Intensified Phycobiliprotein Extraction from *Spirulina Platensis* by Freezing and Ultrasound Methods

Najafian, Nahid; Arabi, Azam*[+]

Department of Food Science and Technology, Shahreza Branch, Islamic Azad University, Shahreza, I.R. IRAN

Nezamzadeh Ejhieh, Ali Reza

Department of Chemistry, Shahreza Branch, Islamic Azad University, Shahreza, Isfahan, I.R. IRAN

ABSTRACT: *In this study, the extraction of phycobiliprotein pigments from Spirulina Platensis dried powder was optimized using ultrasound-assisted extraction (ultrasonic water bath) combined with the freezing-thawing method. Response surface methodology (RSM) using Central Composite Designs (CCD) was employed to investigate the combined effect of two process variables of ultrasound time (30-90 min) and freezing-thawing time (5-12 h) on the amount of allophycocyanin (A-PC), C-phycocyanin (C-PC), phycoerythrin (PE), C-PC purity (EP), and the yield of C-PC. The purification of C-PC extracted from optimal and control conditions (extracted via freeze-thawing method without ultrasound) was performed, and the C-PC powder was evaluated by FT-IR spectroscopy and XRD. Results showed that at the optimal conditions of sonication time (55.5 min) and freezing time (5h), the average values of PE, A-PC, C-PC concentration, C-PC purity, and C-PC yield were (0.095±0.009), (0.051mg/g), (0.109±0.051mg/g), (0.498±0.25), and (2.32±0.21 %), respectively. The comparison of scanning electron microscopy images of algae biomass indicated that freezing alone and for a long time had caused more destruction of cells. The damage caused by ice crystals and large pores led to the production of extract with low purity. The ultrasonic pretreatment (5.5 min) for extracting the C-PC pigment from Spirulina Platensis compared to the control sample (12 h freezing without ultrasound) did not destroy the functional groups in the pigments.*

KEYWORDS: *Phycobiliproteins; Phycocyanin; Spirulina Platensis; Response surface, Optimization.*

INTRODUCTION

Nowadays, bioactive compounds are extensively utilized in food industries and pharmaceuticals as food additives and natural drugs to substitute chemicals that harm the health of humans. Many types of research have shown that microalgae have very high amounts of functional food components compared to plant and animal food products [1-3]. *Spirulina Platensis*is very interesting among these microalgae since it contains all nutrients and bioactive components needed daily by the organisms [4-5].

Spirulina Platensis is one of the critical multicellular

** To whom correspondence should be addressed.*
+ E-mail: aazam9531@yahoo.com & aarabi@iaush.ac.ir
1021-9986/2023/2/601-617 17/$/6.07

blue-green cyanobacteria that contains phycobilisomes as light-harvesting protein–pigment complexes. These pigments are mainly composed of 80 to 85% of brilliantly colored polypeptides named phycobiliproteins [5-6]. In general, phycobiliproteins (PBPs) consist of the chromophore that contains a and b subunits, which have a molecular weight of about 20 kDa [7-8] and are divided into three particular subclasses: allophycocyanin (A-PC), C-phycocyanin (C-PC), and phycoerythrin (PE).

The C-PC is an intense pigment since it comprises about half of the phycobiliprotein's weight [9], and it represents up to 20% of the Spirulina dry weight [7,10,11]. PBPs have a high fluorescence quantum field, so they have been used for fluorescence microscopy, immunoassays, and fluorescence-activated cell sorting [12]. C-PC provides green-blue color in the Spirulina algae [12] and is a nutraceutical compound with biological activity isolated or purified from sources. This pigment is used as a dietary supplement due to its high protein, vitamins, minerals, and essential fatty acids [13,14]. C-PC food-grade type is generally recognized as safe (GRAS) and has been used as a food colorant for ice sherbets, chewing gum, candies, milkshakes, gum, jellies, ice cream, and in cosmetics and medicine [12,15]. Furthermore, phycocyanin (C-PC) has been proven to have curative properties, including anticancer, anti-inflammatory, and antioxidant activities [16, 17].

Many methods have been performed to separate and purify PC from microalgae. Such non-thermal processes as freezing and thawing, acidification, and ultrasound treatment can be used to extract C-PC from *Spirulina Platensis;*moreover, other innovative techniques such as Pulsed Electric Fields (PEF) and High Hydrostatic Pressure (HHP) [5,18], and extraction with various solvents [2,19,20] can be employed in this regard. Unfortunately, most have not shown a good stability, high production yield, and high efficacy, although some processes require great equipment and lengthy processing times. Several researchers previously reported the extraction of PC from microalgae by using ultrasound technique with another common method (alone and in combinations) [2,3,21,22]. However, employing only one method will not achieve high efficiency of cell disruption. Hence, a combined method can be more effective in extracting phycobiliprotein [23].

Freezing-thawing has been mentioned as the most common method for extracting phycocyanin. This method involves freezing at -20°C and thawing (at room temperature). Freezing leads to the damage of algae cell wall. This cycle is reproducible and does not interfere with the protein's biological function [24]. However, the repetition of the freezing and thawing cycle is time-consuming and energy-intensive. Accordingly, although it can be done on a laboratory scale, it is not widely accepted on an industrial scale. On a laboratory scale, the ultrasonic technique is one of the more common techniques for cell disruption and extraction of many compounds from various matrices (microbial, plant, etc.…). It is an efficient extraction method for the cell lysis of microalgae [25].

Ultrasound is a sound wave with a frequency above the range audible to humans (above 20 kHz). The propagation of the ultrasonic acoustic wave in a liquid under certain conditions results in the collapse of gas bubbles called cavitation that induces shockwave. When the cavitation occurs near the surface of the sample matrix, the shockwave disrupts the cell wall sample and improves the mass transfer rates of intercellular material into t he solvent [27].

Previous studies have been conducted for extracting phycocyanin from Spirulina Platensis through ultrasound techniques. Results have shown that the ultrasound technique has a better effect on extracting phycocyanin from the cell structure [4,28-30]. However, no study has compared the combined effect of the freezing-thawing process and the ultrasonic process. Also, no research has been done on the effect of freezing and ultrasonic process on algal structure and structural properties of extracted phycocyanin.

The present study aimed to evaluate the effect of ultrasound-assisted methods as innovative green technologies in combining the freezing-thawing method for the effective extraction of Phycobiliproteins from *Spirulina Platensis*. Therefore, Response Surface Methodology (RSM) was used to investigate the effects of the two-process technique of ultrasound time (30-90 min) and freezing-thawing time (5-12 h) on the extraction of PE, C-PC, A-PC, extract purity (EP), and the extraction yield of C-PC. Also, the optimization of extraction conditions was performed using the response surface method in Central Composite Design (CCD). The effect of the extraction process on residue algae biomass and phycocyanin structure was evaluated.

EXPERIMENTAL SECTION

Materials

Microalgae *Spirulina Platensis* biomass with 10% moisture content was obtained from Sina Rizjolbake Qeshm company, Iran. The biomass was stored under dry and dark conditions. Ammonium Sulfate and all chemicals used in the experiments were provided from Merck Co., Germany.

Characteristics of algal powder

Before Phycobiliproteins extraction, the characteristics of the *Spirulina Platensis* biomass were analyzed. The dry solid and ash content were analyzed as the dry residue at 105 ºC and the combustion residue at 600 ºC, respectively, based on (AOAC 2000 and AOAC 923.03 methods). The protein and lipid content of *Spirulina Platensis* powder were evaluated based on the Association of Official Analytical Chemists (AOAC 954.01 and AOAC 920.39). Crude protein was calculated by multiplying total nitrogen by the conventional conversion factor of 6.25 [32]. Carbohydrate levels were evaluated by subtracting the sum of ash, moisture, protein, and lipid percentages from 100. All measurements were performed in triplicates and noted as dry basis weight (%).

Phycobiliproteins extraction

To extract phycobiliproteins (C-PC, A-PC, and PE) from *Spirulina Platensis*, the dry powder of this microalgae was mixed with distilled water (1:25 w/v). The first step, ultrasound pretreatment, was performed in the condition 80 kHz frequency and 100 W power of ultrasound bath (Elmasonic, P 60 H Ultrasonic cleaning unit, Germany), and the temperature of 40ºC. These parameters were constant for all samples, and the time of sonication was varied according to the experimental design treatment condition presented in Table 1.

To release C-PC, after ultrasonic treatment, the microalgae solution was placed in a freezer with a temperature of -20°C (according to Table 1), followed by thawing at 4°C for 24 h in a refrigerator. After freezing-thawing treatment, the supernatant was separated and centrifuged at 10000g for 15 min (using Centrifuge (KOKOSAN, H-11N, Tokyo, Japan)) at 4°C to remove the cell debris and the supernatant crude extract was collected [33,34]. The schematic diagram of Phycobiliproteins ultrasound-assisted extraction from *Spirulina Platensis* biomass is shown in Fig. 1.

Measurement of C-PC, A-PC, and PE Content

The quantity of three major phycobiliproteins in *Spirulina Platensis* extracts, C-PC, A-PC, and PE, were measured using the following method:

First, 100 mg of extracts were placed in a 15 ml tube and mixed with 10 ml of 0.1 M phosphate buffer (pH 7.0) for 1 min. The supernatants from centrifugation at 2000g for 5 min were collected, and the absorbance was measured at 562 nm, 615 nm, and 652 nm with UV-Vis Spectrophotometer (model: UNICO UV/VIS2100S, manufactured by the United States.). The amounts of C-PC, A-PC, and PE were calculated as the specific Optical Density (OD) using the following equations [19, 35]:

$$\left[\text{C-PC}\ (\text{mg/mL})\right] = \left[\text{O.D}_{615} - 0.474\ (\text{O.D}_{652})\right] / 5.34 \quad (1)$$

$$\left[\text{A-PC}\ (\text{mg/mL})\right] = \left[\text{O.D}_{652} - 0.208\ (\text{O.D}_{615})\right] / 5.09 \quad (2)$$

$$\left[\text{PE}\ (\text{mg/mL})\right] = \left[\text{O.D}_{562} - 2.41\ (\text{C-PC}) - 0.849\ (\text{APC})\right] / 9.6 \quad (3)$$

After measuring the contents of three phycobiliproteins components, each treatment results were analyzed by design expert software version 11, and optimal conditions by a 3-D contour or surface plots were predicted.

Extract Purity (EP) Ratio of C-PC

Purification of C-PC was done by using Ammonium sulfate. Ammonium sulfate was gradually added in 100 ml crude extracts to achieve 50% saturation with continuous stirring. The resulting solution was kept for five h and centrifuged at 10,000g for 20 min at 4°C. The obtained blue residue was dissolved in Na-phosphate buffer (0.005 M; pH- 7.0). Bennett and Bogorad's method was used to calculate the purity of C-PC extracts, according to Equation (4) [35].

$$\text{EP} = \frac{\text{OD620}}{\text{OD280}} \quad (4)$$

Concentration of C-PC

The concentration of C-PC was determined according to Equation (5)[35]:

Table 1. Central composite experimental design for the two independent variables with experimental (Exp.) values of the dependent variables

	Independent variables			Dependent variables			
Run	(X_1) Ultrasou. time (min)	(X_2) Freezing time (h)	C-PC (mg/ml)	A-PC (mg/ml)	PE (mg/ml)	EP (A620/A280)	C-PC Yield(%)
1	60.0	8.5	0.074	0.033	0.008	0.500	1.850
2	60.0	8.5	0.061	0.030	0.008	0.440	1.530
3	60.0	5.0	0.138	0.070	0.015	0.540	3.450
4	90.0	12.0	0.068	0.033	0.006	0.600	1.700
5	30.0	8.5	0.080	0.033	0.008	0.430	2.000
6	30.0	5.0	0.085	0.040	0.010	0.380	2.130
7	30.0	12.0	0.084	0.036	0.009	0.400	2.100
8	60.0	12.0	0.059	0.026	0.005	0.590	1.480
9	60.0	8.5	0.080	0.042	0.012	0.460	2.000
10	90.0	5.0	0.093	0.062	0.014	0.410	2.330
11	60.0	8.5	0.093	0.050	0.014	0.450	2.330
12	60.0	8.5	0.098	0.036	0.014	0.650	2.450
13	90.0	8.5	0.130	0.076	0.013	0.560	3.250
Control sample	-	12	0.079	0.031	0.0076	0.49	2.13

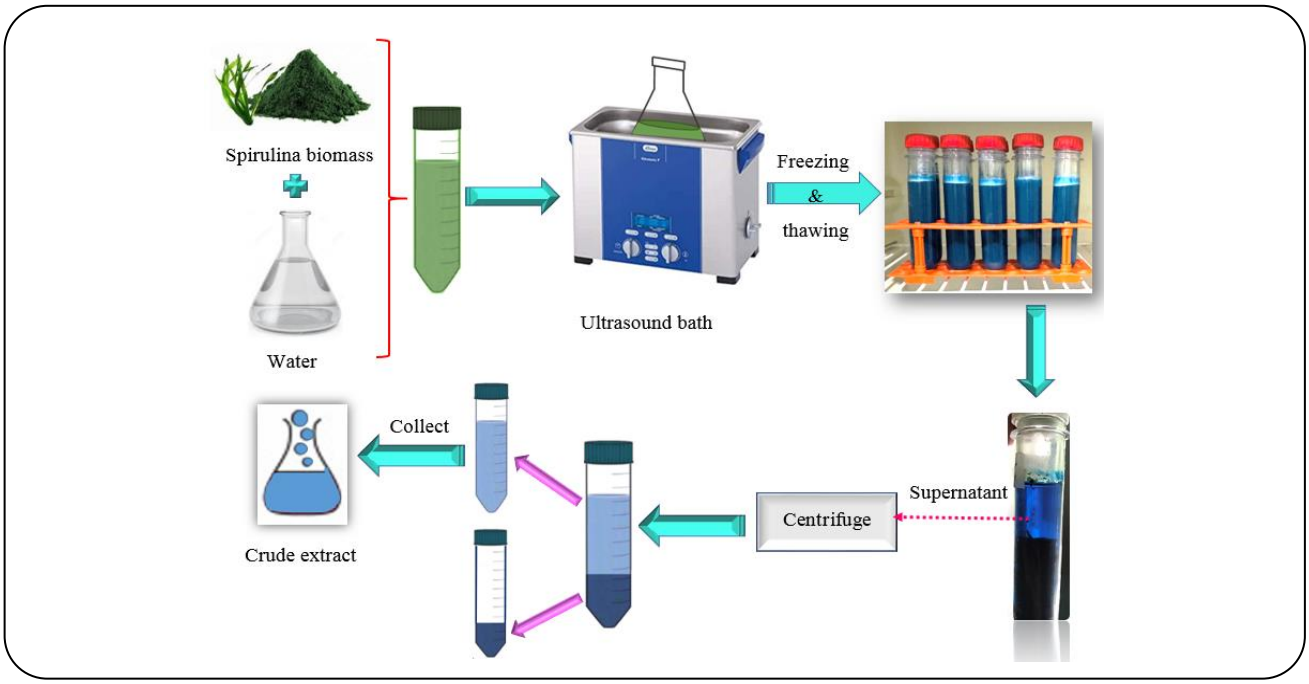

Fig. 1: The schematic diagram of Phycobiliproteins ultrasound-assisted extraction from Spirulina Platensis biomass.

$$\text{EC-PC}= \frac{\text{OD620}-0.474\text{OD652}}{5.34} \quad (5)$$

Where C-PC is the C-phycocyanin concentration (mg/mL), OD_{620} is the optical density of the sample at 620 nm, OD_{652} is the optical density of the sample at 652 nm.

Determining yield of C-PC extraction

To calculate the yield of the C-PC extraction, the following equation was applied [19].

$$\text{C-PC yield (\%)}= \frac{\text{C-PC}\times V}{DB} \quad (6)$$

Where V is the solvent's volume (mL), DB is the biomass (dry weight-g), and C-PC is the C-phycocyanin concentration (mg/mL).

Scanning electron microscopy (SEM)

To study the effect of the extraction method on *Spirulina Platensis* microstructure, the dried residues of *Spirulina Platensis* cellular structure changes were assessed by Scanning Electron Microscopy (SEM). SEM images of the *Spirulina Platensis* after two selective extractions (optimum treatment condition (ultrasonic (55.5 min) + freezing time (5 h)) and freezing time of 12 h) were taken by a scanning electron microscope (scanning electron microscope Philips XL30 ESEM FEG, Netherland). To obtain the SEM image, a thin layer of gold was covered on the surface of samples using a gold sputter coating machine. The microscope voltage was 12 kV, and images were provided with the magnification of 650 and 2000 times [37]. All experiments were done in triplicates.

Fourier transform infrared (FT‑IR) spectroscopy and X-ray diffraction (XRD) patterns

Fourier Transform InfraRed (FT-IR) spectroscopy (Model Spectrum PerkinElmer Spectrum 65, Norwalk, Connecticut, PerkinElmer, U.S.A) was used to investigate the molecular changes resulting from the ultrasonic and freeze-thawing treatment in the phycocyanin functional groups. The FT-IR spectra images of the samples were obtained by PerkinElmer Spectrum 65 FT-IR spectrometer (Model Spectrum 65, Norwalk, Connecticut, PerkinElmer, U.S.A) in the frequency range of 400-4000 cm-1 by KBr disk [38].

X-ray diffraction (XRD) patterns for samples were obtained using X-ray diffractometer with Cu K_radiation. The diffractometer was operated at 40 kV and 30 mA. All measurements were performed at room temperature, 25 ◦C, within a diffraction range of 0–60◦ at 1◦/s speed. The PANalytical X'Pert HighScore software (Almelo, The Netherlands) was also used to analyze different peaks.

Experimental design and optimization of process

RSM was used to determine optimum conditions for phycobiliproteins extraction from *Spirulina Platensis*. The experiments were designed according to the CCD, the most widely used form of RSM. There were two variables, including freezing-thawing time (X_1) and sonication time (X_2). Each factor was mentioned at three levels (−1, 0, 1). All variables were taken at a central coded value (Table 2). In this study, for the experimental design alpha=1 as the face center was obtained.

After experiments and tests, extract purity, PE, APC, C-PC concentration, and C-PC yield were evaluated as responses (Table 1). Eq. (7) shows the two-variable mathematical model:

$$yi=\beta 0\sum_{i=1}^{2}\beta ixi\sum_{i=1}^{2}\sum_{j=1}^{2}\beta ijxixj \quad (7)$$

Where y_i is the response, β_0, β_i, β_{ij} are the regression coefficients, and X_i *and* X_j is the independent parameter. The adequacy and fitting quality of equations obtained for dependent variables were tested using analysis of variance (ANOVA) with a p-value ≤ 0.05 considered significant.

The process optimization was performed using RSM with CCD and aimed to find the levels of independent variables that could give maximum C-PC and yield. The extraction studies were performed in triplicate under optimal conditions to verify the validity of the independent variables predicted by the polynomial model. The average values of the results were compared with the predicted value for each response variable to determine the accuracy and suitability of the model.

RESULTS AND DISCUSSION

Algal powder chemical composition

The chemical composition of algal powder (Spirulina Platensis) was mentioned in Table 3. All weights were calculated on a dried basis. The chemical composition of Spirulina Platensis depends on its growing conditions and

Table 2: Independent variables and their coded.

parameters	unit	symbols	Coded value of variables		
			-1	0	1
Ultrasound time	min	X_1	30	60	90
Freezing-thawing time	hour	X_2	5	8.5	12

Table 3: Proximate analysis of Spirulina Platensis powder (%).

Composition (w/w)	Dried basis
Ash	9.42 ± 0.03
Fat	1.28 ± 0.09
Protein	58.10 ± 0.09
Carbohydrate	29.0 ± 0.01
Fiber	1.96 ± 0.01

its production techniques. The culture media of Spirulina Platensis used in this work was Zarrouk's. The moisture of algae powder was 9.62 ± 0.04%; this value is higher than those reported by *Bensehaila et al.* (2015), (5.42 ± 0.031) and *Marrez et al.* (2016) (8.16 ± 0.03). The protein and carbohydrate of algae powder were 58.10 ± 0.09% and 29.00 ± 0.01%; respectively, these values are higher than those reported by *Marrez et al.* (2016), who reported that protein and carbohydrate of algae powder represented 51.92 ± 1.27% and 13.12 ± 0.69% of Spirulina dry weight, respectively. A similar finding of protein content (58.20 %) was reported by *Alvarenga et al.* (2011). Similar to our study, *Yucetepe et al.* (2018) found that the ash content of Spirulina was 9.50 ± 0.02% of dry weight. In comparison, it was lower than those reported by *Marrez et al.* (2014) (13.00 ± 0.43%). The fat content of algae powder was 1.28 ± 0.09% of dry weight. *Marrez et al.* (2014) describe that the total lipid of Spirulina dry weight was 6.57 ± 0.22% of Spirulina dry weight. The fiber content was 1.96 ± 0.01% of dry weight, which is lower than that obtained by *Marrez et al.* (2014) (5.23 ± 0.22%).

Model fitting by Response Surface Methodology(RSM)

The effect of two parameters as ultrasonic and freeze-thawing time on the quantity of three major phycobiliproteins in *Spirulina Platensis* extracts (C-PC, A-PC, PE), purity of Phycocyanin extract, and phycocyanin extraction yield were measured. According to a central composite design, a total of 13 treatments were obtained (Table 1) with five replicates at the center point to determine the pure error and repeatability of all data. Analysis of variance (ANOVA) of the fitted second-order polynomial model and coefficients of determination (R^2) for each dependent variable are shown in Table 4. The R^2-values were 0.90, 0.89, 0.95, 0.98, and 0.91 for EP, PE, C-PC, A-PC, and C-PC yield, respectively.

Models for all the responses were developed according to the ANOVA technique (Table 4). Various parameters, including model F-value, coefficient of determination (R^2), F-value (disproportion), and coefficient of variability (CV), were used to evaluate the adequacy of the model. A significant model has been suggested based on the comparison of F-values with the critical F-values ($F_{0.05,2,10}$=0.749, $F_{0.05,3,9}$=0.852) as indicated in Table 4. On the other hand, the proposed model can process more than 95% of the data. The value of P, model type, and model incompatibility were assessed at a significance level of 0.05, and the final equation for the model was proposed. A lower p-value indicates the higher significance of the corresponding coefficient, and $p \leq 0.05$ shows that the corresponding coefficient is highly significant, to expand the response surface models, all nominal terms, with $p > 0.05$, were eliminated. The final regression equations were developed (Eqs.(8–12)):

$$C-PC:(y) = -0.0237x_2 + 0.0922 \quad (8)$$

$$A-PC:(y) = 0.012x_1 - 0.0141x_2 + 0.0454 \quad (9)$$

$$PE:(y) = -0.034x_2 + 0.0111 \quad (10)$$

$$\text{Extract purity}\,(y) = -0.0405x_1 - 0.1318x_2 + 0.4869 \quad (11)$$

$$C-PC\ \text{Yield}:(y) = -0.3578x_2 - 0.25x_1x_2 + 2.16 \quad (12)$$

A significant lack of fit shows that the models failed to represent the data in the experimental domain, at which points were not included in the regression. The lack of fit was not significant for all variables ($p > 0.05$), meaning that all models accurately predicted the related responses (Table4). The smaller F-values can also confirm the

Table 4: Regression coefficients of predicted second order polynomial models for of the dependent variables.

Factor	Coefficient (β)				
	EP	PE	C-PC	A-PC	YIELD
Intercept	0.4869	0.0111	0.0922	0.0454	2.12
X_1: Ultrasound time	0.0583	0.0015**	0.0142*	0.0124*	0.1133
X_2: Freezing time	0.0433*	-0.0034**	-0.0237*	-0.0141*	-0.3652
Interaction					
x_1x_2	0.0420	-0.0022	-0.0168	--------	-0.2500
Adj. R^2	0.9095	0.8929	0.9539	0.9872	0.9101
F-value (model)	4.49*	6.77*	6.49*	7.73**	4.69*
Critical F-values	$F_{0.05,3,9}$=0.852	$F_{0.05,2,10}$=0.749	$F_{0.05,3,9}$=0.852	$F_{0.05,3,9}$=0.852	$F_{0.05,2,10}$=0.749
F-value (lack of fit)	0.2658	0.9487	0.2878	0.1510	0.8730
Critical F-values	$F_{0.05,5,4}$=1.04	$F_{0.05,5,4}$=1.04	$F_{0.05,6,4}$=1.06	$F_{0.05,5,4}$=1.04	$F_{0.05,6,4}$=1.06
P-value (model)	0.0345*	0.0378*	0.0154*	0.0455*	0.0275*

The β subscripts of 1 and 2 are related to ultrasonic time and freezing and thawing time.
** Significant at $p \leq 0.05$.*
*** Significant at $p \leq 0.01$.*
**** Significant at $p \leq 0.001$.*

insignificant LOF than the critical F-values reported in Table 4 ($F_{0.05,5,4}$=1.04 and $F_{0.05,6,4}$=1.06). The results suggest that the models used in this study were able to identify the extraction conditions for Phycobiliproteins extraction from *Spirulina Platensis*.

C-PC Concentration

The measure of C-PC concentration is presented in Table 1. The concentration of C-PC of samples was between 0.059 and 0.138 mg/g, and there were significant differences among the C-PC concentrations of the different treatments ($p < 0.05$). The maximum C-PC concentration was observed in run 10, with 90 min and 5-hour conditions in an ultrasound bath and freezing time, respectively (Table 1). As shown in Table 4, the results showed that the C-PC concentration was influenced linearly by the time of freezing and ultrasound processing ($p < 0.05$). The interaction of variables (freezing time ×ultrasound time) was not significant in this response. According to the coefficients obtained, the proposed model for C-PC concentration was defined following Eq. (8).

As can be seen in the equation Eq. (8), the negative coefficient of freezing time (x_1) and the interaction of variables ($x_1 \times x_2$) revealed that freezing time has a reverse effect on the extraction of C-PC. The interaction effect of ultrasound time and freezing time on C-PC concentration is presented in the 3-D surface plot of Fig. 2(a). The C-PC concentration of treatments rose by increasing the sonication time (min) and decreasing the freezing time (Fig. 2. A), so that the lowest concentration of phycocyanin is seen in the values of high freezing time (12 hours) and minimum ultrasonic time (30 min). Our results were similar to the data reported by *Aouir et al.* (2015), indicating that the C-PC of Phycopiliproteins from Arthrospira Platensis was raised by increasing ultrasonic time from 0 to 60 min. However, with additional extraction time from 60 to 90 min, the C-PC yield decreased after the ultrasonic time rose to 90 min [5]. This indicates that phycocyanin was destroyed by ultrasonic irradiation as long as the sonication time was appropriate in length. There was, therefore, almost a complete extraction of phycocyanin. *Hrishikesh* and co-authors (2018) evaluated the effect of ultrasound parameters besides amplitude (10–70%) and time (0–3.0 min). They reported that the yield increased with increasing ultrasound time and the results of their research were consistent with the results of this study [28].

Extracting the totality of a specific component from microalgae is often prevented by the intrinsic rigidity of

its cell wall. To overcome this barrier, an initial operation unit of cell disruption is required to permit complete access to the internal components and facilitate the extraction process.

As mentioned above, blue-green algal phycobiliproteins exist in supermolecular pigment aggregates and phycobilisomes, assembled in subunits composed of trimmers of each phycobiliprotein [40].

The Phycocyanin structure consists of a monomer formed by two helix-shaped subunits, called alpha (α) and beta (β), with one bilin chromophore attached to the α subunit and two of them to the β subunit. The monomers gather together to form a ring-shaped trimer (αβ), resulting in hexameric structures [(αβ)3]2 [41]. The chromophore, called phycocyanobilin, is responsible for the blue color of the molecule, and it consists of an open-chain tetrapyrrole group that binds proteins through a thioether bond (Fig. 2) [41]. In other words, both α and β subunits have a bilin chromophore. The bilin contains linear tetrapyrrole rings attached to the cysteine amino acid of the apoprotein by thioether linkages [42].

As mentioned above, blue-green algal phycobiliproteins exist in supermolecular pigment aggregates and phycobilisomes, assembled in subunits composed of trimmers of each phycobiliprotein. With sonication, the extraction behavior of phycocyanin from *Spirulina Platensis* follows the first-order kinetics concerning irradiation time of ultrasonic waves [40].

In microalgae extraction processes, ultrasonic waves assist in cell disruption and particle size minimization to enhance the release of its cellular contents. Also, the ultrasonic irradiation can improve the protein extraction efficiency after cell lysis [43].

Impact waves caused by cavitation, which include nucleation, growth, decay, and the formation of tiny bubbles in the fluid, all caused by changes in the bulk pressure due to ultrasonic technique, destroy the membrane of algal cells and release cellular material. Cavity results are affected by the physical effects of fine perturbations and velocity/pressure shock waves. Micro-turbulence caused a severe mixing, while shock waves disrupted the cell wall [44].

The other researchers have observed similar behaviors. Işılltera and co-authors optimized phycocyanin extraction from *Spirulina Platensis* using three methods (classical (freeze-thawing), ultrasonic, and microwave). They reported that PC was significantly influenced by the extraction time in all extraction methods. PC increased with time, as the temperature was constant throughout the experiment [20].

ANOVA results indicated that the effect of freezing time on C-PC concentration was greater than ultrasound time treatment (table 4). In the freeze-thawing process, the internal structure of *Spirulina Platensis* is altered and simultaneously facilitates the penetration of water to dissolve more proteins [40,45]. Safi et al. (2013) reported that the C-PC extraction from the cell breakage caused by freezing and biomass was quickly released. Also, he said that the ultrasound extraction destroyed the cell walls and, all biological components in the biomass were removed rapidly [45].

Forouki and co-authors reported that increasing the ultrasound time did not reduce the amount of phycocyanin and played an essential role in increasing its release and complete extraction [40]. According to these results, it can be said that in the current study, a vital factor for reducing the amount of phycocyanin is the freezing time; this is confirmed by the F-value of this variable (F-value = 11.52) and the negative sign of the coefficient in the proposed model (Eq. (8)) .

Results of A-PC concentration

According to the experimental data (Table 1), the A-PC ranged from 0.0265 to 0.0768 mg/g. ANOVA results (Table 4) revealed that the linear effects of variables (sonication time and freezing time) were significant ($p < 0.05$). Moreover, the maximum F-value corresponded to freezing time, which indicated the binding effect of freezing on the A-PC concentration. In addition, the P-value of variables was significant ($p < 0.05$), but their interaction was not significant. The lack of fit of these models was insignificant, which means these models were also suitable mathematically.

Based on Eq. (9), the ultrasound time has a positive effect, and the freezing time negatively affects the A-PC parameter. Fig. 2 (b) shows the interaction of the freezing time and ultrasound time on the A-PC concentration. As shown in this figure, at a freezing time of 5 h, the amount of A-PC increases gradually with increasing the ultrasound time from 30 to 90 min. *Aouir et al.* (2015) stated that the A-PC of Phycopiliproteins increased with increasing ultrasonic

time from 0 to 90 min. The present result agrees with what they reported [5].

The ultrasound power has influenced the number of cell disruptions during extraction. The formation of bubbles due to the cavitation process of ultrasonic gives considerable energy around it that disrupts the cell wall. The cell disruptions will release the A-PC from the cell. A longer extraction time will increase the rate of mass transfer, and it is also improves the level of A-PC concentration [40]. Ultra-sonication provokes cavitation that further results in cell swelling, more solvent uptake, and pore enlargement present on the cell walls, thus increasing diffusivity across the cell walls. As the ultrasound extraction destroyed the cell walls, all biological components in the biomass were quickly released [20].

Phycoerythrin (PE) Concentration

The experimental conditions and the results of the phycoerythrin concentration (PE) as a response to the experimental design are shown in Table1. As expected, PE was obtained in the lowest amounts in most extraction conditions, similar to other reported results for these Pigments [2, 46]. Measurements revealed that the PE concentration ranged from 0.0057 to 0.0165 mg/g. Also, results in the ANOVA table showed that the 2FI effect of variables on PE was significant ($p < 0.05$), and the highest F-value was the one for the freezing time.

As shown in Table 4, a linear model was established (Eq. (10)). From the proposed model for PE (Eq.(10)) and considering the presence of a negative coefficient for the X_2 variable (freezing time), it was inferred that time of freezing of algae suspension had a reverse effect on PE concentration and that the variables had an antagonistic effect on the concentration of this pigment. The same trend was observed for C-PC and A-PC. Fig. 2(C) illustrates a 3-D surface plot correlating sonication and the freezing amounts to PE amounts isolated under various extraction conditions.

An increase in sonication time from 30 to 90 min. led to a rise in the PE concentration, on average, 0.0165 mg/g. A change in the freezing time from 5 to 12 hours reduced the PE concentration (Fig. 2. c), probably due to denaturing of phycoerythrin. Also, results showed that the combining effect of variables resulted in reducing the phycoerythrin concentration.

Following this study, *Aouir et al.* (2015) studied the extraction of phycobiliprotein PE using ultrasound technology. The result shows that, with the increase of the ultrasonic time from 30 to 90 min, the concentration of PE increased. The present result agrees with what they reported [5].

These data also show that ultrasound irradiation increases the cell lysis during the extraction process. In particular, micro-bubbles that form due to cavitation deliver enormous amounts of energy to surrounding biomasses, disrupting algal cell walls and increasing protein release.

Phycocyianin purity

C-PC is considered a food grade when A620/A280 is greater than or equal to 0.7, as a reagent grade when A620/A280 is between 0.7 and 3.9, and as an analytical grade when A620/A280 is greater than or equal to 4 [34]. According to the experimental data, as shown in Table 1, the purity of phycocyanin extract was ranged from 0.300 to 0.598%. The values obtained in this study were lower than the results reported by Silveira and co-authors. They evaluated the effect of temperature and biomass-solvent on the extract purity of phycocyanin.

There are some difficulties in phycocyanin extraction because of multilayered cell walls and large amounts of contaminants [34]. According to Table 4, the quadratic term of ultrasound time (X_1^2) has a significant influence on the EP ratio of phycobiliprotein ($p < 0.05$). Also, ANOVA results showed that the purity of phycocyanin extracts was affected by the linear term of freezing time(X_2). The coded model generated the response surface plot (Fig.2. (d)). The 3-D surface plot illustrates the combined effect of ultrasound time and freezing time on the amount of EP. It can be seen from Fig. 2 (d) that the maximum amount of EP was obtained at the middle level of ultrasound time and freezing time (60 min and 8.5), respectively. Decreasing the purity of extract by increasing the processing time (both the sonication time and freezing time) can be related to the increased denaturation of protein subunits and diffusion ratio [47].

The highest purity of CPC was found using 60 min. of the sonication before the freezing time. Further sonication (60 to 90 min) resulted in a lower purification factor of CPC in which CPC was denatured during sonication. The least CPC was obtained using the lowest sonication time

(30 min) due to insufficient sonication to break down the cell wall completely.

Chia et al. (2020) extracted C-PC from dried *Spirulina* using an ultrasound probe ranging from 3 to 12 min, as increasing the ultrasound time led to a decrease in the purity level of the extract [48].

Mittal et al. (2017) extracted phycobiliproteins from *Gelidium* using a similar working range from 1 to 10 min. A further increase in sonication time led to an increment of the purity and stability of phycobiliproteins [4]. In evaluating the effect of freezing time on the purity of the C-PC extract, based on Fig. 2 (d), it can be seen that with increasing the freezing time, the purity decreases, and the maximum purity is when the freezing of the algal suspension is done for 5 hours (run 12). Although more incredible extraction times of freezing can also increase transfer rates, continuous ultrasound radiation may lead to saturation of solutes with solvents and decreased protein purity and yields [30].

In a long time of freezing, even though a slight increase in C-PC concentration was obtained, purity was reduced, indicating the release of contaminant proteins. These results were in accordance with Hrishikesh Tavanandi and co-author [28].

C-PC Yield

The measured C-PC yield is presented in Table 1. The sample yield was between 1.58 to 2.73%, and there were significant differences among the C-PC yield of the different treatments ($p < 0.05$). The maximum yield was observed in run 10, with 90 min sonication and 12 h freezing time in extraction processing (Table 1). Ultrasound waves facilitate cell swelling, leading to improved dissolution and mass transfer of lipids and proteins through enlarged pores, and enhanced extraction Times [30, 49].

Using ultrasonic technique for biological materials, including microalgae in a liquid medium, mainly uses the pore-forming process to induce cell breakdown. In particular, cavitation is a complex phenomenon involving the nucleus, growth, and collapse of the microbubble request in the fluid caused by the bulk pressure due to ultrasound waves. In addition, cavitation can result in the physical effect of micro-turbulence and the release of large amounts of heat and shock waves. It becomes a disorder of the cell wall [20,50].

As shown in Table 4, results showed that the yield was influenced linearly by sonication time and time of algae freezing ($p < 0.05$). The interaction of variables (sonication time × freezing time) was significant on the yield. According to the coefficients obtained, the proposed model for C-PC yield was defined by Eq. (12).

The C-PC yield of extracts rose by increasing the level of sonication time only at the lowest level of freezing time (Fig. 2(e)). Also, the increase in freezing time led to a decrease in this response. Our results were similar to the data reported by *Hadiyanto* and *Norhadi* (2016). These researchers found that the increasing ultrasonic time from 5 to 20 min leads to an increase of C-PC yield [51].

As the phycobiliproteins are intracellular, cell disruption is required for their efficient release during extraction. The extraction mechanism involves two physical phenomena: diffusion through the cell walls and washing out (rising) the cell contents once the wall is broken. Both phenomena are significantly affected by ultrasound irradiation [50]. The minuscule C-PC yield was obtained using the highest freezing time and sonication time due to two parameters that completely break down the cell wall and the C-PC structure denaturized.

Mittal et al. (2017) extracted phycobiliproteins from *Gelidium* using a sonication range from 2 to 10 min in a combination of conventional methods (freezing and thawing, homogenization, and maceration). It was observed that a sonication time of 2 minutes gave a yield of 0.04 mg/g dry basis, while a sonication of 10 minutes resulted in a yield of 0.1 mg/g dry basis [4]. As a result, the tissue degradation increases, and consequently, the complex structure of phycocyanins changes. In other words, protein denaturation occurs at a high level, and C-PC is diminished [52,53]. These degradation reactions are probably related to modifications on secondary, tertiary, and quaternary structures promoted by the freezing temperature.

Other authors have studied the effects of ultrasounds on carotenoid recovery from microalgae. In this regard, Cha and co-author [54] reported a high recovery of lutein and b-carotene from C. Vulgaris compared to the classic maceration method. Similarly, *Ruen-Ngam et al.* [55] found a significant astaxanthin yield (up to 55–60 %) when they applied ultrasonic-assisted extraction in *Haematococcus Pluvialis* compared to the conventional maceration method.

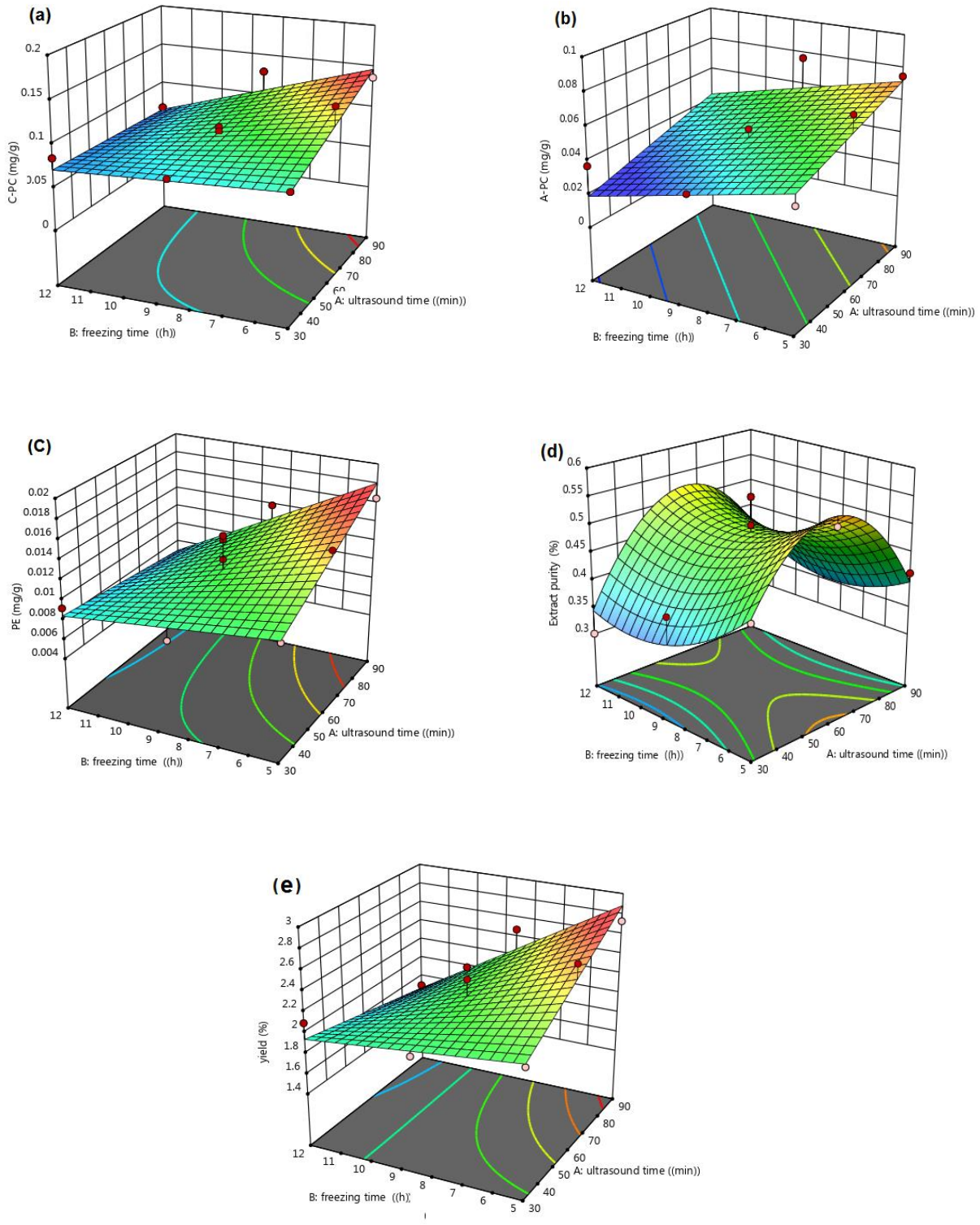

Fig. 2: Response surface diagrams illustrating the effects of ultrasound time and freezing-thawing time on (A) C-PC Concentration, (B) Allophycocyanin (A-PC), (C) Phycoerythrin (PE), (D) Extract purity (EP) Ratio, (E) phycocyanin (C-PC) yield, extracted from Spirulina Platensis. (x_1=A: Ultrasound time x_2= B: freezing time).

Optimization conditions and model validation

To define optimal process conditions for phycocyanin extractions using freezing and sonication, we used the response surface methodology of Statistica11 software. The optimal extraction conditions for ultrasound time (min) and freezing (h) were evaluated, targeting the maximum C-PC concentration, C-PC purity, and C-PC yield. The desirability function is widely used in RSM to determine a combination of variables to optimize multiple responses. Conformity tests were carried out with the same experimental conditions to examine the accuracy of the mathematical model correlations.

By applying the desired function methodology, the optimum level of process time was obtained at the sonication time 55.5 min and 5 hour freezing of algae with overall desirability of 1.0. The above-mentioned optimal conditions were experimentally validated to confirm the adequacy of the models. Under these conditions, the predicted PE , A-PC, C-PC concentration, C-PC purity, and C-PC yield values were 0.0137 mg/g , 0.055 mg/g , 0.114 mg/g, 0.425%, and 2.61%, respectively (Fig.3), wherein experimental tests, results showed that at the optimum condition of sonication time (55.5 min) and freezing time (5 h), the average value of PE , A-PC, C-PC concentration, C-PC purity, and C-PC yield were (0.095±0.009), (0.051mg/g), (0.109±0.051mg/g), (0.498±0.25), and (2.32±0.21 %), respectively.

Comparing the optimal and control samples, it can be said that the combined method of freezing and ultrasound had a better performance in the extraction of phycobiliprotein. According to Table 1, in the control sample with 12 hours of freezing, the concentration of CPC, APC, PE, EP (extract purity), and the C-PC yield were 0.079, 0.031, 0.0076, 0.49, and 2.13, respectively, which were reported in lower values than the optimal method.

SEM imaging

The *Spirulina Platensis* morphological changes during the extraction condition (freezing (12 h) and optimum condition (ultrasonic (55.5 min) and freezing time (5 h) were observed using SEM and presented in Fig. 3. (A-D), respectively. It can be seen in the figure that the *Spirulina Platensis* residue, which was obtained by applying the freezing method (3. A and C) has a coarse surface with large pores compared to another sample that was treated with ultrasonic and freezing (Fig. 3. B and D). The comparison of the two treatments indicates that the use of freezing alone and for a long time has caused more destruction of cells and due to the damage caused by ice crystals, large pores and cracks have been created in the structure of algae.

Small cracks and crevices are seen in the sample structure treated with ultrasonic and freezing in short times. (Fig. 3. A & C) shows that using the freezing process for a long time resulted in considerable damage to the cell walls of the sample and deep cavities in the sample.

The comparison of the two treatments shows that a longer freezing processing time led to further deep cavities in the sample. Based on the images presented in Fig. 3, it can be concluded that the combined use of ultrasonic and freezing techniques in short time can cause proper extraction of phycocyanin with higher purity. The high efficiency of phycocyanin at high freezing and sonication times (3. E) can be attributed to this reason. In other words, the high efficiency and low purity of the extracted phycocyanin during long freezing times can be explained by the creation of large holes in the filamentous structure of spirulina algae, which causes the release of more protein and other compounds such as polysaccharide compounds. This factor causes phycocyanin to be low in purity despite its high yield.

Fourier Transform InfraRed (FT-IR) spectroscopy

The FT-IR spectroscopy was performed to identify the characteristics of the functional groups of extracts obtained from the optimum treatment and the control treatment (only freezing 12h), (Fig. 4). In addition, FT-IR spectroscopy was used to examine the possible changes in these functional groups during the mentioned extraction treatments. As shown in Fig. 4, the FT-IR spectra resulting from the two treatments are overlapping.

The two spectra of FT-IR in this figure had the absorption peaks at the specified wavenumbers; the bands of 1656 cm^{-1} represent an amide I group, bands of 1540 cm^{-1} and 1446 cm^{-1} represent an amide group (amide II((stretching C=O bond)) and (CN) signals, respectively. During the PC purification process, the band with a quite sharp absorption in the region of 1000–1100 cm^{-1} was an inorganic sulfate derived from ammonium sulfate. In addition, the presence of bands at 1500 to 1600 cm^{-1} indicates aromatic rings [37]. Thus, by comparing

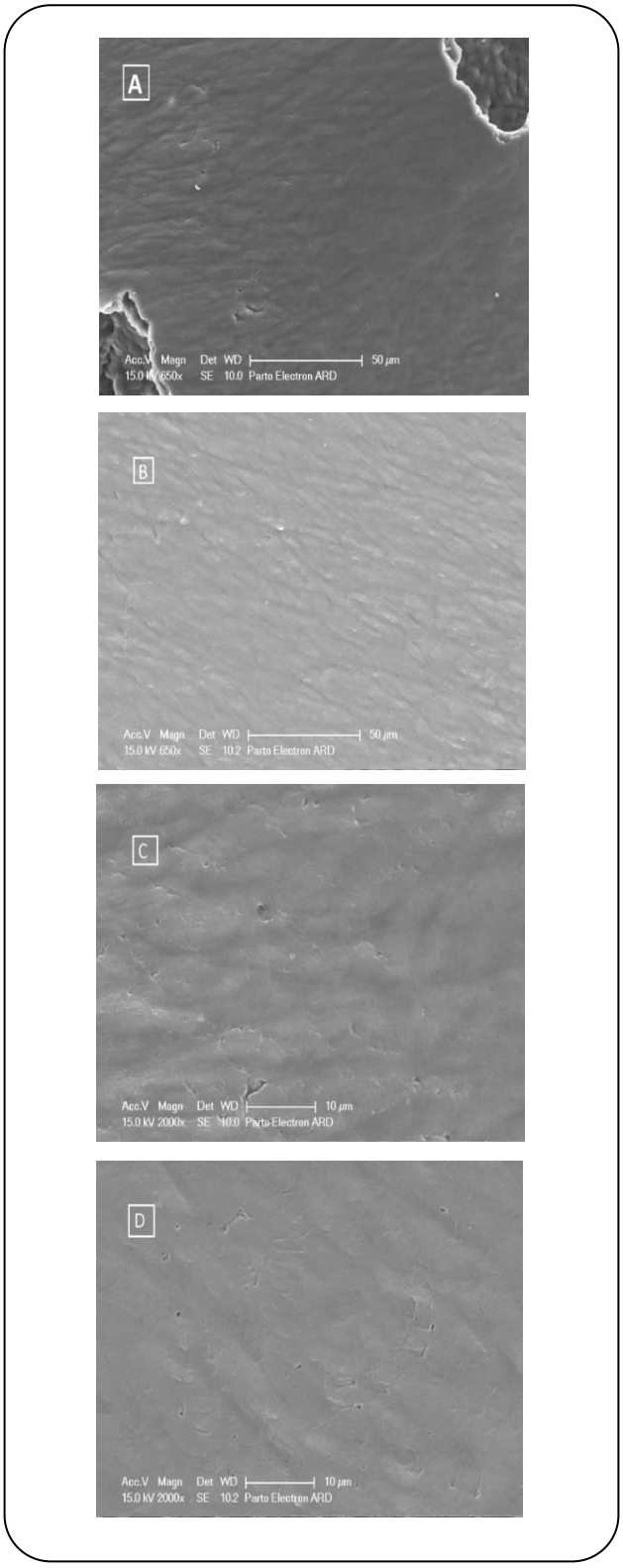

Fig. 3: The SEM image of Spirulina Platensis filament obtained after freezing (A and C) and optimum condition treatment (ultrasonic (62.6 min) and freezing time (5.6 h)) (B and D) extraction of phycocyanin.

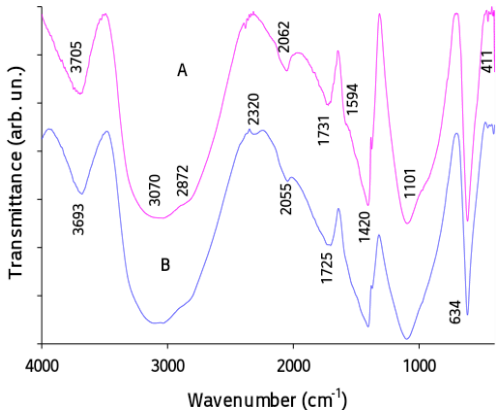

Fig. 4: FT-IR spectra of the phycocyanin obtained by optimum condition treatment (ultrasonic (62.6 min) and freezing time (5.6 h) extraction (a) and control sample extraction only by freezing (b).

the spectra of FT-IR, it can be concluded that the use of the ultrasonic pretreatment (5 min) to extract the C-PC pigment from *Spirulina Platensis* compared to the non-use of this pretreatment (control sample, 12 h freezing without ultrasonic), did not lead to any destruction of the mentioned functional groups in the pigments.

XRD pattern of C-PC

Fig. 5 shows XRD patterns for phycocyanin samples, prepared (A) and the samples prepared after 90 min ultrasonication- 12 h freezing processes (B). As far as we know, no significant published work has been reported on XRD patterns of phycocyanins. Only one report has been published to describe the XRD data of some phycocyanins [41]. It is mentioned in a reference that some phycocianin samples such as Spirulina, Phormidium, and Lyngbya have been analyzed by XRD and the crystal phases of Hexagonal (Unit-cell parameters (Å): a = b = 154.97, c = 40.35) and Monoclinic (Unit-cell parameters (Å): a = 107.33, b = 115.64, c = 183.26, β = 90.03) have been reported for Spirulina while a Monoclinic (Unit-cell parameters (Å): a = 107.87, b = 115.76, c = 183.54, β = 90.3) crystallite phase for Phormidium. Further, two crystallite phases of Hexagonal (Unit-cell parameters (Å): a = b = 151.96, c = 39.06) and Monoclinic (Unit-cell parameters (Å): a = 107.45, b = 115.33, c = 183.36, β = 90.08) have been also obtained for Lyngbya.

The XRD pattern in Fig. 5. revealed typical diffraction peaks at 2θ-positions of 16.68, 20.23, 23.62, 28.34, 29.26, 33.05, 35.74, 38.80, 40.96, 45.82, 46.71, 48.87, 51.40,

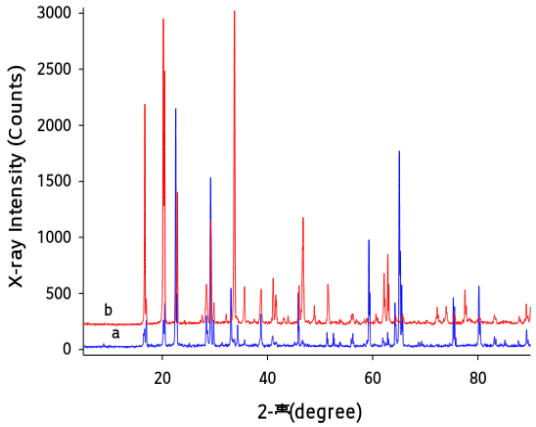

Fig. 5. XRD patterns of Phycocyanin were produced from optimum condition (A) and 12 h freezing (B).

52.48, 56.08, 59.31, 61.83, 62.92, 64.35, 65.24, 72.45, 74.06, 72.51, 77.84, 80.36, 83.24, 85.22, and 89.36 degree. After the treatment of the sample during 90 min sonication and 12 h. freezing, some changes occurred in the XRD pattern (pattern B). Firstly, the overall peak intensity of the sample was increased, confirming a higher crystalline degree of the frozen sample. Further, some changes in the intensity of the individual peaks were also observed. For example, the intensity of the peaks positioned at 2θ-degree of 16.68, 20.23, 33.05 (shifted to 33.74), 35.75, 40.96, 45.82, 46.71, 51.41 (with the shift to 51.57), 61.83, and 62.92 were increased. The highest peak is located at 16.68, 20.23, and 33.05 degrees among the high peaks. The intensity of some peaks was decreased, including the peaks located at 23.62 and 64.35 °, and some at positions of 52.48, 59.31, 65.24, 72.51, 80.36, 85.22, and 89.36º were disappeared. Besides, some new peaks positioned at 2θ-degree of 28.34, 72.45, 74.06, and 77.84 º were also observed. These changes in the XRD patterns confirm that during the ultra-sonication and freezing processes, the crystallite phase may be changed.

CONCLUSIONS

In the current study, the extraction of phycobiliproteins from dried biomass of *Spirulina Platensis* by ultrasonic combined with the freezing-thawing method was optimized using RSM. As expected, C-PC was obtained in most extraction conditions in the highest amounts, followed by APC, and the amount of PE as the lowest. Increasing the ultrasound time and the decrease in the freezing time improved the purity and efficiency of the extracted phycocyanin. By applying the methodology of the desired function, the optimum level of process time was obtained at a sonication time 55.5 min and 5 hours of freezing of algae. Comparing the optimal and control samples, it can be said that the combined method of freezing and ultrasound had a better performance in the extraction of phycobiliprotein. Also comparing the spectra of phycocyanin obtained from optimum treatment and control treatment (only freezing 12h) indicated that the ultrasonic pretreatment to extract the C-PC pigment did not lead to any destruction of the functional groups in the pigments.

Acknowledgment

The authors would like to thank Dr. Mohsen Shahrokhi, an assistant professor in Applied Linguistics at the English department of Shareza Branch of Islamic Azad University, for his comments regarding the English language of an earlier draft of the current paper.

Received :May 24, 2022 ; Accepted : Aug. 22, 2022

REFERENCES

[1] Bachchhav M.B., Kulkarni M.V., Ingale A.G., An Efficient Extraction of Phycocyanin by Ultrasonication and Separation Using 'Sugaring Out', *J. Phycol. Soc. India*, **47**: 19-24 (2017).

[2] Hoi W., Lee H., Effect of Ultrasonic Extraction on Production and Structural Changes of C-Phycocyanin from Marine Spirulina Maxima, *International Journal of Molecular Sciences*, **19(1)**: 220 (2018).

[3] Zhang, X.-F., X. Wang, and G.-H. Luo, Ultrasound-Assisted Three-Phase Partitioning of Phycocyanin From Spirulina Platensis,*European Journal of Pure and Applied Chemistry*, **4(1)** (2017).

[4] Mittal R., et al., Ultrasound-Assisted Methods for Enhanced Extraction of Phycobiliproteins from Marine Macro-Algae, Gelidium Pusillum (Rhodophyta), *Ultrasonics Sonochemistry*, **38**: 92-103 (2017).

[5] Aouir A., Amiali M., Kirilova-Gachovska T, Benchabane A., Arezki Bitam A., "The Effect of Pulsed Electric Field (PEF) and Ultrasound (US) Technologies on The Extraction of Phycopiliproteins from Arthrospira Platensis", *in 2015 IEEE Canada International Humanitarian Technology Conference (IHTC2015)*. IEEE (2015).

[6] Bhosle D., Janghel A., DeoSaurav, Alexander A., Emerging Ultrasound-Assisted Extraction (UAE) Techniques as Innovative Green Technologies for the Effective Extraction of the Active Phytopharmaceuticals, *Research Journal of Pharmacy and Technology*, **8(7)**: 963-970 (2015).

[7] Chaiklahan R., Chirasuwan N., Loha V., Suvit Tia S., Boosya Bunnag B., Separation and Purification of Phycocyanin from Spirulina Sp. Using a Membrane Process. *Bioresource Technology*, **102(14)**: 7159-7164 (2011).

[8] Abalde J., Betancourt L., Torres E., Cid A., Barwell C., Purification and Characterization of Phycocyanin from the Marine Cyanobacterium Synechococcus sp. *IO9201*. *Plant Science*, **136(1)**: 109-120 (1998).

[9] Rodríguez-Sánchez R., Ortiz-Butrón R., Blas-Valdivia V., Hernández-García A., Cano-Europa E., Phycobiliproteins or C-phycocyanin of Arthrospira (Spirulina) Maxima Protect Against HgCl2-Caused Oxidative Stress and Renal Damage, *Food Chemistry*, **135(4)**: 2359-2365(2012).

[10] Vonshak, A. Spirulina Platensis (Arthrospira). Taylor & Francis (1997).

[11] Jaouen, P., B. Lépine, N. Rossignol, R. Royer, and F. Quéméneur. Clarification and Concentration with Membrane Technology of a Phycocyanin Solution Extracted from Spirulina Platensis. *Biotechnology Techniques*, **13(12)**: 877-881(1999).

[12] Fernández-Rojas B., Hernández-Juárez J., Pedraza-Chaverri J., Nutraceutical Properties of Phycocyanin. *Journal of Functional Foods*, **11**: 375-392 (2014).

[13] De Jesus Raposo M.F., de Morais R.M.S.C., de Morais A.M.M.B., Health Applications of Bioactive Compounds from Marine Microalgae, *Life sciences*, **93**(15): p. 479-486(2013)

[14] Habib M.A.B., Review on Culture, Production and Use of Spirulina as Food for Humans and Feeds for Domestic Animals and Fish, *Food and Agriculture Organization of the United Nations* (2008).

[15]- Mishra, S.K., A. Shrivastav, and S. Mishra. Effect of Preservatives for Food-Grade C-PC from Spirulina Platensis, *Process Biochemistry*, **43(4)**: 339-345 (2008).

[16] Romay C., Gonzalez R., Ledon N., Remirez D., Rimbau V., C-Phycocyanin: A Biliprotein with Antioxidant, Anti-Inflammatory, and Neuroprotective Effects, *Current Protein and Peptide Science*, **4(3)**: 207-216 (2003)

[17] Eriksen, N.T. Production of Phycocyanin—A Pigment with Applications in Biology, Biotechnology, Foods, and Medicine, *Applied Microbiology and Biotechnology*, **80(1)**: 1-14 (2008).

[18] Suwal S., Perreault V., Marciniak A., Tamigneaux É., Deslandes É., Bazinet L., Jacques H., Beaulieu L., Doyen A., Effects of High Hydrostatic Pressure and Polysaccharidases on the Extraction of Antioxidant Compounds from Red Macroalgae, Palmaria Palmata, and Solieria Chordalis, *Journal of Food Engineering*, **252**: 53-59(2019).

[19] Moraes C.C., Sala L., Cerveira G.P., Kalil S.J., C-Phycocyanin Extraction from Spirulina Platensis Wet Biomass, *Brazilian Journal of Chemical Engineering*, **28(1)**: 45-49(2011).

[20] İlter I., Akyıl S., Demirel Z., Koç M., Conk-Dalay M., Kaymak-Ertekin F., Optimization of Phycocyanin Extraction from Spirulina Platensis Using Different Techniques, *Journal of Food Composition and Analysis*, **70**: 78-88(2018).

[21] Yucetepe A., Saroglu O., Bildik F., Ozcelik B., Daskaya-Dikmen C., Optimisation of Ultrasound-Assisted Extraction of Protein from Spirulina Platensis Using RSM, *Czech Journal of Food Sciences*, **36(1)**: 98-108 (2018).

[22] Sutanto H., Suzery M., Phyocyanin Extraction from Microalgae Spirulina Platensis Assisted by Ultrasound Irradiation: Effect of Time and Temperature. *Songklanakarin Journal of Science & Technology*, **38(4)** (2016).

[23] Liu M.Q., Yang X.Q., Qi B., Li L.H., Deng J.C., Hu X., Study of Ultrasonic-Freeze-Thaw-Cycle Assisted Extraction of Polysaccharide and Phycobiliprotein from Gracilaria Lemaneiformis. In Advanced Materials Research, *Trans. Tech. Publ.* (2013).

[24] Vernès L., Granvillain P., Chemat F., Vian M., Phycocyanin from Arthrospira Platensis. Production, extraction, and analysis. *Current Biotechnology*, **4(4)**: 481-491 (2015).

[25] Da Costa Ores J., de Amarante M.C.A., Kalil S.J., Co-Production of Carbonic Anhydrase and Phycobiliproteins by Spirulina Sp. and Synechococcus Nidulans, *Bioresource Technology*, **219**: 219-227 (2016).

[26]- Ma Y.-Q., Chen J.-C., Liu D.-H., Ye X.-Q., Simultaneous Extraction of Phenolic Compounds of Citrus Peel Extracts: Effect of Ultrasound. *Ultrasonics Sonochemistry*, **16(1)**: 57-62 (2009).

[27] Al Jitan S., Alkhoori S.A., Yousef L.F., Phenolic Acids from Plants: Extraction and Application to Human Health, *Studies in Natural Products Chemistry*, 389-417. (2018).

[28] Hrishikesh A. Tavanandi, R.M., Jampani Chandrasekhar, K.S.M.S. Raghavarao. Simple and Efficient Method for Extraction of C-Phycocyanin from Dry Biomass of Arthospira Platensis, *Algal Research*, **31**: 239-251 (2018).

[29] Hadiyanto H., Suttrisnorhadi S., Response Surface Optimization of Ultrasound-Assisted Extraction (UAE) of Phycocyanin from Microalgae Spirulina Platensis, *Emirates Journal of Food and Agriculture*, **28(4)**: 227 (2016).

[30] Hadiyanto H., Adetya N.P., Response Surface Optimization of Lipid and Protein Extractions from Spirulina Platensis Using Ultrasound-Assisted Osmotic Shock Method, *Food Science and Biotechnology*, **27(5)**: 1361-1368 (2018).

[31] Chemists A.O.A.O.C., *Official Methods of Analysis*, (1920).

[32] Jones, D.B. Factors for Converting Percentages of Nitrogen in Foods and Feeds into Percentages of Proteins. (1931)

[33] Kamble S.P., Gaikar R.B., Padalia R.B., Shinde K.D., Extraction and Purification of C-phycocyanin from Dry Spirulina Powder and Evaluating its Antioxidant, Anticoagulation, and Prevention of DNA Damage Activity, *Journal of Applied Pharmaceutical Science*, **3(8)**: 149 (2013).

[34] Kumar D., Dhar D.W., Pabbi S., Kumar N., Walia S., Extraction and Purification of C-phycocyanin from Spirulina Platensis (CCC540), *Indian Journal of Plant Physiology*, **19(2)**: 184-188 (2014).

[35] Bennett A., Bogorad L., Complementary Chromatic Adaptation in a Filamentous Blue-Green Alga, *The Journal of Cell Biology*, **58(2)**: 419-435(1973).

[36] Muthulakshmi M., Saranya A., Sudha M., Selvakumar G., Extraction, Partial Purification, and Antibacterial Activity of Phycocyanin from Spirulina Isolated from Freshwater Body Against Various Human Pathogens, *J. Algal. Biomass Util.*, **3(3)**: 7-11 (2012).

[37] Khashayary S., Aarabi A., Evaluation of Physico-mechanical and Antifungal Properties Of Gluten-based Film Incorporated with Vanillin, Salicylic Acid, and Montmorillonite (Cloisite 15A), *Food and Bioprocess Technology*, (2021).

[38] Aarabi A., Mizani M., Honarvar M., The Use of Sugar Beet Pulp Lignin for the Production of Vanillin, *International Journal of Biological Macromolecules*, **94, Part A**: 345-354(2017).

[39] Marrez D., Naguib M.M., Sultan Y., Daw Z.Y., Higazy A.M., Evaluation of Chemical Composition for Spirulina Platensis in Different Culture Media, *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, **5**: 1161-1171(2014)

[40] Furuki T., Maeda S., Imajo S., Hiroi T., Amaya T., Hirokawa T., Ito K., Nozawa H., Rapid and Selective Extraction of Phycocyanin from Spirulina Platensis with Ultrasonic Cell Disruption, *Journal of Applied Phycology*, **15(4)**: 319-324 (2003).

[41] Ferraro G., Imbimbo P., Marseglia A., Lucignano R., Monti D.M., Merlino A., X-Ray Structure of C-Phycocyanin From Galdieria Phlegrea: Determinants of Thermostability and Comparison with a C-Phycocyanin in the Entire Phycobilisome. *Biochimica et Biophysica Acta. Bioenergetics*, **1861(9)**: 148236 (2020).

[42] Safaei M., Maleki H., Soleimanpour H., Norouzy A., Zahiri H.S., Vali H., Noghabi K.A., Development of a Novel Method for the Purification of C-Phycocyanin Pigment from a Local Cyanobacterial Strain Limnothrix sp. NS01 and Evaluation of its Anticancer Properties, *Scientific Reports*, **9(1)** (2019).

[43] Luo J., Fang Z., Smith R.L., Ultrasound-Enhanced Conversion of Biomass to Biofuels, *Progress in Energy and Combustion Science*, **41**: 56-93 (2014).

[44] Barba F.J., Grimi N., Vorobiev E., New Approaches for the Use of Non-conventional Cell Disruption Technologies to Extract Potential Food Additives and Nutraceuticals from Microalgae, *Food Engineering Reviews*, **7(1)**: 45-62 (2014).

[45] Safi C., Ursu A.V., Laroche C., Zebib B., Merah O., Pontalier P.-Y., Vaca-Garcia C., Aqueous Extraction of Proteins from Microalgae: Effect of Different Cell Disruption Methods, *Algal Research*, **3**: 61-65(2014).

[46] Patel A., Mishra S., Pawar R., Ghosh P.K., Purification and Characterization of C-Phycocyanin from Cyanobacterial Species of Marine and Freshwater Habitat, *Protein Expression and Purification*, **40(2)**: 248-255 (2005).

[47] Vali Aftari R., Rezaei K., Mortazavi A., Bandani A.R., The Optimized Concentration and Purity of Spirulina Platensis C-Phycocyanin: A Comparative Study on Microwave-Assisted and Ultrasound-Assisted Extraction Methods, *Journal of Food Processing and Preservation*, **39(6)**: 3080-3091(2015).

[48] Chia S.R., Chew K.W., Leong H.Y., Manickam S., Show P.L., Nguyen T.H.P., Sonoprocessing-Assisted Solvent Extraction for the Recovery of Pigment-Protein Complex from Spirulina Platensis, *Chemical Engineering Journal*, **398**: 125613(2020).

[49] Tavanandi H.A., Vanjari P., Raghavarao K.S.M.S., Synergistic Method for Extraction of High Purity Allophycocyanin from Dry Biomass of Arthrospira Platensis and Utilization of Spent Biomass for Recovery of Carotenoids, *Separation and Purification Technology*, **225**: p. 97-111(2019).

[50] Donsì F., Ferrari G., Pataro G., Emerging Technologies for the Clean Recovery of Antioxidants from Microalgae, M*icroalgae*, 173-205 (2021).

[51] Hadiyanto H., Suttrisnorhadi S., Response Surface Optimization of Ultrasound-Assisted Extraction (UAE) of Phycocyanin from Microalgae Spirulina Platensis, *Emirates Journal of Food and Agriculture*, 227-234 (2016)

[52] Pearce R.S., *Plant Freezing and Damage.* Annals of Botany, **87(4)**: 417-424(2001).

[53] Lupatini A.L., de Oliveira Bispo L., Colla L.M., Costa J.A.V., Canan C., Colla E., Protein and Carbohydrate Extraction from S. Platensis Biomass by Ultrasound and Mechanical Agitation, *Food Research International*, **99**: 1028-1035 (2017).

[54] Cha K.H., Lee H.J., Koo S.Y., Song D.-G., Lee D.-U., Pan C.-H., Optimization of Pressurized Liquid Extraction of Carotenoids and Chlorophylls from chlorella vulgaris. *Journal of Agricultural and Food Chemistry*, **58(2)**: 793-797 (2010).

[55] Ruen-Ngam A., Shotipruk D., Pavasant P., Comparison of Extraction Methods for Recovery of Astaxanthin from Haematococcus Pluvialis. *Separation Science and Technology*, **46(1)**: 64-70 (2011).