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Optimization of Spirulina's Phycocyanin Extraction Yield Using Response Surface Method

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Authors' contributions

This work was carried out in collaboration between both authors. Author TPN designed and implemented the research, performed the statistical analysis, wrote the manuscript with input from all authors. Author MTD designed and implemented the research, wrote the manuscript with input from all authors, managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Besides cultivation, extraction is also a critical stage in enhancing the yield of phycocyanin production - a highly valuable compound from Spirulina biomass. In this study, the combined effect of three important variables in the ultrasonic-assisted extraction process on phycocyanin extraction yield, namely extraction temperature, sonication time, and solvent pH were investigated through a central composite design experiment. Furthermore, the response surface method was applied in order to define an optimal condition to achieve the highest extraction yield. The results showed that when temperature ranged from 35ºC to 45ºC, sonication time from 20 to 50 minutes, and solvent pH from 6 to 8, the average yield of 30.135±1.552 mg/g was obtained with an average purity of 0.871±0.043. A regression model was also successfully developed, which allowed a good prediction of extraction yield based on the three mentioned variables. On the other hand, an optimal

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condition for extraction was also proposed with sonication time = 43.57 minutes, extraction temperature = 37.6^{o}C, and solvent pH = 6.7. These results were practically valuable for the improvement of phycocyanin extraction from Spirulina biomass.

Keywords: Phycocyanin; response surface method; Arthrospira platensis; ultrasound-assisted extraction.

1. INTRODUCTION

Phycocyanin (PC), a water-soluble blue pigment, is a typical phycobiliprotein present in *Arthrospira,* with a content up to 20% of total protein of the algae [1]. Characterized by its strong antioxidant ability, this natural pigment is widely utilized in various fields, ranging from the food to pharmaceutical and cosmetic industries [2]. Therefore, producing a large amount of good-quality PC at a reasonable price to meet the market demand is currently a big practical question for PC producers. Specifically, not only a high production rate of algal biomass but also a good extraction yield of PC from *A. platensis* biomass should be achieved.

Regarding PC extraction, there are many approaches to breaking the algal cell wall to obtain the internal phycocyanin pigment, including homogenization, freezing and thawing, lysozyme treatment, and acid treatment [3]. A common disadvantage of these methods is that they are time-consuming [4]. To shorten the procedure, the ultrasound-assisted extraction method (UAE) was proposed, which can accelerate the cell breakdown efficiency [3]. The ultrasonic wave stimulates the widening of the membrane pores, allowing the solvent to easily penetrate deeper into the cell wall, increasing the diffusivity, and thus resulting in higher extraction efficiency. It was reported that the phycocyanin extraction efficiency by the UAE method was 57% higher than the freezing and thawing method, which was assessed to be the best phycocyanin extraction method [3]. Moreover, this method has other advantages, including requiring cheap and simple equipment and easy implementation steps. Therefore, the ultrasound-assisted extraction method (UAE) is currently widely applied to extract natural compounds, including phycocyanin [5,6], astaxanthin [7], and β-carotene [8].

Main factors that often affect the extraction efficiency of UAE are temperature, time, and pH of solvent [3]. Temperature has a great influence on the content of protein pigment in general and phycocyanin in particular [6,9]. For example, phycocyanin can be damaged and lose antioxidant properties when extracted at temperatures above 80°C for 24 h [10]. The extraction time is also an important parameter often of interest in the extraction process since the longer extraction time generally can lead to more yield of the required compounds. Besides, the pH of the solvent also needs to be adjusted to increase the stability and durability of the phycocyanin molecules [11,12]. However, it should be noted that there might be interactions between these factors that affect yield and purity of PC obtained. Furthermore, if combined with high temperature, the effect can be opposite [13], and thus optimization of these three factors is essential.

The response surface method (RSM) uses statistical, graphical, and mathematical techniques to study, improve, or optimize a process where the response variable is influenced by many variables more than one independent variable. The advantage of this method is that it can investigate not only the single effects but also the combined effects of the independent variables on the response variable and present the results in the form of a mathematical model as well as visual charts. The RSM also allows the improvement of the experimental process by proposing new sets of experiments [14]. Therefore, this study aims to (1) investigate the influence of factors: temperature, ultrasonic treatment time, and the pH of solvent on the yield and the purity of phycocyanin from the microalgae spirulina; and (2) to optimize the extraction conditions for the highest productivity using the response surface method.

2. MATERIALS AND METHODS

2.1 Materials

Dried and milled biomass of *Arthrospira* platensis with a humid content of 1.6% was provided by the Algal technology laboratory - Biology and Environmental Science Faculty - The University of Danang.

2.2 Experiment Design

A central composite design (CCD) was applied for the experiment with three factors: pH of the solvent (X_1), temperature (X_2), and ultrasonic treatment time (X_3). Based on our preliminary results, we defined central values and steps of three variables as follows: pH= 7±1, Temp = 40±5°C, and Time = 35±10 minutes.

The productivity prediction model is represented by a full quadratic equation:

$$Y = \beta + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32}$$

Where: Y is the productivity (mg/g); X_1, X_2, X_3 are respectively pH, temperature, and time; β_1, β_2, β_3 are the corresponding first-order coefficients; β_{12}, β_{13}, β_{23} are the interaction coefficients of each pair of corresponding factors; β_{11}, β_{22}, β_{33} are the corresponding quadratic coefficients.

2.3 Determination of Phycocyanin Content and Purity

Dried biomass was mixed in Ammonium chloride 0.05M solvent (w:v ratio was 1:50). Ultrasound treatment was then performed at frequency 37 kHz. Factors including temperature of ultrasonic bath (35-45°C), treatment duration (20-50 min) and pH levels of solvent (6-8) were adjusted accordingly as shown in Table 1. The blue supernatant is obtained after a refrigerated centrifuge at 10,000 rpm in 30 minute. Optical density (OD) of the extract is determined at 615 nm and 652 nm using UV-VIS (Jasco V750). Phycocyanin content (PC [mg/ml]) is calculated according to the equation of Bennett and Bogorad [15]:

$$PC = \frac{OD_{615} - 0.474 \times OD_{652}}{5.34}$$

Extract purity (EP) and extraction yield (Y [mg/g]) is obtained as followed equations [16]:

$$Y(mg/g) = \frac{PC \times V}{DB};\ EP = \frac{OD_{615}}{OD_{280}}$$

with V is volume of solvent (mL), DB is dried biomass (g) and OD615, OD280 are optical density of extract measured at 615 nm and 280 nm accordingly.

2.4 Data Analysis

Central composite design experiment and response surface model are developed using *package {rsm}* in R [17]. The process of descriptive statistics and data analysis are all performed on the R platform [18].

3. RESULTS AND DISCUSSION

3.1 Extraction of Phycocyanin under Various Conditions

Yield and purity of phycocyanin extracted from *A. platensis* using UAE method were displayed in Table 1.

Table 1. Yield and purity of PC extract from *A. platensis* under different conditions

Coded levels			Actual values			Yield of extraction (mg/g)	Purity
pH	T (^{0}C)	t (min.)	pH	T (^{0}C)	t (min.)		
0	0	0	7	40	35	31.48	0.878
1	-1	-1	8	35	25	26.91	0.857
0	0	0	7	40	35	30.56	0.828
-1	1	-1	6	45	25	28.33	0.792
1	1	1	8	45	45	28.04	0.891
0	0	0	7	40	35	31.51	0.916
-1	-1	1	6	35	45	31	0.879
0	0	0	7	40	35	31.42	0.843
1.5	0	0	8.5	40	35	28.14	0.908
0	1	0	7	45	35	29.02	0.801
-1.5	0	0	5.5	40	35	30.12	0.908
0	0	0	7	40	35	31.83	0.913
0	0	-1.5	7	40	20	30.13	0.822
0	-1	0	7	35	35	30.89	0.930
0	0	0	7	40	35	31.41	0.872
0	0	1.5	7	40	50	31.37	0.896
Average						**30.14 ± 1.55**	**0.87 ±0.04**

The highest yield of PC extraction (31.83 mg/g) was achieved when pH of solvent was 7 and ultrasound was applied at 40°C in 35 minutes. Meanwhile, the extraction yield was the lowest (26.91 mg/g) in treatment with pH = 8, temperature = 35°C, and time = 25 minutes. The average value was 30.14 ± 1.55 mg/g. The purity of PC extract changed slightly when extraction factors varied, ranging from 0.79 to 0.93, with the average value being 0.87 ± 0.04.

Ultrasound-assisted extraction was proved to be an efficient, cost-effective, and simple method to obtain phycocyanin extract from *A. platensis* biomass. Izadi and Fazilati utilized this approach to extract PC from Spirulina using distilled water, achieving a yield of 5.95 mg/g, which is much lower than our result (30.135 mg/g). Kissoudi et al. applied an additional step of ultrasonication after freeze-thaw cycles and reached very high yields of 58.84 - 66.44 mg/g with EP = 0.67-0.95 [19]. Extraction yield of this study was much higher than ours while extract purity was quite equivalent. This could be attributed to the difference in quality of microalgal biomass as well as solvent used.

Purity of PC extract from *A. platensis* reported in current publications had a wide range of fluctuation. The average value in this study was 0.871, higher than results from studies of Martelli et al., 2014 (EP=0.45) [20], Santiago-Santos et al., 2004 (EP = 0.4) [21]; Soni et al., 2008 (EP = 0.42) [22]; Kumar et al., 2014 (EP = 0.75) [23] but lower compared to those of Prasanna et al., 2007 (EP=1.18) [24] and Manirafasha et al., 2017 (EP=1.5) [25]. A research study by Devi et al., 2020, achieved an impressive result on both extraction yield and extract purity, being 44.5 mg/g and 2 respectively. In that study, phycocyanin was derived from the dried biomass of *Arthrospira maxima*, with extraction in phosphate buffer being ultrasonic assisted in 4 minutes [26].

A. platensis is a cyanobacteria having a multilayered cell wall, which makes extraction difficult [1,27]. The ultrasonic cell disruption step was thus proposed to increase PC extraction efficiency. In fact, the effectiveness of the UAE method is still controversial, for example, Vuong found that UAE possessed no positive effect on extraction efficiency [28], while Kadam et al. reported the opposite result [13]. Overall, our research findings were in agreement with the latter, which demonstrated the potential of this ultrasound-assisted method, because of its simplicity, efficiency, and time-savings, while still obtaining a good yield of moderate-purity phycocyanin. It should be emphasized that the purity of the extract can still be enhanced by the purification steps after.

3.2 Optimization of Extraction Yield by Response Surface Method

Based on the experimental result, a full quadratic regression model (*) was built with 3 variables including ultrasonic temperature and time, and pH of solvent. Regression coefficients and statistical test values were shown in Table 2.

$$Y = 31.38 - 0.66x1 - 0.94x2 + 0.41x3 + 0.54x1x2 + 0.55x1x3 - 0.44x2x3 - 1.01x1^2 - 1.48x2^2 - 0.29x3^2 \quad (*)$$

It was noticeable that 93.67% of the variation in extraction yield can be explained by the change in the three investigated variables, namely solvent pH, sonication time, and extraction temperature, with a confidence level being 99.99% (p-value = 0.0004 << 0.05). The lack of fit value = 0.804 > 0.05 demonstrates that the difference between the model-predicted yield and the experimental yield is not significant, indicating that this model can be used to predict the extraction yield when changing pH, temperature, and time.

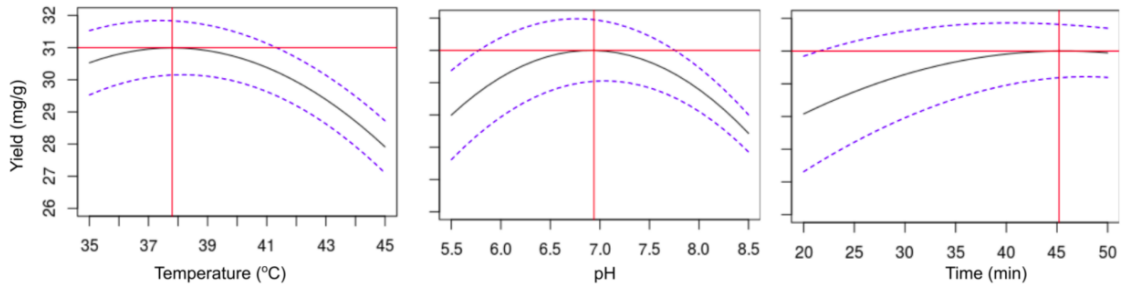

Fig. 1. Single effect of temperature, pH and time on phycocyanin extraction yield

Table 2. Regression coefficients for quadratic model of phycocyanin extraction yield

Parameters	Predicted coefficients	p - value
β	31.38417	7.868*1E-13 ***
pH (x1)	-0.66	0.01159 *
Temp (x2)	-0.935	0.014775 *
Time (x3)	0.41333	0.06595.
pH:Temp (x1: x2)	0.5367	0.0926.
pH:Time (x1 :x3)	0.55	0.1552
Temp:Time (x2 :x3)	-0.435	0.1563
pH^2 $(x1)^2$	-1.01241	0.0002125 ***
$Temp^2$ $(x2)^2$	-1.48262	0.0003619 ***
$Time^2$ $(x3)^2$	-0.29241	0.0617.
Adjusted R-squared	**p-value**	**Lack of fit**
0,9367	0,0004079***	0,804316

***Value is statistically significant with confidence (.): 90%; (*): 95%; (**): 99%; (***): 99.99%*

In our model, temperature (x_2) had the greatest influence on the yield of phycocyanin extraction, with coefficients being -1.483 for the second-order function and -0.935 for the first-order function with 95% confidence (Table 2). If the time is fixed at 45.2 minutes and the solvent pH is 6.94, the phycocyanin yield will increase from 30.5 to 31 mg/g when the temperature is increased from 35°C to 37.8°C, then gradually decrease to 27.9 mg/g as temperature is further increased to 45°C (Fig. 1). Temperature is considered a very important factor in the extraction process, not only for phycocyanin in Spirulina [6,9] but also for bioactive compounds in other plant species [28–30]. An increase in temperature can lead to a higher yield of bioactive compounds since temperature increases the cell damage as well as the solubility of the compounds in the solvent [29]. However, overheating solution during extraction can also cause denaturation of target compounds. For example, temperatures above 50°C will denature proteins and reduce the diffusion rate of solvents into cells, leading to a significant reduction in the phycocyanin content obtained during extraction [8]. This was also noted in our study. The structure of phycocyanin found to be destroyed by coagulation of phycobiliproteins at high temperature was observed in studies of Sarada et al. and Antelo et al.'s study [10,12].

pH also had an obvious effect on extraction yield. The regression constants corresponding to variables pH^2 and pH in the yield prediction model were -1.012 and -0.66 with p-value < 0.05, respectively. The neutral pH range (7±0.5) gave the highest phycocyanin extraction yield, while the solvent becoming alkaline or acidic reduced the amount of phycocyanin extracted. Sarada has stated that phycocyanin is stable at pH range of 5 to 7.5 at a temperature range of 27±2°C, and at pH above or below this range, the phycocyanin solution will lose its color [12]. According to Duangsee, at low pH, the soluble phycocyanin molecules are altered due to the breakage of hydrogen bonds and salt bridges [31]. Similarly to temperature, too high or too low pH can also lead to protein denaturation.

Regarding time, the extraction yield increased proportionally with the duration of sonication, reaching the maximum value at around 45 minutes. Longer extraction time did not significantly increase the yield and may even cause a reduction (Fig. 1). In the study of Tavanandi, which evaluated the yield and purity of phycocyanin extract from Spirulina dry biomass, the author recommended extraction for more than 8 minutes to achieve higher extraction efficiency [32]. Hadiyanto and Suttrisnorhadi reported that phycocyanin yield gradually increased with increasing extraction time up to 20 minutes [33]. Since the degree of cellular disruption caused by ultrasonic waves is stable throughout the sonication period, a sufficiently long time is required to ensure that the target compound will be continuously extracted from the cell into the solvent until saturation is reached [8]. According to Şahin and Samlı, the extraction process occurs in two consecutive stages: first "washout", in which the compound is rapidly dissolved into the solvent, and then "slow diffusion" - the diffusion of a bioactive compound into a solvent at a slower rate [34]. Increasing extraction time, in general, results in a higher content of the required compounds extracted, yet when combined with high temperature, the effect can be opposite [13].

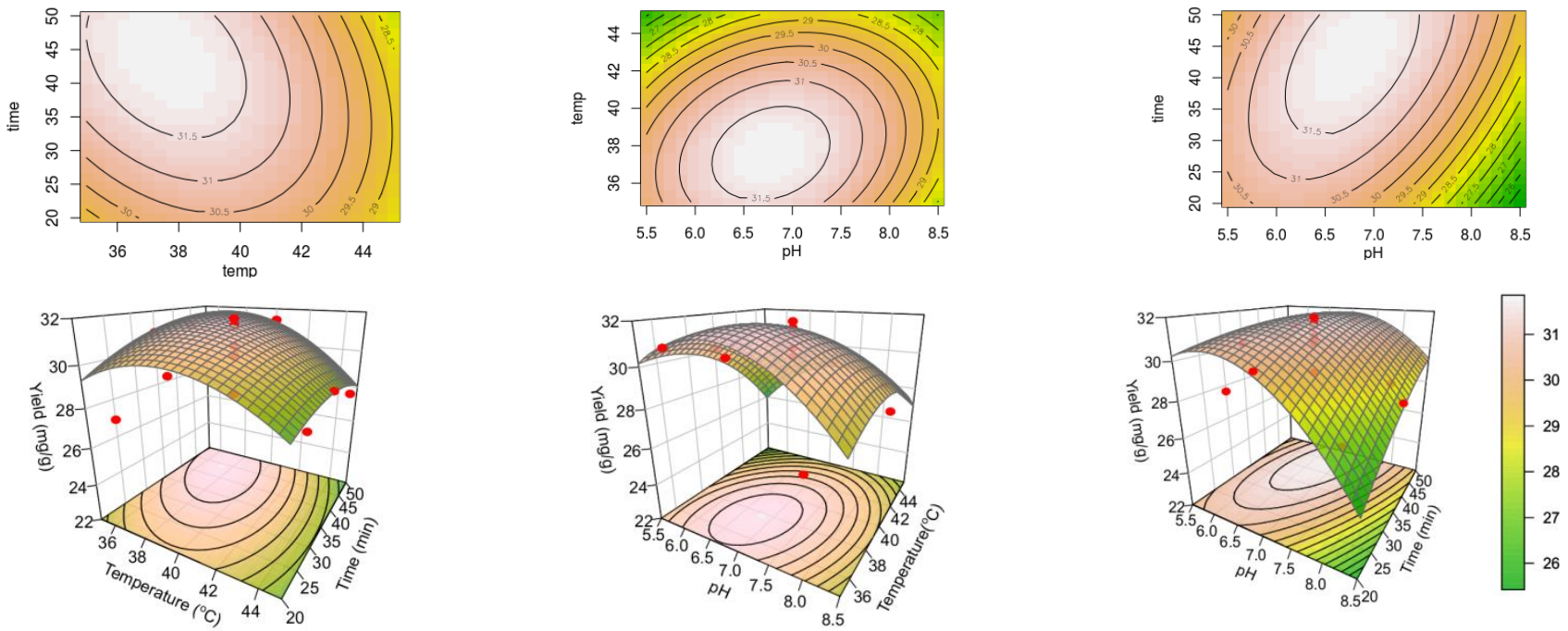

Fig. 2. 2D and 3D Response surface of phycocyanin extraction yield under phycocyanin combined effects of temperature, solvent pH and sonication time

Table 3. Phycocyanin extraction yield obtained from predictive model and from experiment

Variable	Optimal value of variables (Xopt)		Optimal yield (Yopt)	
	Coded value	Actual value	Predicted	Experiment
pH	-0.22	6.8		
Temperature	-0.48	37.5 (^{0}C)	Y = 31.85	Y = 31.72 ± 1.07
Time	0.86	44 (min)	(mg/g)	(mg/g)

An optimal set of extraction conditions had been achieved, namely x_1=-0.221, x_2=-0.481, and x_3= 0.857, corresponding to pH=6.78, temperature= 37.6^0C, and time=43.57 minutes. With these values, the maximum extraction yield predicted based on equation (*) is 31.85 mg/g.

To verify this prediction, an additional experiment with the optimal combination of variables was performed with 3 replicates. The average extraction yield from the experiment was 31.72±1.07 mg/g (Table 3), being approximate to the predicted value.

4. CONCLUSION

This study found that phycocyanin extraction yield ranged from 26.91 to 31.83 mg/g (average 30.14 ± 1.55) with a purity of 0.792-0.930 (average 0.87 ± 0.04), when the extraction temperature ranged from 35ºC to 45ºC, sonication time was 20–50 minutes, and solvent pH was 6–8. The response surface model showed that these three factors significantly affected the extraction yield, with a coefficient of determination of 0.937 (p-value << 0.001). An optimal set of conditions for achieving maximum yield was proposed: pH = 6.78, temperature = 37.6ºC, and time = 43.57 minutes, corresponding to a yield of 31.85 mg/g.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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