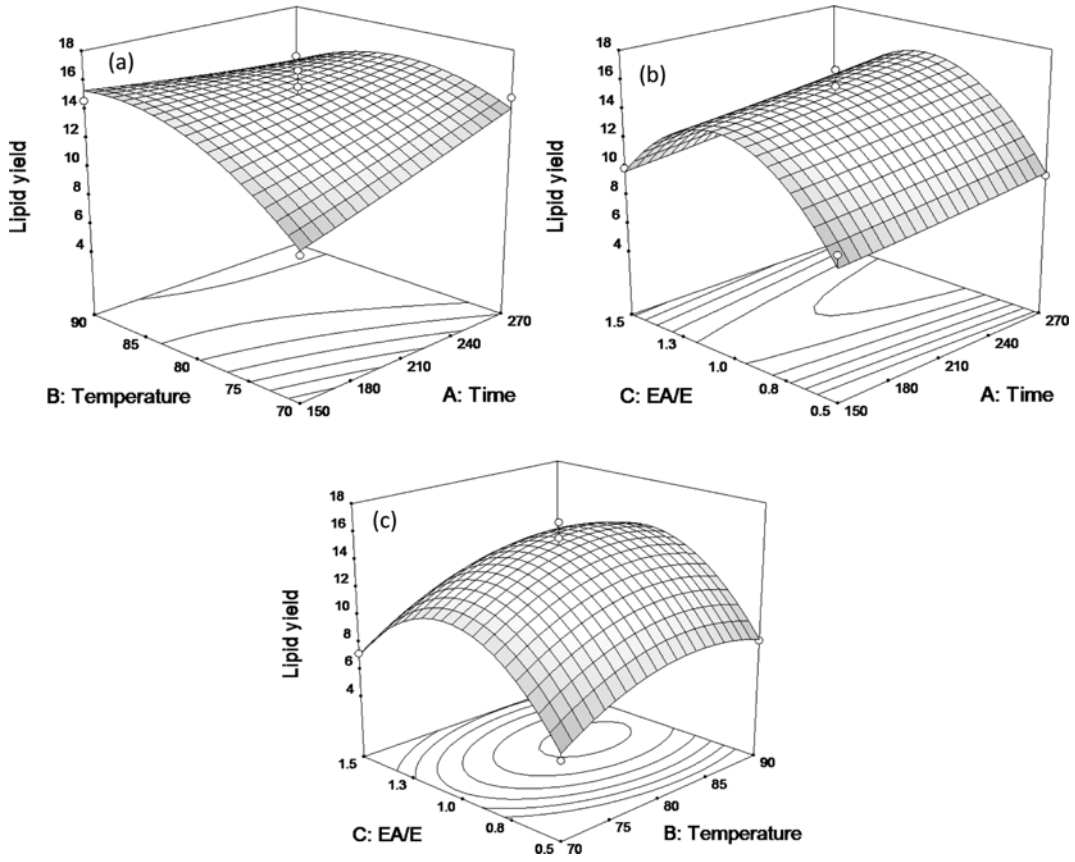

Fig. 2. Response surface plots (3D) and contour plots (2D) showing the mutual effect of (a) extraction temperature and time; (b) EA/E and extraction time; (c) EA/E and extraction temperature on the lipid extraction yield.

Table 3 illustrates the regression data for lipid yield for the developed predictive model. The determination coefficient (R^2=0.9582) by analysis of variance (ANOVA) of the fitted model, implies that only 4.18% of the total variation could not be explained by the model. The value of the adjusted determination coefficient (Adj R^2=0.9045) also verifies the high significance of the model, indicating acceptable deviations between the experimental value and the predicted data. Moreover, the fitness of the model was studied through lack of fit test. The *F* value of 0.9 and *p* value of 0.5138 confirm the validity of the model . Thus the model is adequate for prediction in the range of experimental variables. Finally, the model p-value was only 0.0005, much less than F value (17.84), indicating that the model terms were significant. As shown in Table 3, the linear coefficient indicated that the extraction temperature (X_2) was the most significant independent variable impacting on lipid yield with a p-value less than 0.05 [25]. The higher the extraction temperature was, the greater lipid yield. The EA/E (X_3) also exerted a positive individual influence on the extraction yield. This implies that an increase in the EA/E could improve the lipid extraction. In addition, the extraction temperature and EA/E exerted significant quadratic effects (Table 3). However, other terms (X_1X_3, X_2X_3) were statistically insignificant, suggesting an absence of interaction between the variables (p>0.05). To understand the mutual effect of the corresponding parameters, the regression model was represented in terms of response surface plots (3D) and contour plots (2D) in Fig. 2. Fig. 2(a) illustrates the combined effects of the extraction time and temperature on the extraction yield. It shows that the interaction between the two selected variables has little influence on the extraction yield. With regard to the correlation between extraction time and EA/E (Fig. 2(b)), it is apparent that the lipid extraction efficiency is dramatically enhanced as the EA/E increases up to 1.0 (v/v) and then decreases rapidly with further increments of this value at a given extraction time. However, only a slight improvement in lipid extraction yield could be seen with greater extraction times, while keeping EA/E volume constant. Moreover, no significant interactions were evident between these two variables, as demonstrated by its *p*-value of 0.8069. Whereas, the interaction between extraction temperature and the EA/E had significant effects on the extraction yield, as illustrated in Fig. 2(c). The maximum predicted value indicated by the surface is confined by the smallest ellipse in the contour diagram. The smallest ellipse on the contour plot indicates that there is a perfect interaction between the independent variables. As shown, the lipid yield increased with an increase extraction temperature up to about 80 °C, followed by a slight decrease at higher temperature. The lipid yield increased with an increase in percentage of ethyl acetate of 50%, followed by a sharp decrease at higher ethyl acetate addition.

2-2. Validation of the Model

According to the above results, the optimum extraction conditions were obtained as follows: extraction time (270 min), extraction

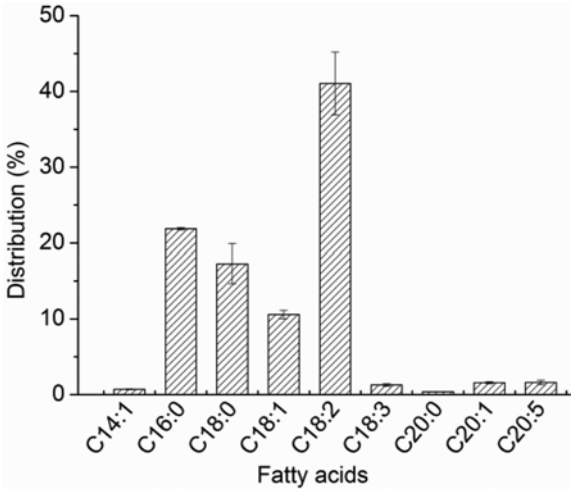

Fig. 3. Fatty acid profile of lipids extracted by EAE.

temperature (80 °C) and EA/E (1.0). The predicted extraction yield obtained from *Chlorococcum* sp. was 16.01% of the dry weight under the optimum conditions, according to the model. To confirm these conclusions, extraction experiments based on the optimal extraction parameters were performed and the extraction yield was determined. The experimental value was 15.74% of the dry weight, which shows good agreement with the predicted value calculated by the model equation (16.04%). Moreover, the verification experiments also proved that the predicted values of lipid yield from the fitted model could be satisfactorily achieved within a 95% confidence interval of experimental values and consequently demonstrating the RSM model is satisfactory.

3. Fatty Acids Profile of Extracting Lipids

The fatty acid profile is an indicator of the suitability of algal lipid for use as a biodiesel feedstock [2]. It is worthwhile to highlight that different extraction solvents not only could lead to different lipid yields, but also affect the fatty acid composition of lipids. It is of great importance to characterize the fatty acid profiles of lipid extracted for the potential evaluation as feedstock for biodiesel production. Fig. 3 presents the fatty acid distribution of lipid extracted at the optimum conditions. It can be observed that linoleic acid (C18:2) was the most abundant component, which was at a proportion of 41.05%, followed by palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), which accounted for 21.88%, 17.29%, 10.6% of the total fatty acids, respectively. The above-mentioned components accounted for more than 90% of the total fatty acids. Other fatty acids were only present in trace quantities. In addition, the total FAME derived from the lipid extracted at the optimum condition was calculated to be (8.73±0.52)% on dry weight basis of algal biomass (Table S1). In conclusion, lipids extracted by EAE from *Chlorococcum* sp. are a suitable feedstock for biodiesel production [13].

CONCLUSIONS

We used an RSM model, validated by experimental results, to show that lipids in *Chlorococcum* sp. can effectively be extracted by EAE. The optimum extraction conditions are as follows: Extraction temperature 80 °C, extraction time 270 min and EA/E 1.0. The distribution of fatty acid profiles of lipids suggests that the lipids extracted by EAE from *Chlorococcum* sp. are a good alternative for conventional oil for biodiesel production. However, further work is required to investigate the extraction of wet biomass and scale up of the extraction process.

ACKNOWLEDGEMENTS

This research was supported by Grants from the National Natural Science Foundation of China (Grant No. 51476177).

SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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Supporting Information

Optimization of algal lipid extraction by mixture of ethyl acetate and ethanol via response surface methodology for biodiesel production

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(*Received 11 January 2016 • accepted 14 April 2016*)

Table S1. FAME yield at optimum extraction conditions

FAME	Concentration (mg/L)			Standard deviation
	Experiment 1#	Experiment 2#	Average	
C14:1	0.06	0.06	0.06	0.00
C16:0	2.06	1.76	1.91	0.21
C16:1	0.23	0.22	0.23	0.01
C18:0	1.46	1.54	1.50	0.06
C18:1	0.97	0.88	0.93	0.06
C18:2	4.16	3.06	3.61	0.78
C18:3	0.11	0.11	0.11	0.00
C20:0	0.04	0.03	0.04	0.01
C20:1	0.14	0.14	0.14	0.00
C20:5	0.23	0.23	0.23	0.00
Total	9.47	8.03	8.75	1.01
FAME yield/%	9.10	8.36	8.73	0.52

Note: Extraction conditions are as follows: Extraction time, 270 min; extraction temperature, 80 °C; EA/E, 1.0 and solvent to biomass ratio 40 : 1. The biomass quantity for experiment 1# and experiment 2# was 104 mg and 96 mg, respectively. The FAMEs were both dissolved in 1 mL hexane (chromatographic pure grade)