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Research Article

Enhancing the yield of chlorophyll a from fresh spirulina using ultrasound-assisted extraction

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Abstract

Spirulina is a spiral cyanobacteria with simple cell structure, making it easier to extract chlorophyll than other materials. Investigation the ultrasound assisted extraction of chlorophyll a was conducted on the following factors: type of solvent, concentration of solvent, ratio of solvent: material, time and temperature. Optimization by Design Expert the extraction of chlorophyll a from spirulina algae indicates the condition to obtain the highest amount of chlorophyll a of 15.07 mg/g was as followings: solvent ethanol concentration 99.7%, ratio solvent / material: 16/1, time 2.5 hours, temperature 52°C, ultrasonic frequency 40kHz.

Keywords: ultrasound-assisted, extraction, *Chlorophyl a*, Spirulina

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Introduction

Spirulina are multicellular, filamentous cyanobacteria. The genus *Spirulina* comprises photosynthetic organisms, with phycocyanin as the main photosynthetic pigment; it also has carotenoids and *chlorophyll a* (Rahim et al. 2021). *Spirulina* has been growing naturally in our environment for millions of years, it is a tough plant which is able to withstand harsh growing conditions. In fact the micro-algae cell never really dies it goes dormant when weather conditions are not favorable (Sánchez et al. 2003) and as soon as this change and the environment is once again suitable for growth, *spirulina* begins growing and reproducing again. Naturally growing *spirulina* can be found in high alkaline lakes (Challouf et al. 2011).

The basic biochemical composition of *spirulina* can be summarized as follow: *Spirulina* contains unusually high amount of protein, between 55-70% by dried weight, depending upon the source (Cohen et al. 1997, Phang et al. 2000, Rosa et al. 2015). It is a complete protein, containing all essential amino acids, superior to all standard plant protein such as that from legumes. *Spirulina* also has a high amount of polyunsaturated fatty acids, 1.5-2.0 % of 5-6% total lipid; several vitamins such as B1, B2, B3, B6, B9, B12, vitamin C, D and E. Especially, *Spirulina* contains many pigments including chlorophylls a, xanthophyll, beta-carotene, echinenone, c-phycocyanin and allophycocyanin (Sánchez et al. 2003).

The blue-green color of *spirulina* is due to two pigments: phycocyanin (blue) and chlorophyll (Rosa et al. 2015). *Spirulina* is an economical bioresource of good-quality natural *chlorophyll a* since most of the *chlorophyll* in *spirulina* exist in the form of *chlorophyll a*, unlike most plants that have mixtures of *chlorophyll a* and *b* (Munawaroh et al. 2019). This finding supports the important role of *chlorophyll a* as an acceptor for photo-chemical conversing light energy for capturing light required for photosynthesis. In addition, *chlorophyll a* shows its biological effects, such as anticancer, anti-aging, anti-inflammatory, and antioxidant activity effects (Choi et al. 2018)

However, the *chlorophylls* are very unstable and difficult to extract in intact forms out of the hard cells of *Spirulina*, which leads to very low extraction yield. The extraction of *chlorophylls* from *spirulina* has not been studied much (Simon and Helliwell 1998). To obtain pure *chlorophyll a* from natural resources has not been easy since its purification steps are more complicated and also require special attention to avoid heat and light damage during the purification process (Hosikian et al. 2010). In addition, phycocyanin, carotenoid and chlorophylls are important bioactive compounds in spirulina. Recent study on the extraction of photosynthetic pigments from different *Spirulina platensis* samples was carried out to evaluate the yield and purity of phycocyanin using ultrasonic irradiation. The highest yield of phycocyanin (2.5 mg/g dw) and purity 0.8 were obtained for 3 hours at 35 kHz sonification (Minchev et al. 2021).

So far, the studies on chlorophylls extraction from *spirulina* have focused mainly on extracting the *chlorophylls* through the supercritical extraction process with different combinations of various parameters such as solvent ratio temperature, pressure (Sanchez et al. 2009). There were also several reports on obtaining fair amounts of chlorophylls from plants resources by optimizing those variables (Wasmund et al. 2006).

Ultrasonic extraction has most often been used to break down cell walls with high efficiency, but without additional heat that could deteriorate heat-labile bioactive substances in the biomass (Kong et al. 2014). In addition, the extraction process efficiency depends on many factors such as type of solvent, temperature, duration of extraction, and ultrasound condition. The present study aims to enhance the efficiency of *chlorophyll a* extraction from *spirulina* grown in Vietnam by optimizing the extraction condition using surface response method with Design Expert 11 software.

Materials and Methods

Fresh sample of spirulina (*Spirulina plantensis*) was purchased from Vietnam National University of Agriculture in 2019. The fresh spirulina has a moisture content of 82%, an ash content of 7.32%, a fiber content of 2.26% and a protein content of 59.45% by dry weight. The fresh spirulina was kept frozen until analysis. Chemical reagents were of analytical grades.

Extraction optimization. About 1g of sample was ultrasound assisted extracted in 10mL ethanol using

Qsonica (model C75E, USA) at 40 kHz, 50°C and 3 hrs. The extract then was centrifuged (6000 rpm in 15 min) to obtain the supernatant. The *chlorophyll* was measured by UV VIS method (Karsten et al. 2005).

Using this procedure, each factor was investigated in turn and choose the appropriate factor for the following experimental steps. Solvent: methanol, ethanol and acetone. Solvent concentration: 70%, 80%, 90%, 99.7%.

Extraction time (x1): 2 hrs.; 2,5 hrs.; 3 hrs.; 3,5 hrs.

Extraction temperature (x2) 30°C, 40°C, 50°C, 60°C. Solvent/material ratio (x3): 5/1, 10/1, 15/1, 20/1.

Optimization of the *chlorophyll a* extraction conditions was determined by a surface response method using Design Expert 11 software. The design of experiments included the factors contributing to the extraction efficiency of *chlorophylls* and the levels of these investigated factors. Time, temperature and ratio of solvent: material was the designed at three levels.

The mathematical model (Munawaroh et al. 2019) describes the effects of independent variables on dependent variables with quadratic polynomial function. The acceptance level of the model is represented by the determination coefficient representing the quality of the regression model (R^2). Optimal parameters obtained by Design Expert were used to perform actual validation to determine the optimum extraction conditions.

***Chlorophyll a* determination.** The quantification of chlorophylls was determined in which the supernatants were measured at different wavelengths.

Chlorophyll a was measured at 645nm.
Chlorophyll a: $C_a = 12{,}7A_{663} - 2{,}69A_{645}$ mg/l
All the experiments were carried out in triplicated. The statistically analysis was calculated at the 5% significance level.

Results and Discussion

Preliminary screening of the extraction. Table 1 shows the result of preliminary screening of the extraction which only based on the variation of one parameter of the *chlorophyll a* extraction condition such as type of solvent, ethanol concentration, ratio of solvent/spirulina, temperature and time of extraction.

Table 1. The influence of extraction condition on the obtained *chlorophyll a* concentration

Parameter		***Chlorophyll a* concentration, mg/g**
Type of solvent	acetone	12.86[a]
	ethanol	12.69[a]
	methanol	11.11[b]
Ethanol concentration, %	70	4.51[d]
	80	7,22[c]
	90	11,69[b]
	99.7	12,69[a]
Ratio of solvent/spirulina	5/1	10.73[c]
	10/1	12.69[b]
	15/1	13.95[a]
	20/1	12.46[b]
Extraction time, h	2.0	13.85[b]
	2.5	15.07[a]
	3.0	13.96[b]
	3.5	13.46[c]
Extraction temperature, °C	30	12.79[c]
	40	13.63[b]
	50	15.07[a]
	60	13.79[b]

** Different letters: Significant difference at 5%*

Type of solvent. *Chlorophyll a* was obtained by acetone (12.86 mg/g), ethanol (12.69 mg/ g) and methanol (11.11 mg/g). The amount obtained of *chlorophyll a* changed depending on the type of solvent, and this is similar to that of Tavanandi and Raghavarao (2019) showing the *chlorophyll* extraction efficiency of different solvents in the order: Acetone > DMSO > Ethanol > Methanol > Dimethyl Ether. However, in the study on the optimization of storage and extraction of *chlorophyll* from *Phaeodactylum* and *Macrocystis*, it was showed that acetone was inferior to ethanol (Wasmund 2006). Similarly, acetone was a suitable solvent for many algal groups such as *Stichococcus sp*. and *Chlorella sp*. (Schumann 2005).

Studies on the structure of *chlorophyll* showed that the difference between *chlorophyll a* and *chlorophyll b* is at the C7 position, in *chlorophyll a* it is the methyl group (-CH_3) and in *chlorophyll b* is the formyl group (-CHO). Acetone has the molecular formula of $(CH_3)_2CO$; meanwhile, ethanol (C_2H_5OH) is a solvent containing methyl group, ethyl (-CH_2) and hydroxyl group (-OH). on the other hand, the solubility of *chlorophyll* differs based on different solvents which also leads to difference in test results.

The extraction efficiency of *chlorophyll a* of acetone was a bit higher than that of ethanol. (12.86 mg/g vs 12.69 mg/g). However, this data did not show significant difference between two test series. Moreover, acetone is a toxic solvent, costly with adversely effects on human health. Ethanol is an inexpensive, safe solvent, popular in food and pharmacy industry, so ethanol was chosen as an adequate solvent for extraction in the next experiments for samples of frozen algae.

Concentration of ethanol on the chlorophyll a extracted. Ethanol of 70%, 80%, 90%, 99.7% concentration were used. For frozen sample, *chlorophyll a* obtained as highest in 99.7% ethanol (12.69 mg/g), much higher than that in extraction by 70% (4.51 mg/g) and other concentration of ethanol with statistically difference of 5%.

Ethanol and spirulina ratio on the chlorophyll a extracted. Ethanol of 99.7% concentration was used at the ratio to material of 5/1, 10/1, 15/1, 20/1. Results are presented in as the following, the *chlorophyll a* obtained at ratio 5/1 was 10.73 mg/g, at 10/1 was 12.69 mg/g, at 15/1 was 13.95 and at 20/1 was 12.46 mg/g *chlorophyll*. *Chlorophyll a* obtained at highest at the ratio of 15/1 (13.95 mg/g) as the pigments are easily exposed to the solvent and concentration gradient of the constituent to be extracted between the raw materials is increased leading to higher permeability. But when the solvent is more added, (20/1) the amount of color obtained tends to decrease, because the volume of solvent is too large, which reduce the amount of *chlorophyll* diffusing into the solvent.

Extraction duration on the chlorophyll a extracted. Ethanol of 99.7% concentration and the ratio of solvent / *spirulina* of 15/1 were used in this preliminary screening. The extraction was conducted for 2; 2.5; 3 and 3.5hours. The amount of 15.07 mg/g *chlorophyll a* obtained was the highest at 2.5 hrs. Theoretically, extending the extraction time will increase the contact time between the raw material and the solvent, thereby increasing the diffusion of the particles to be extracted from the raw material into the solution and increasing the extraction efficiency. However, the extraction process will slow down until the equilibrium difference between the concentrations of the extracts in the material and in the solution. Therefore, decreasing in *chlorophyll a* extracted was observed after 2.5 hrs. of extraction.

Extraction temperature on the chlorophyll a extracted. Ethanol solvent of 99.7%, ratio of solvent / material of 15/1 for 2.5 hours was the condition for extraction temperature investigation. *Chlorophyll a* extracted as highest at 50ºC (15.07 mg/g), and as lowest at 30°C (12.79 mg/g). Higher than 60°C, when the extraction temperature increases, the concentration of *chlorophyll a* tends to decrease (from 15.07 mg/g to 13.79 mg/g).

Optimization of the extraction. The experiments were carried out according to Box-Behnken design (Table 2). The quadratic regression equation with three factors (X_1, X_2, X_3) of the target function (Y, *chlorophyll a* content):

$$Y = 14.98 + 0.0087X_1 + 0,3825X_2 + 0.7663X_3 + \\ + 0.7250X_3X_2 – 1.09X_1^2 – 1.31X_2^2 – 1.94 X_3^2 \qquad (1)$$

The results of variance analysis in Table 3 showed that the regression model for *chlorophyll a* content is statistically significant (p = 0.0001, R^2 = 0.97, $R^2_{Adjusted}$ = 0.93). Lack of fit was not statistically significant (p > 0.05). This means that 97% of the response values can be explained using the aforementioned model, as well as suggesting that the selected model's high compatibility.

Fig. 1 showed that the solvent/material ratio is the most important factor affecting the *chlorophyll a* extracted content, followed by the extraction temperature and the least influencing factor is extraction duration.

Correlation between treatment values in the optimized model of *chlorophyll a* extraction from s*pirulina* is shown in Fig. 2. Correlation between experimental and predicted values shows that: the values of the treatments in the model are optimal for the experimental concentration of *chlorophyll a* and the prediction closely with the regression line.

Table 2. Experimental results according to the Box-Behnken design

No	Code			Factor			Y Chlorophyll a, mg/g
	X_1	X_2	X_3	Time, hrs.	Temp., °C	Ratio, v/w	
1	-1	-1	0	2	40	15	12.68
2	1	-1	0	3	40	15	12.24
3	-1	1	0	2	60	15	12.57
4	1	1	0	3	60	15	12.85
5	-1	0	-1	2	50	10	11.12
6	1	0	-1	3	50	10	11.57
7	-1	0	1	2	50	20	12.68
8	1	0	1	3	50	20	12.46
9	0	-1	-1	2.5	40	10	10.90
10	0	1	-1	2.5	60	10	10.73
11	0	-1	1	2.5	40	20	11.29
12	0	1	1	2.5	60	20	14.02
13	0	0	0	2.5	50	15	15.07
14	0	0	0	2.5	50	15	14.85
15	0	0	0	2.5	50	15	15.02

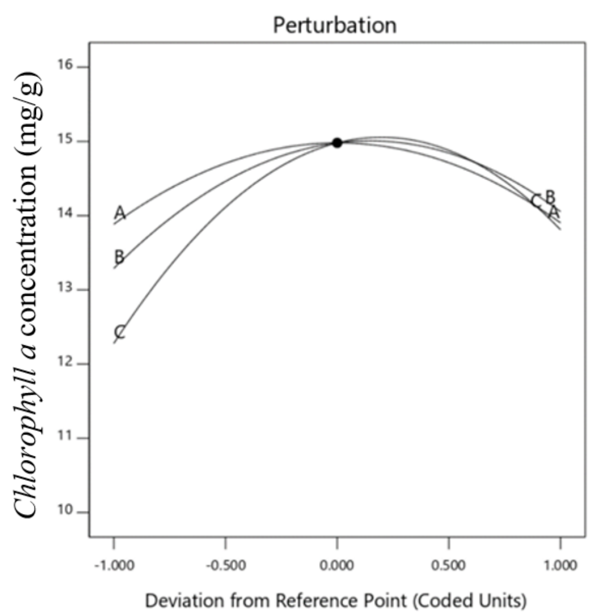

Figure 1. The variation of chlorophyll a concentration within the investigation range

Table 3. Regression coefficients from the experiments

RC	Regression analysis	
	RC	p-value
b_0	14.98	0.0001*
b_1	0.0087	0.9498
b_2	0.3825	0.0246*
b_3	0.7663	0.0007*
b_{11}	-1.09	0.0009*
b_{22}	-1.31	0.0003*
b_{33}	-1.94	<0.0001*
b_{23}	0.7250	0.0065*
R^2	0.97	
$R^2_{Adjusted}$	0.93	
Lack of fit		0.0647

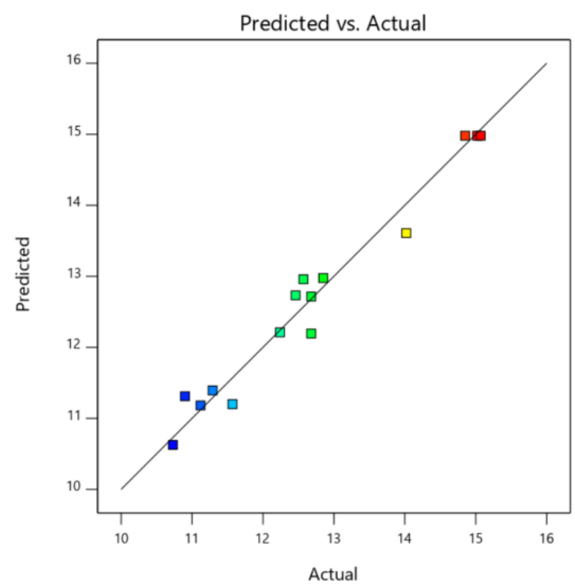

Figure 2. *Chlorophyll a* extraction experimental against the predicted values of the response surface

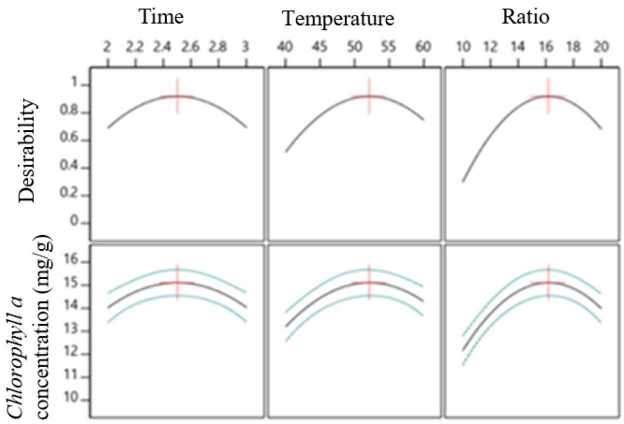

Figure 3. Optimization of factors affecting *chlorophyll a* extraction from *Spirulina*

Based on the quadratic equation and the results shown in Fig. 3, the optimum parameters of *chlorophyll a* extraction from spirulina under the influence of ultrasonic waves under laboratory conditions were determined. At the ratio of solvent (v)/material of 16/1 at 52°C, for 2.5 hours, the predicted amount of *chlorophyll a* extracted is 15.11 mg/g. The optimal parameters for extraction of *chlorophyll a* from Spirulina is expressed with equation (1).

Conclusions

15.07 mg/g *chlorophyll a* obtained as highest with 99.7% ethanol at the ratio solvent/raw material of 16/1 and extracting for 2.5 hrs. at 52°C under 40 kHz of ultrasound. With this optimal condition, the concentration of *chlorophyll a* obtained according to the model is 15.11 mg/ g and in experimental was 15.07 mg/g, proving that the optimal model is compatible and expectation is 99.7%. The results support upgrading the scale to extract purified chlorophyll for the next transformation steps if needed for application in pharmaceuticals and functional foods.

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