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Box-Behnken Optimization and Characterization of Ultrasonic-Assisted Extraction of C-Phycocyanin from *Spirulina platensis*

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Abstract

C-phycocyanin is a natural pigment widely used as food, solar cells, feed additive, cosmetic and pharmaceutical products. The current study investigates the simple, economic and rapid ultrasonic-assisted extraction (UAE) procedure was developed and response surface methodology (RSM) based to optimize C-phycocyanin from *Spirulina platensis*. Several variables that can potentially use to extraction of pigments, namely solid: liquid ratio, ultra-sonication time (h), microwave power and the number of cycles were optimized by means of a RSM approach. The predicted optimal conditions for the highest pigment yield were found at 1:10 (g/mL) solid: liquid ratio, 12.5 min sonication time, 100% microwave power, and 5 cycles. FT-IR spectroscopy analysis used to identify the chemical changes of the before and after pigment extraction. Therefore, this methodology could be used for extracting C-phycocyanin from algae in a faster and effective manner.

Keywords

Spirulina platensis; C-phycocyanin; ultrasonic- assisted extraction; response surface methodology.

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INTRODUCTION

Natural pigments derived from plants, algae, bacteria have created a Centre of attraction for their usefulness in various industries such as food, cosmetics, pharmaceuticals and pharmaceuticals. Colorants are extensively used in the food industry (Setyoningrum et al. 2015; Prakash Marana et al. 2015). *Spirulina platensis*, a blue-green microalga (cyanobacteria), is an important representative of these microorganisms. *S. platensis* is a free-floating filamentous cyanobacterium characterized by cylindrical, multicellular trichomes in an open, left-hand helix. They occur naturally in tropical and subtropical lakes with high pH and high concentrations of carbonate and bicarbonate (Mahdieha et al. 2012). *S. platensis* possess a wide range of colored compounds, including chlorophyll, carotenoids and phycobilli proteins. The phycobilliproteins such as C-phycocyanin (C-PC), allo-phycocyanin and phycoerythrin, are made up of dissimilar α and β polypeptide subunits (Rachen et al. 2009; Raja et al. 2008). C-PC has been widely used in commercial applications in the food, solar energy conversion and cosmetic industry as a natural blue dye (Setyoningrum and Nur 2015).

In recent years, various novel extraction methods have been introduced for the extraction of active components from algae, such as ultrasonic-assisted extraction (UAE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) accelerated solvent extraction (ASE) and enzymatic extraction (Vinatoru 2001; Khoddami et al. 2013; Sivasankari et al 2014). Among these methods, the UAE is an efficient extraction technique due to shorter extraction time stimulated by its acoustic cavitation power. The ultrasound irradiation generates a mechanical effect which increase solvent penetration into the plant material and increase the contact between solvent and plant tissue which accelerate diffusion of extracted solid compound to the solvent (Rostagno et al. 2003; Barba et al. 2015). Therefore, the UAE has been widely employed to extract natural compounds, including extraction of phycocyanin (Hadiyanto and Suttrisnorhadi 2016;

Setyoningrum and Nur 2015), astaxanthin (Zou et al. 2013), β-carotene (Dey et al. 2013), betacyanin (Prakash Maran and Priya 2015).

Response Surface Methodology (RSM) based Box- Behnken design (BBD) is a statistical method to which serves as an accurate, effective, and simple tool for optimizing the experimental process (Govarthanan et al. 2014); Selvam et al. 2014) and is widely used in agriculture, biology, food, chemistry and other fields (Jixiang et al. 2013). The RSM is time and cost effective tools to describe influences and interactions of variables to the responses and to evaluate the key parameters (Box and Wilson 1951; Wang et al. 2011). This present study focuses to investigate the effect of UAE variables: liquid: solid ratio, time, microwave power, number of cycles and frequencies in extraction of phycocyanin from microalgae *S. platensis* and then to employee RSM as a useful engineering tool to optimize process conditions in order to achieve high yield of phycocyanin.

MATERIALS AND METHODS

Materials

Spirulina platensis was obtained from *Spirulina* cultivation centre, Mahendra institution, Kalippatti, Namakkal, Tamil Nadu, India. Methanol (99.8%) and other chemicals used were of analytical grade were obtained from Merck. De-ionized water was utilized throughout the study.

Spirulina Cultivation

Spirulina platensis was cultivated with medium *spirulina* ($NaHCO_3$ - 4.5g, $K2HPO_4$ - 0.5g, $NaNO_3$ - 1.2g, K_2SO_4 – 1g/L; pH 11) at 28 °C under 3000 LX light and 10 L/12 Days photoperiods and was trained for 20 days (Olguin 2003) for the experiment.

Ultrasound assisted extraction (UAE)

Pigments were extracted from *S. platensis* under ultrasonic assisted extraction (UAE) method. Take 1g of *S. platensis* powder was mixed with various volumes of methanol Solid: liquid ratio ranging from 1:5 to 1:15 (g/mL). The suspension was sonicated using a sonicator with various times (5-20 min), microwave power (30-100%) and number of cycles ensuring complete breakdown of cell and complete extraction of pigments. The sample was subsequently centrifuged at 3000 rpm for 15 min. The supernatant containing the pigments was used for characterization. All the experiments were performed in triplicate and the average value was used for the determination of PC content from *S. platensis*.

Experimental design and statistical analysis

Box- Behnken design was used to evaluate the optimum conditions for the extraction process. Statistical analysis was conducted by using Design Expert software 9.0.1. Four parameters selected were solid: liquid ratio, sonication time (h), microwave power and number of cycles were studied at three levels (+1, 0, -1) (Table 1). A total of 29 runs were performed to optimize the process parameters, and experiments were carried out in accordance with the experimental design matrix.

A generic form of the second-order polynomial equation was given as follows

$$Y = \beta_0 \Sigma \beta iXi + \Sigma \beta iXi \beta ij + \Sigma XiXj \quad (1)$$

Where, Y represents the response variable, β_0, βi, and βij is a constant is the linear, quadratic and interactive coefficients, respectively. *Xi* and *Xj* are the levels of the independent variables. The software uses this quadratic model to build the response surface. The adequacy of the model was determined by evaluating the lack of fit. Coefficient of determination (*P*-value) and the Fisher test value (*F*-value) obtained from the analysis of variance (ANOVA) that was generated by the software. 3D plots were generated by varying two variables within the experimental range and holding the other constant at the centre point.

Table 1: Independent variables, their factors and actual values used for the optimization process.

Variables	Factors	Levels		
		-1	0	1
Solid: liquid ratio	A	1:5	1:10	1:15
Sonication time (min)	B	5	12.5	20
Microwave power (%)	C	30	65	100
No. of cycle	D	1	3	5

Determination of total phycocyanin yield (PC)

The extracted concentration of PC was determined according to Bennet and Bogorad (1973) and Patel et al. (2005);

$$PC = \frac{(OD620 - 0.474 OD652)}{5.34} \quad (2)$$

Where, PC is the PC concentration (mg/mL), OD620 is the optical density of the sample at 615 nm, OD652 is the optical density of the sample at 652 nm using an Elico - 164 UV/VIS Spectrophotometer.

The yield of the extraction was defined as (Silveira et al. 2007):

$$\text{PC Yield } \left(\%\frac{g}{g}\right) = \frac{PC \times V}{DB} \times 100\% \quad (3)$$

Where, V is the solvent's volume (mL), DB is the biomass (dry weight-g).

Determination of protein

The total protein contents were determined according to Lowry's method (Lowry et al. 1951).

Fourier-transform infrared spectra (FT-IR) analysis

FT-IR spectroscopy used to determine the functional groups present on the pigment surface. Dried samples of *S. platensis* and *S. platensis* based PC were mixed with spectroscopic-grade germanium-coated potassium bromide (KBr) powder and then ground and pressed into pellets for FT-IR measurement. FT-IR spectra were recorded with a Nexus 470 FT-IR spectrometer (Shimadzu, Japan) at the frequency range of 4000–400 cm^{-1} (Ying et al. 2016).

Determination of C-PC using High Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique that can separate a mixture of compounds and is used to identify, quantify and purify the individual component of the mixture. HPLC analysis was carried out on Shimadzu LC-20AD Prominence (Shimadzu Corporation, Japan) equipped with LC-20AD pump, RF-10A-XL fluorescence detector, column oven (CTO-10AS VP), auto-sampler SIL-20AC HT, and LC solution version 1.24 SP1. HPLC analysis of PC and its fraction was performed using the methodology of Anjum et al. (2012). About 1 mg of concentrated sample was dissolved in 1 mL of HPLC grade methanol and 20 µl was injected to determine the C-PC.

Statistical analysis

The Stat-Ease software (Design expert 9.0.1 software trial, Delaware, USA) was employed to analyze experimental data and to calculate the predicted response.

RESULTS AND DISCUSSION

Response surface optimization

Response surface optimization (RSM) is more advantageous than the traditional single parameter optimization in that it reduces time, cost and raw materials (Selvam et al. 2014). A total of 29 runs were needed for optimizing the four individual parameters in the current Box-Behken design. Table 2 shows the experimental conditions and PC extraction yield results according to the BBD design. The maximum PC extraction yield of (19.4 mg/g) was recorded under the experimental conditions of ultrasonic assisted extraction (UAE) extraction, solid: liquid ratio 1:10 (g/mL), ultrasonic extraction time 12.5 min, microwave power 100% and number of cycles 5.

Table 2: Box-Behnken design conditions and measured yield of pigment.

Run	Solid: Liquid radio(g/ml)	Sonication time (min)	Microwave power (%)	No. of cycle	Pigment yield (mg/g)
1.	1:15	5	65	3	13
2.	1:5	12.5	30	3	14.3
3.	1:5	20	65	3	15.6
4.	1:10	12.5	100	1	16.8
5.	1:10	12.5	65	3	17
6.	1:15	20	65	3	14.8
7.	1:15	12.5	65	1	13.6
8.	1:10	12.5	65	3	17
9.	1:10	12.5	100	5	19.4
10.	1:15	12.5	30	3	15.6
11.	1:10	12.5	65	3	17
12.	1:10	12.5	65	3	17
13.	1:10	12.5	65	3	17
14.	1:10	5	65	5	13.5
15.	1:10	5	30	3	12.8
16.	1:10	20	30	3	14.1
17.	1:15	12.5	65	5	16.5
18.	1:10	12.5	30	5	14.8
19.	1:10	5	65	1	12.6
20.	1:10	5	100	3	17.4
21.	1:10	20	100	3	16.7
22.	1:5	12.5	100	3	16.8
23.	1:10	20	65	1	15.3
24.	1:5	12.5	65	5	16.3
25.	1:5	5	65	3	13.6
26.	1:10	12.5	30	1	14.6
27.	1:10	20	65	5	16.2
28.	1:5	12.5	65	1	15.2
29.	1:15	12.5	100	3	18.3

Model Fitting

Based on the RSM model, the empirical relationship between the input variables and experimental results obtained were reflected in a second-order polynomial equation with interaction terms. By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following quadratic equation:

$$\text{PC yield} = 17.00 + 0.000\,A + 0.82B + 1.60C + 0.72D - 0.050AB + 0.050AC + 0.45AD - 0.50BC + 0.000BD + 0.60\,CD - 0.87A^2 - 1.87B^2 + 0.12\,C^2 - 0.73\,D^2 \quad (4)$$

Where, Y A is solid: liquid ratio 1:10 (g/mL), B is an ultrasonic extraction time 12.5 min, C is microwave power 100% and D is the number of cycles 5. Analysis of variants (ANOVA) was used to evaluate the significance of the coefficients of the models (Yuan et al. 2008). The regression coefficient values of an equation are given in Table 3. The significance of the developed models was concluded through ANOVA, where the $p < 0.0001$ indicates the high significance. Values greater than 0.1000 indicate the model terms are not significant. Along with very low probability value ($p<0.0001$) for the Fisher's values (*F* values) were found to be 10.70 for PC, which in turn exhibits the adequacy of quadratic models (Prakash Maran et al. 2013). The *P*-values were invoked as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The lack of fit 0.71 and its associated *p*-value < 0.0001 was significant which showed that the fit of the proposed statistical models.

Table 3: Analysis of variance (ANOVA) for the fitted second order polynomial model.

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
Model	75.90	14	5.42	10.70	< 0.0001
A-Solid:liquid radio	0.000	1	0.000	0.000	1.0000
B-Sonication time	8.00	1	8.00	15.80	0.0014
C-Microwave power	30.72	1	30.72	60.63	< 0.0001
D-No of cycles	6.16	1	6.16	12.16	0.0036
AB	0.010	1	0.010	0.020	0.8903
AC	0.010	1	0.010	0.020	0.8903
AD	0.81	1	0.81	1.60	0.2267
BC	1.00	1	1.00	1.97	0.1819
BD	0.000	1	0.000	0.000	1.0000
CD	1.44	1	1.44	2.84	0.1140
A^2	4.97	1	4.97	9.80	0.0074
B^2	22.80	1	22.80	45.01	< 0.0001
C^2	0.10	1	0.10	0.20	0.6615
D^2	3.41	1	3.41	6.73	0.0212

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
Residual	7.09	14	0.51		
Lack of Fit	7.09	10	0.71		
Pure Error	0.000	4	0.000		
Cor Total	82.99	28			

The predicted R^2 (0.5077) and adjusted R^2 (0.8291) values for optimal conditions were in reasonable agreement with the value of R^2 (0.9145), which is closer to 1.0, indicating the better fit of the model in the experimental data (Govarthanan et al. 2014).

Analysis of response surfaces

Response surface methodology plays an essential role in identifying the optimum values of the independent variables efficiently, under which dependent variables could achieve a maximum response. In the 3D plot, the extraction yield of PC was obtained along with two continuous variables, while the other two variables were detained constant at their respective zero level (center value of the testing ranges) (Prakash Maran & Priya 2013). The Fig. 1 depicts the response surface plots of UAE based extraction of PC. These plots can be useful in understanding both the main and interaction effects of the independent variables on the response (Ghitescu et al. 2015).

Fourier Transform Infrared (FT-IR) spectral analysis

The FT-IR spectral analysis needed to be done to identify the major functional groups present in the extracted pigment (Amit Kumar et al. 2013). In (Fig. 2 a, b) the FT-IR spectrum of the raw extract and pigment obtained from *S. platensis* is presented. The FT-IR spectra of pigment situated 3429.23 cm^{-1} corresponds to the stretching vibrations of amine (protein) groups (N-H) and the deformation vibration of the C–H bonds in the aliphatic groups absorb in the region 2924 cm^{-1}. The signal 1640 cm^{-1} may be allocated to amide (C=O stretching) vibrations (Patel et al. 2005). The band at 1610 cm^{-1} can be assigned to symmetrical and asymmetrical stretching vibration for the carboxyl ion (COO) indicating the existence of carboxylic acid ester or carbonyl groups.

High Performance Liquid Chromatography (HPLC) analysis

The qualitative and quantitative analysis of C-PC by HPLC of *S. platensis* were evaluated. The major C-PC identified the peak area, retention time (RT) was shown in Fig. 3. The RT for 1.94 min it indicates the presence of C-PC compounds.

Figure 1. 3D surface plot.

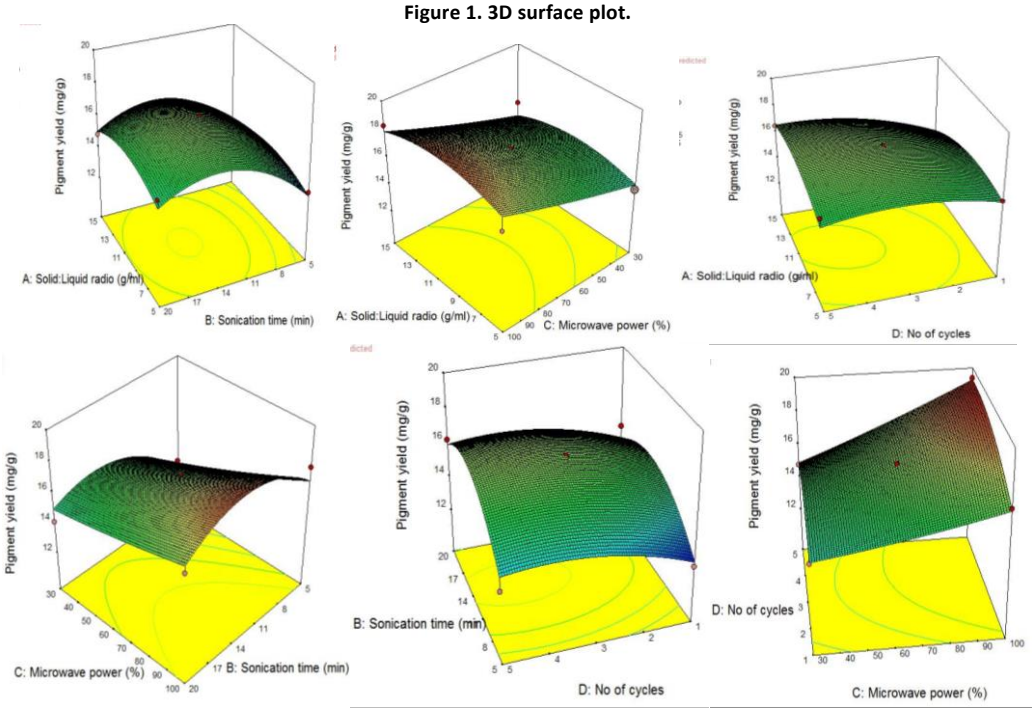


Figure 2. FTIR analysis spectrum for the blue pigment extracted from *S. platensis.*

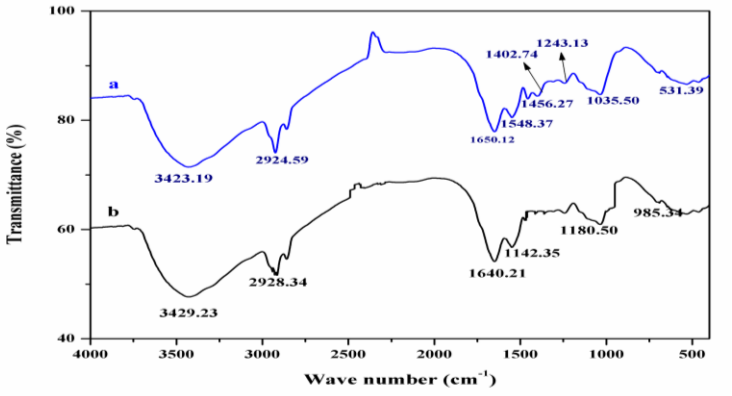
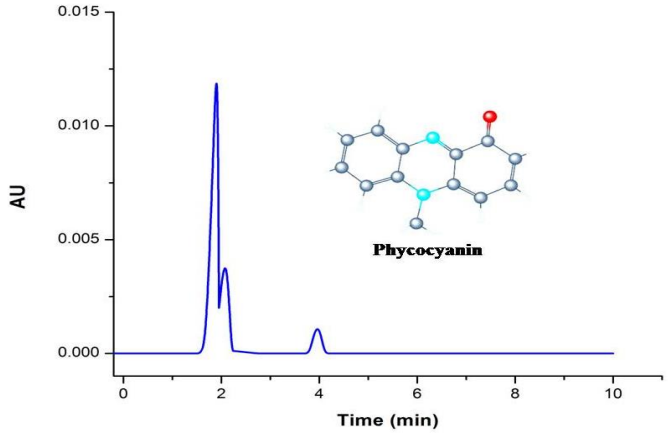


Figure 3: HPLC profile of C-phycocyanin from *Spirulina platensis.*



CONCLUSIONS

The C-phycocyanin (PC) was extraction assisted by ultrasonic assisted extraction (UAE) and its optimization has been studied for *S. platensis*. UAE was just an efficient tool to improve the PC extraction through response surface methodology (RSM). The maximum yield PC obtained for optimum process conditions for solid: liquid ratio (1:10 g/mL), sonication time (12.5 min); microwave power (100%); number of cycles (5). Under these optimal conditions, the yield of PC was 19.4 mg/g. FT-IR analysis of pigment showed the presence of nitrogen (N–H) amine group and confirmed that the extracted pigment belongs to phycocyanin.

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