Optimization of ultrasound-assisted extraction parameters of chlorophyll from *Chlorella vulgaris* residue after lipid separation using response surface methodology

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Abstract An investigation into ultrasound-assisted extraction (UAE) was conducted for the extraction of chlorophyll from *Chlorella vulgaris* residue after lipid separation. The best possible combination of extraction parameters was obtained with the response surface methodology (RSM), at a three-variable, three-level experiment Box–Behnken design (BBD). The optimum extraction parameters were as follows: extraction temperature, 61.4 °C, extraction time, 78.7 min, ethanol volume, 79.4 %, at a fixed ultrasonic power of 200 W. Under the modified optimal conditions, the model predicted a total chlorophyll content of 30.1 mg/g. Verification of the optimization showed that chlorophyll extraction of 31.1 ± 1.56 mg/g was observed under the optimal conditions, which well matches with the predicted value. Under these conditions, two stage extraction could sufficiently reach the maximal chlorophyll yield (35.2 mg/g), and the extraction rate reached up to 88.9 %. The present paper provides a feasible technology route for comprehensive utilization of bioactive substances from *Chlorella* and microalgal biomass biorefinery.

Keywords Chlorophyll · *Chlorella vulgaris* · Ultrasound-assisted extraction · Response surface methodology · Optimization

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Introduction

Chlorophylls are the most common green pigments found in plants. As an integrated part of vegetable foodstuffs, chlorophylls have been a natural component of the human diet throughout history. In vivo, these pigments play a key role in photosynthesis (Schoefs 2002).

Microalgae produce of wide variety of biomaterials, one of them is chlorophyll. Most algae cultured under optimum condition were reported contained about 4 % dry weight of chlorophyll from overall cell weight (Chen and Jiang 1999). Cyanobacteria typically contain chlorophyll-a while species of green algae mostly have chlorophyll-b (Deng et al. 2008). *Chlorella* is a type of edible algae. It's a single-celled organism that grows in water and has a high nutritional value. Two species are typically grown for human consumption: *Chlorella vulgaris* and *Chlorella pyrenoidosa*. *Chlorella* is over 50 % protein (dry weight) and contains a spectrum of fatty acids, carbohydrates, vitamins, amino acids and trace minerals (Chen and Jiang 1999). It also contains high amounts of chlorophyll. The high chlorophyll content of *chlorella* is credited with giving the algae many of its beneficial properties (Nakanishi 2001).

Chlorophyll provides a chelating agent activity which can be used in ointment, treatment for pharmaceutical benefits especially liver recovery and ulcer treatment. Besides that, it repairs cells, increases haemoglobin in blood and faster the cell growth (Zheng et al. 2006). Chlorophyll has also been investigated as source of pigments in cosmetics. Furthermore, in food industry, chlorophyll is used as natural pigment ingredient in processed foods (Humphrey 2004). Because of its strong green pigment and consumers demand for natural foods, chlorophyll is gaining importance as a food additive.

However, to the best of our knowledge, there is limited literature on efficient extraction of chlorophyll from microalgae. In order to seek more environmentally friendly methods, decrease the solvent consumption, shorten the extraction time, and increase the extraction yield, various novel extraction techniques have been developed for the extraction of nutraceuticals from plants, including ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), and microwave-assisted extraction (MAE) (Wang and Weller 2006). Among these, UAE is an inexpensive, simple and efficient alternative to conventional extraction techniques. UAE has been employed as an efficient technique for plant pigments, phenolics and antioxidants extraction (Tiwari et al. 2009; Annegowda et al. 2011), as well as treatments on texture of chickpea (Yildirim et al. 2011). UAE was also adopted to extract carotenoids, chlorophyll a and lipids from microalgae, such as *Dunaliella salina*, *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp. (Macías-Sánchez et al. 2009; Lee et al. 2010). The enhancement in extraction obtained by using ultrasound is mainly attributed to the effects of acoustic cavitations produced in the solvent by the passage of an ultrasonic wave. Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid and liquid phase (Wang et al. 2008).

Response surface methodology (RSM), an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization. This method had been successfully applied in the optimization of fermentation processes (Li et al. 2007; Cui et al. 2006; Gangadharan et al. 2008), enzymatic hydrolysis (Kunamneni and Singh 2005; Nilsang et al. 2005), synthesis parameters for polymers (Shieh and Lai 2000), and food processing operations (Huang et al. 2008; Liu et al. 2009).

The main objective of this study was to apply statistical methods to optimize the UAE parameters of chlorophylls from green algae *Chlorella vulgaris* residue after lipid separation using solvent extraction for microalgal biomass biorefinery. In this paper, the optimum parameters including UAE temperature, time and ethanol volume, were obtained by RSM in order to determine an optimal set of operational conditions. The Box–Behnken design (BBD) was employed to evaluate the coefficients in a quadratic mathematical model. We hope the investigation provides a feasible way to production of biofuel low costly through biorefinery of active ingredients in microalgae, especially the proteins, carbohydrates, natural pigment and other nutrients.

Materials and methods

Algae material and chemicals Dried green algae (*C. vulgaris*) sample was obtained from Qian Xuesen Deserticulture

Center Laboratory (Gansu, China). The sample were sealed in polyethylene bags and stored at $-20\text{ }^{\circ}\text{C}$ until ready for sampling. All chemicals and solvents were of the analytically pure and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Lipid separation and UAE Dried alga samples were finely mechanical ground 20 min for homogenization and powder process in a laboratory mill (Jiangsu, China) and extracted with *n*-hexane for lipid separation at darkness, room temperature and agitation until the solvent did not show any colouration. Then the extract was separated from the pellet and recovered by centrifugation at $5000 \times g$ for 10 min (Shanghai, China). The supernatant liquid was evaporated on a rotary evaporator (Tokyo, Japan) and the alga lipid was recovered, and the pellet dried at $50\text{ }^{\circ}\text{C}$ as the chlorophyll extraction samples.

100 mg of homogenized dry sample was put in a sealed vessel, which attached to a reflux condenser, and extracted (extraction conditions varied depending on the particular experiment) in a conventional water bath with magnetic agitation or in an ultrasonic extractor (KQ 250DE, 40.0 kHz, 250 W, Jiangsu, China) at a fixed ultrasonic power of 200 W. An in-water pipe was added to the opposite of out-water pipe in the bath, and the flux ratio between in-water and out-water was regulated to keep solution temperature stable in the test. The mixture was centrifuged after extraction and the supernatant used for chlorophyll determination.

Chlorophyll content determination Absorption measurements of chlorophyll were made on a Hitachi U-1800 spectrophotometer. The modified equation proposed by Wellburn (1994) was used for the determination of chlorophyll concentration in the samples of *C. vulgaris*. The absorbance was recorded at 649 and 665 nm simultaneously in order to determine chlorophyll a (Ca) and b (Cb) contents when methanol and ethanol as extraction solvent. For acetone extract, the absorbance was recorded at 645 and 663 nm. The total chlorophyll content equal to sum of Ca and Cb, and the chlorophyll content was expressed as mg/g dry weight.

Experiment design and statistical analysis

Selection of extraction solvent Different concentrations (anhydrous solvent and 80 % organic solvent +20 % water) of methanol, ethanol and acetone were applied for choice of solvent suitable for *C. vulgaris* chlorophyll extraction. The experiments were carried out at conventional agitation leaching ($50\text{ }^{\circ}\text{C}$, 30 min, 200 rpm and the solvent: sample ratio of 1:5 (ml:mg)).

Selection of extraction method Comparison of conventional and UAE of chlorophyll from *C. vulgaris* residue was

Table 1 The variables levels of OED for UAE of total chlorophyll from *C. vulgaris* residue

Factor Levels	x_1 /Temperature (°C)	x_2 /Ethanol volume (%)	x_3 /S-Lr (mg: ml)	x_4 /Time (min)
–1	50	70	25 : 10	30
0	60	80	50 : 10	60
1	70	90	100 : 10	90

OED orthogonal experimental design; UAE ultrasound-assisted extraction; S-Lr Solid–liquid ratio

investigated for selection of an efficient extraction method. The conventional agitation extraction was carried out at 50 °C, 30 min, 200 rpm and the solvent: sample ratio of 1:5 (ml:mg). UAE was carried out at 50 °C, 30 min, 200 rpm, solvent: sample ratio of 1:5 (ml:mg), ultrasonic power of 200 W. The selected solvent (ethanol) was used in both extraction methods.

Identifying the significant variables using orthogonal experimental design (OED) The present study was aimed at screening of the important factors with respect to their main effects by OED. Table 1 illustrated the experimental design with four factors as well as the variables. Table 2 displayed the OED matrix and results for evaluating factors influencing chlorophyll extraction content from *C. vulgaris* residue.

Box–Behnken design (BBD) The levels of the significant parameters and the interaction effects between various extraction parameters which influence the chlorophyll content significantly were analysed and optimized by Box–Behnken methodology (Box and Behnkin 1960). In this study, the experiment design contains 17 trials and the values of the responses were the mean of triplications. The experimental design used for the study is shown in Tables 3 and 4. The second-order polynomial coefficients were calculated and

analyzed using the “Design Expert” software (Version 7.0, Stat-Ease Inc., Minneapolis, USA) statistical package.

Statistical analysis Data were expressed as mean $\pm$ standard deviation (SD, $n=4$) from two independent parallel experiments for chlorophyll extraction and duplicate tests in each time. Significant differences among the means of samples were analyzed by Tukey’s test and statistical analysis of the model was performed by the analysis of variance (ANOVA).

Results and discussion

Selection of extraction solvent for *C. vulgaris* chlorophyll The wavelength scanning spectrum and chlorophyll content of different extraction solvents from *C. vulgaris* residue were summarized in Figs. 1 and 2. We can conclude from the results that extraction of *Chlorella* chlorophyll with 80 % organic-aqueous solvents were better than anhydrous organic solvents (Fig. 1). In the tested solvents, the 80 % methanol-aqueous solution was the most efficient solvent for chlorophyll extraction, and obtained the maximum total chlorophyll content (16.8 mg/g). Extraction efficiencies for *Chlorella* total chlorophyll were significantly different ($p<0.05$) for all the tests except 100 % ethanol and 100 % acetone. The extraction contents of chlorophyll b were more than chlorophyll a when the methanol, ethanol and acetone as the extracting solvents (Fig. 2a).

In Jespersen and Christoffersen (1987) showed that ethanol is as efficient as methanol for extraction of chlorophyll. Kinzie (1993) used methanol: tetrahydrofuran (80: 20, v/v) as the extracting solvent and a modified dichromatic equation to calculate the concentration of chlorophyll a. Wright and Mantoura (1997) recommended methanol rather than acetone for routine marine samples. Schumann et al. (2005) observed that from the various methods tested for chlorophyll a extraction

Table 2 OED matrix for evaluating factors influencing UAE of total chlorophyll from *C. vulgaris* residue

Trials	x_1 (Temperature)	x_2 (Ethanol volume)	x_3 (S-Lr)	x_4 (Time)	TCC (mg/g) ^a
1	1 (50 °C)	1 (70 %)	1 (25:10)	1 (30 min)	16.1 $\pm$ 0.21
2	1 (50 °C)	2 (80 %)	2 (50:10)	2 (60 min)	19.6 $\pm$ 0.44
3	1 (50 °C)	3 (90 %)	3 (100:10)	3 (90 min)	17.5 $\pm$ 0.31
4	2 (60 °C)	1 (70 %)	2 (50:10)	3 (90 min)	24.7 $\pm$ 0.14
5	2 (60 °C)	2 (80 %)	3 (100:10)	1 (30 min)	21.5 $\pm$ 0.29
6	2 (60 °C)	3 (90 %)	1 (25:10)	2 (60 min)	20.3 $\pm$ 0.34
7	3 (70 °C)	1 (70 %)	3 (100:10)	2 (60 min)	25.6 $\pm$ 0.26
8	3 (70 °C)	2 (80 %)	1 (25:10)	3 (90 min)	29.1 $\pm$ 0.43
9	3 (70 °C)	3 (90 %)	2 (50:10)	1 (30 min)	21.6 $\pm$ 0.27
Mean value 1	17.713	22.140	21.823	19.740	
Mean value 2	22.167	23.387	21.980	21.817	
Mean value 3	25.447	19.800	21.523	23.770	
Range	7.734	3.587	0.457	4.030	

OED orthogonal experimental design; UAE: ultrasound-assisted extraction; S-Lr Solid–liquid ratio; TCC Total chlorophyll content.

^a $n=4$

Table 3 The variables and levels of BBD for UAE of total chlorophyll from *C. vulgaris* residue

Factor Levels	X_1 / Temperature (°C)	X_2 / Time (min)	X_3 / Ethanol volume (%)
–1	50	30	70
0	60	60	80
1	70	90	90

BBD Box–Behnken design; UAE ultrasound-assisted extraction

from green microalgal, isolates dimethyl formamide (DMF) gave higher concentrations than did acetone in almost all treatments. While, Wasmund et al. (2006) suggested that the chlorophyll extraction efficiency of the various solvents seems to depend on other factors, such as the taxonomic composition of the algal community. However, taking into account the safety of solvent in food additives and cost control, ethanol was the best green solvent for *C. vulgaris* chlorophyll extraction as the raw material for further production of sodium salt chlorophyllin, which was then used throughout.

The effects of ethanol volume on *C. vulgaris* total chlorophyll content are significantly from 70 % to 100 % (Fig. 2b), as well as the chlorophyll a and b. The extraction contents of chlorophyll b were more than the chlorophyll a in the all experimental groups. The maximum total chlorophyll content (14.2 mg/g), chlorophyll a (4.76 mg/g) and b (9.48 mg/g) were obtained at the 80 % ethanol concentration. Meanwhile, anhydrous ethanol reduced the extraction of total chlorophyll (8.61 mg/g). The differences between

80 % and 100 % ethanol volume were significant when examined by single factor ANOVA analysis of total recovery ($p < 0.05$). This behaviour can be attributed to the fact that chlorophyll is heterogenically bound to other compounds in the chloroplast and at least two or even three fractions of chlorophyll exist in the chloroplast. Therefore, the different polarities of organic solvent and water lead to the extraction of different types of chlorophyll and this gives rise to variations in the extraction yield of this substance (Öquist and Samuelsson 1980; Macías-Sánchez et al. 2009). Besides, the relative polarity and the increase in effective swelling of the algae by water, which helped increase the surface area for solute–solvent contact (Hemwimol et al. 2006). Furthermore, the mixture of water and organic solvent decrease the solution viscosity and the resistance of liquid mass transfer. Therefore, suitable water content in extracting solvent was more conducive to extraction of chlorophyll from the dried algae sample.

Selection of extraction method for *C. vulgaris* chlorophyll The extraction results indicated that the total chlorophyll extraction content under UAE (13.2 mg/g) higher than conventional cheating (8.32 mg/g), a remarkable increase (59.0 %) of the chlorophyll yield and highly significant difference ($p < 0.01$) were observed. UAE significantly improved the extraction yield. Since ultrasound could accelerate swelling and hydration and caused an enlargement in the pores of the plant cell walls, it resulted in a better mass transfer of solute constituents from the plant materials to solvent. The disruption of plant

Table 4 BBD with three independent variables for UAE of total chlorophyll from *C. vulgaris* residue

Run	Factor 1		Factor 2		Factor 3		TCC (mg/g)	
	X_1 (°C)	Code X_1	X_2 (min)	Code X_2	X_3 (%)	Code X_3	Actual value ^a	Predicted value
1	50	–1	30	–1	80	0	24.6±1.86	24.7
2	70	1	30	–1	80	0	26.4±0.89	26.7
3	50	–1	90	1	80	0	28.0±1.32	27.8
4	70	1	90	1	80	0	28.6±1.10	28.5
5	50	–1	60	0	70	–1	24.6±1.34	24.6
6	70	1	60	0	70	–1	26.1±1.61	25.9
7	50	–1	60	0	90	1	23.7±1.35	23.9
8	70	1	60	0	90	1	25.4±1.04	25.4
9	60	0	30	–1	70	–1	24.8±1.35	24.7
10	60	0	90	1	70	–1	27.1±0.70	27.3
11	60	0	30	–1	90	1	24.6±1.54	24.3
12	60	0	90	1	90	1	26.5±1.50	26.6
13	60	0	60	0	80	0	30.4±1.02	29.6
14	60	0	60	0	80	0	29.5±1.10	29.6
15	60	0	60	0	80	0	30.0±1.55	29.6
16	60	0	60	0	80	0	28.8±1.08	29.6
17	60	0	60	0	80	0	29.4±1.21	29.6

BBD: Box–Behnken design; UAE: ultrasound-assisted extraction; TCC: Total chlorophyll content.

^a $n=4$

Fig. 1 Wavelength scanning of different chlorophyll extraction solvents from *C. vulgaris* residue

cells by microjet after the cavitation bubble collapsed could increase the rate of solvent penetration into plant tissue (Toma et al. 2001). Therefore, UAE is an efficient technique for the extraction of chlorophyll from algae.

Optimization of *C. vulgaris* chlorophyll UAE conditions using OED The UAE conditions for *C. vulgaris* chlorophyll were optimized by orthogonal experiments (L_3^4) based on our preliminary single factor experiments (temperature, ethanol volume, ratios of the solvent/sample and time). The results from the Table 2 indicated that the chlorophyll yield improved with the increase of extraction temperature from 50 to 70 °C, and time from 30 to 90 min. The effects of ethanol volume and solid–liquid ratio on chlorophyll yield were different with the temperature and time. The highest chlorophyll yields were obtained at 80 % ethanol volume

and 50:10 (mg: ml). The ANOVA for orthogonal experimental results for *C. vulgaris* total chlorophyll are listed in Table 5. We can conclude from the results that the effects of extraction temperature (x_1 , $p<0.01$), ethanol volume (x_2 , $p<0.05$) and time (x_4 , $p<0.05$) on the total chlorophyll content were significant. But the affect of solid–liquid ratio (x_3) was not significant. Thus, the effect of solid–liquid ratio (x_3) was not considered and optimized in the further RSM experiment.

Optimization of *C. vulgaris* chlorophyll UAE conditions using BBD

Analysis of Box–Behnken experiment The significant factors obtained from the orthogonal experiments were further optimized by using the RSM in order to establish the mathematical model and equation for UAE of *C. vulgaris* chlorophyll. The optimal level of the key factors (temperature, time and ethanol volume) and the effect of their interactions on total chlorophyll content were further explored by the BBD. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation (equation in terms of coded factors) was established to explain the UAE of *C. vulgaris* chlorophyll:

$$Y = 29.62 + 0.70X_1 + 1.23X_2 - 0.27X_3 - 0.32X_1X_2 + 0.04X_1X_3 - 0.095X_2X_3 - 1.76X_1^2 - 0.95X_2^2 - 2.92X_3^2$$

where Y is the predicted total chlorophyll content; X_1 , X_2 and X_3 are the coded values of temperature, time and ethanol volume, respectively.

Fig. 2 Effects of extraction solvents and ethanol concentrations on chlorophyll contents of *C. vulgaris* residue (mean $\pm$ SD, $n=4$). The different letters on same style bars are significantly different ($p<0.05$). **a** extraction solvents; **b** ethanol concentrations

Table 5 ANOVA for orthogonal experimental ($L3^4$) results for UAE of total chlorophyll from *C. vulgaris* residue

Factors	Statistics				
	SS	DF	MS	F-Value	Significance
x_1 / Temperature	90.34	2	45.17	277.41	** a
x_2 / Ethanol volume	19.89	2	9.94	61.07	* b
x_3 / S-Lr	0.33	2	0.16	1.00	
x_4 / Time	24.34	2	12.17	74.75	* b

ANOVA Analysis of variance; UAE ultrasound-assisted extraction; S-Lr Solid-liquid ratio

SS Sum of squares; DF Degree of freedom; MS Mean square

^a Significant at $p < 0.01$, ^b Significant at $p < 0.05$

Table 6 shows the ANOVA for response surface quadratic model. The model F -value of 33.56 implies the model is significant. There is only a 0.01 % chance that a "Model F -value" this large could occur due to noise. Values of "Prob $> F$ " less than 0.05 indicate model terms are significant. The p -values are used as a tool to check significance of each variables, which also indicate the interaction strength between each independent variable. The smaller the p -values, the bigger the significance of the corresponding variables. p -values in this study less than 0.05 indicate model terms are significant. In this case X_1 , X_2 , X_1^2 , X_2^2 , X_3^2 are significant model terms. In this case values greater than 0.10 indicate the model terms are not significant. The "Lack of Fit F -value" of 0.36 implies the lack of fit is not significant relative to the pure error. There is a 78.53 % chance that a "Lack of Fit F -value"

Table 6 ANOVA for response surface quadratic model

Source	SS	DF	MS	F-Value	p -value (Prob $> F$)	
Model	74.54	9	8.28	33.56	< 0.0001	significant
X_1 -Temperature	3.86	1	3.86	15.66	0.0055	
X_2 -Time	12.03	1	12.03	48.74	0.0002	
X_3 -Ethanol volume	0.60	1	0.60	2.43	0.1631	
X_1X_2	0.40	1	0.40	1.63	0.2419	
X_1X_3	0.01	1	0.01	0.02	0.8843	
X_2X_3	0.04	1	0.04	0.15	0.7135	
X_1^2	13.03	1	13.03	52.79	0.0002	
X_2^2	3.81	1	3.81	15.45	0.0057	
X_3^2	35.94	1	35.94	145.62	< 0.0001	
Residual	1.73	7	0.25			
Lack of Fit	0.37	3	0.12	0.36	0.7853	not significant
Pure Error	1.36	4	0.34			

ANOVA Analysis of variance; SS Sum of squares; DF Degree of freedom; MS Mean square

this large could occur due to noise. Non-significant lack of fit is good.

Effects of temperature, duration and ethanol volume on chlorophyll extraction The response-surface graphs were obtained using the experimental design shown in Fig. 3. Figure 3a shows the effect of the extraction temperature

Fig. 3 Response surface plot of the combined effects of UAE temperature, time and ethanol volume on total chlorophyll content of *C. vulgaris* residue

and time on the total chlorophyll content at a fixed ethanol volume (80 %, v/v). At a definite extraction temperature, the chlorophyll yield increased rapidly with the increase of the extraction time, and nearly reached a peak at the extraction time about 78.7 min. However, the extraction temperature showed a quadratic effect on the response (chlorophyll yield). The maximum chlorophyll yield was obtained at 61.4 °C, followed by a decline with the further increase of the extraction temperature.

Figure 3b shows the effect of the extraction temperature and the ethanol volume on the chlorophyll yield at a fixed extraction time of 60 min. The results indicated that the quadratic effect of the ethanol volume was striking, and the highest value at 79.4 %. The quadratic effect of the extraction temperature was significant, and the chlorophyll yield reached the highest value also at 61.4 °C.

The interaction between the extraction time and ethanol volume is shown in Fig. 3c at the fixed extraction temperature of 60 °C. The chlorophyll yield increased with the extraction time and the ethanol volume increasing, and the maximum response value was obtained at 78.7 min and 79.4 %, respectively. Those results may attribute to the oxidation and degradation of chlorophyll under higher temperature and longer duration time (Schumann et al 2005).

Validation of the model According to the results of the statistical design, the optimized parameters for chlorophyll UAE from *C. vulgaris* residue after lipid separation were prepared as follows: temperature 61.4 °C, time 78.7 min, and ethanol volume 79.4 % (v/v). Under the above optimized conditions, the maximum chlorophyll yield of *C. vulgaris* was estimated as 30.1 mg/g.

A repeat UAE of chlorophyll from *C. vulgaris* residue under modified optimal conditions (temperature 61 °C, time 79 min, ethanol volume 79 %) was carried out for verification of the optimization, and the maximal chlorophyll content was 31.1 ± 1.56 mg/g ($n=4$). The measured total amount of chlorophyll from *C. vulgaris* residue was 39.6 mg/g. The extraction rate of 78.5 % was achieved. This value was found to be 3.43 % more than the predicted value. This discrepancy might be due to the slight variation in experimental conditions. The excellent correlation between predicted and measured values verifies the model validation and existence of an optimal point.

Effect of extraction times on the chlorophyll yield The effects of extraction times on the yield of chlorophyll were examined under the above modified optimal conditions. The results shown that two stage extraction produced significantly ($p<0.05$) higher chlorophyll yield (35.2 ± 1.83 mg/g, $n=4$) than one-time extraction (31.1 ± 1.56 mg/g, $n=4$). But the chlorophyll yield at three-time extraction (36.8 ± 1.06 mg/g, $n=4$) did not had significant difference with two stage

extraction. Hence, the optimal extracting times were fixed at two stage extraction. The extraction rate of chlorophyll was 88.9 % under the two stage extraction.

Conclusion

In this paper, RSM (BBD) was adopted to identify UAE parameters which enhanced the chlorophyll extraction from *Chlorella* sp. residue after lipid separation. The results suggested that UAE method is an efficient technique for the extraction of chlorophyll from microalgae, and statistical design methodology offers an efficient and feasible approach for extraction parameters optimization. The modified optimum extraction parameters from BBD were extraction temperature 61 °C, extraction time 79 min, ethanol volume 79 % at a fixed ultrasonic power of 200 W. Under these conditions, two stages extraction could sufficiently reach the maximal chlorophyll content (35.2 mg/g) and extraction rate (88.9 %). The present paper provides feasible technology route for comprehensive utilization of biologically active components from *Chlorella* and its biorefinery.

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