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Original Article

Phyocyanin extraction from microalgae *Spirulina platensis* assisted by ultrasound irradiation: effect of time and temperature

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Abstract

This research was aimed to extract phycocyanin from microalgae *Spirulina platensis* using an extraction assisted by ultrasound irradiation. The extraction was conducted by using variable of extraction time, temperature and ultrasound frequency, while ethanol was used as solvent. The results show that the yield of phycocyanin extract was 15.97% at constant frequency of 42 kHz and 11.24% at constant frequency of 28 kHz, while the soxhlet extraction method obtained yield at 11.13%. The ultrasound could reduce the extraction time from 4 hrs (conventional) to 20 minutes, while the optimum temperature of extraction was found at 55°C.

Keywords: *Spirulina platensis*, phycocyanin, ultrasound, extraction, solvent, anti-oxidant, yield

1. Introduction

Currently the demand of functional foods is growing rapidly due to their beneficial effects on health. In the modern era, food is not only used as a source of energy and nutrients, but also for the maintenance and improvement of the body's immune system. Functional foods can be produced by adding ingredients that have special health functions into food products (Hadiyanto *et al.*, 2014). Moreover, the increased attention to functional products will also support a healthier lifestyle (Christwardana *et al.*, 2013).

On the other sides, it shows that the world is experiencing a foods crisis because of the difficulty of getting proteins, minerals, vitamins, and pigments in food products. This crisis of functional food can be resolved by searching for alternative compounds from natural resources, i.e. microalgae. An advantage of microalgae is the rapid harvesting process. Further, they are the most efficient plants in receiving and absorbing solar energy as well as CO_2 and nutrients for photosynthesis process with a very high growth rate (Seo *et al.*, 2013). In addition, microalgae are also studied for fuel generation and as a source of feed and pharmaceutical products (Henrikson, 2009).

Several microalgae have been identified to have a protein content above 30%, such as *Scenedesmus, Chalamydomonas, Chorella, Dunaliella, Euglena gracilis, Primnesium parvum, Tetraselmis maculata, Spirulina, Anabaena cylindrica* (Chisti and Moo-young, 1986; Tang *et al.*, 2011). *Spirulina platensis* is rich in active compounds such as proteins, minerals, vitamins, pigments (phycocyanin and β-carotene) and polyunsaturated fatty acids (γ-linolenic acid) (Seo *et al.*, 2013; Coca *et al.*, 2014). Among others,

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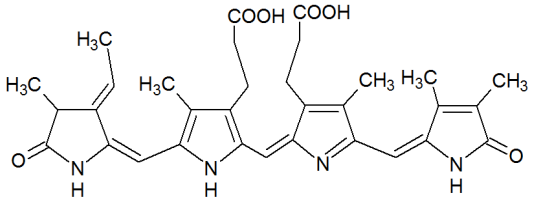

Figure 1. Phycocyanin chemical structure.

Spirulina platensis has been widely used as a source of functional compounds such as protein and pigments (Saranraj and Sivasakthi, 2014). Moreover, *Spirulina platensis* also produce large quantities of high value compounds, such as phycobiliproteins, which consists of chromophore-polypeptides, α and β subunits soluble in water and thus form phycobilisomes compounds. Phycobilisomes is composed of allo-phycocyanin (APC) in the core and surrounded by phycocyanin pigment (CPC) (Antelo *et al.*, 2008)

Phycocyanin is a natural pigment (Figure 1) that has a high economic value. These compounds are available in *Spirulina platensis* of up to 20% of the dry weight (Antelo *et al.*, 2008). The blue-green pigment can be used as a natural coloring agent in foods such as chewing gums, ice cream, soft drinks, confectionery, gel, etc. This dye gives a more attractive appearance and increase the nutritional value of food. This compound also can replace synthetic dyes (Chaiklahan *et al.*, 1999).

Phycocyanin has been proven to have therapeutic properties including antioxidant, anti-inflammatory, anti-cancer activities (Romay *et al.*, 2003; Silveira *et al.*, 2007) and it could help AIDS patients to have longer lives (Rabadiya and Patel, 2010). Recently, more research activities have explored phycocyanin as an antioxidant, anti-mutagenic, anti-viral, anti-cancer, anti-allergic, hepatoprotective compound, with possible positive effects towards diabetes, cardiovascular diseases, blood vessel-relaxing, and with blood lipid-lowering effects (Chaiklahan *et al.*, 2012; Thangam *et al.*, 2013). Several studies show that extracts of phycocyanin can prevent or inhibit cancer in humans and animals. In vitro studies suggests that extract of phycocyanin from *Spirulina* can enhance cell nucleus enzyme activity and DNA repair synthesis (Estrada *et al.*, 2001), inhibited the growth of bacterial, preventing a host of degenerative organ diseases by increasing immunity, repair cells damaged by toxic chemicals or radiation and improving their ability to function in spite of stresses from environmental toxins and infection agent (Rabadiya and Patel, 2010).

Phycocianin is mostly extracted from microalgae *Spirulina platensis*. Generally, the extraction affects the yield and releases the compounds from the cell wall of microalgae due to several factors such as type of solvent, solvent to biomass ratio, the length of extraction, and also temperature (Moraes *et al.*, 2011; Prabuthas *et al.*, 2011). Microalgae *Spirulina platensis* with phycobiliproteins as the main components has a relatively fragile cell walls (Sa *et al.*, 2014). Various attempts of cells disruption have been applied to separate the pigment phycocyanin which is still bound to the thylakoid membrane, such as water extraction, homogenization in mortar and pestle, freezing and thawing, homogenization in virtimex, acid extraction, lysozyme treatment (Sarada *et al.*, 1999) and enzymatic method (Moraes *et al.*, 2011). However, several drawbacks have been pointed out from these methods such as, long extraction time, ice formation in the freezing and thawing methods (Acker and Mc Gann, 2003). To overcome those problems, ultrasound was introduced to accelerate the cell disruption (Moraes *et al.*, 2011). Furthermore, Moraes *et al.* (2011) stated that ultrasound assisted extraction methods has a 57% higher efficiency than the freezing and thawing methods. The selection of the method to extract phycocyanin with maximum yield and highest activity antioxidant mainly depends on the nature of compounds and thermal stability for extraction. Soxhlet extraction has limitations due to involvement of more than one phase in the system. These methods are time consuming and may require longer time depending on the diffusion rates of solvents. High energy consumption is also one of the disadvantage of the conventional extraction processes while phycocyanin are active compounds of heat sensitive for extraction carried out at higher temperatures, may damage the quality of the extract. In certain cases, the active molecules may be destroyed due to severe pressure and temperature conditions. Solvents which are used in conventional methods exhibit lower efficiency because the molecular affinity between the solvent and solute is decreased (Shirsath *et al.*, 2012; Dey and Rathod, 2013).

Method of ultrasound assisted extraction (UAE) is effectively used to damage or to break the cell walls, reducing the particle size, increase the yield and the rate of mass transfer (Shirsath *et al.*, 2012). In addition, the UAE can damage microcytin found in *Spirulina platensis* soluble in water (Wu *et al.*, 2011). For UAE method, the selection of solvents is very important for extraction. Sen *et al.* (2005) reported that the most suitable solvent for phycocyanin extraction was ethanol since it has highest affectivity as compared to hexane, petroleum ether, ethanol and water.

The different substances that have been extracted using ultrasound include essential oils, aromatic compounds, citrus compounds, sugars, proteins, acids, natural dyes, pigments, etc. (Vilkhu *et al.*, 2007; Shirsath *et al.*, 2012). In addition to the beneficial effect of ultrasound on the yield and kinetics of extraction, it has been observed that extraction can be carried out at much lower temperature and pressure, which can result in substantial decrease in the overall cost of the operation. However, the extraction of phycocyanin from *Spirulina platensis* has not being optimized particularly for its frequency of ultrasound and effect of temperature of extraction to yield. In order to extract phycocyanin from *Spirulina platensis* cells, selection of ultrasonic frequency was important. The yield of phycocyanin in crude extract was depended on ultrasonic frequency (Vilkhu *et al.*, 2007). In this study, the extraction of phycocyanin from wet biomass

using UAE method was presented. Two ultrasound frequencies was used (28 kHz and 42 kHz) to evaluate the effect of ultrasound frequency to the obtained yield and antioxidant activities, while the effect of temperature and time of extraction were also described.

2. Materials and Methods

2.1 Materials

Microalgae (*Spirulina platensis*) biomass contained 16% moisture content was obtained from CV. Microalgae (Sukoharjo, Indonesia). The biomass was stored under dry and dark conditions.

2.2 Experimental design

The experimental design used a central composite design (three levels) with two variables: temperature and time of extraction. The total run was 12 trials: nine points on design factors and three repeated variables at a central point. The temperature was set at three different levels: 30, 45 and 60°C and extraction time of 20, 35 and 50 minutes. Variable responses are yield and EC_{50} values (measuring antioxidant activity).

2.3 Phycocyanin extraction and analysis

In this study ultrasound frequency of 28 kHz and 42 kHz with a power of 200 W were used to determine the effect of the frequencies on phycocyanin yield and antioxidants activity of microalgae *Spirulina platensis.* The conventional extraction method was also conducted to compare yield and antioxidant activity data obtained by UAE method.

***Conventional (soxhlet) extraction*:** The conventional was carried out in a soxhlet extractor and used ethanol 95% as a solvent. The extraction was done for 4 hours, the ratio of biomass and solvent was set at 15 gr biomass /250 mL solvent and temperature was set at 50°C.

***Ultrasound Assisted Extraction*:** About 15 grams of biomass were mixed with 250 mL solvent (ethanol 95%). The biomass was then sonicated at frequencies of 28 kHz and 42 kHz in an ultrasound bath with power of 200 W at the defined variables (Table 1). Samples were settled for two hours in an Erlenmeyer coated with aluminum foil. The supernatant was separated from the sediment and then was evaporated in a rotary vacuum evaporator to separate the solvent in order to obtain phycocyanin extract. Spectrophotometer analysis was used to determine the concentration of phycocyanin and according to Bennet and Bogorad (1973); the phycocyanin concentration was defined as:

$$PC = \frac{OD_{620} - 0.474OD_{652}}{5.34} \quad (1)$$

where PC is the 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 wavelength 652 nm.

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

$$Yield = \frac{PC \times V}{DB} \quad (2)$$

where yield is the extraction yield of phycocyanin in terms of mg phycocyanin/gr of dried biomass (in the next discussion yield will be expressed as %-g/g), V is the volume of solvent (mL) and DB is the dried biomass (g).

2.4 Antioxidant activity determination

The antioxidant activity of all the extracts was measured by using a method described by Sen *et al.* (2005). About 23.5 mg DPPH was dissolved in 100 mL of methanol and then was stored at 4°C. For the measurement of antioxidant activity, this stock solution was diluted by 1:10 in methanol. Approximately 0.1 mL of the extract was added/diluted in 3.9 mL of DPPH solution so that the total final solution was 4 mL which was characterized by discoloration. A blank solution was prepared and consisted of 0.1 mL which was added to 3.9 mL of methanol. This reaction was completed after 4 hours at room temperature, and the absorbance was measured at wavelength of 516 nm using spectrophotometer UV/VIS sp 300. Antioxidant activity of the extract solution was then calculated by Equation 3:

$$EC_{50} = \frac{Abs.blank - Abs.extract}{Abs.\ blank} \times 100\% \quad (3)$$

EC_{50} values represent the amount of concentration of extract solution needed to reduce DPPH free radicals by 50%.

2.5 Optimization of ultrasound assisted extraction (UAE)

In order to determine the optimum process conditions of UAE method, the mathematical model is required. The 2nd order of polynomial model was derived from linear regression based on experimental data obtained in section 2.2. The 2nd

Table 1. Variables used in the experiment of ultrasound assisted extraction method.

Variable fixed	Independent variable
Biomass size of 0.5 mm	Temperature (°C) : 30-60
Biomass/solvent ratio: 15 gr/250 mL	Time (min) : 20-50
Concentration of ethanol : 96%	Frequency of ultrasound : 28 kHz and 42 kHz

order linear correlation (Equation 4) was employed to determine the correlation between response and variables: temperature (X_1) and extraction time (X_2):

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{1,1} X_1^2 + \beta_{2,2} X_2^2 + \beta_{1,2} X_1 X_2 + \text{error} \quad (4)$$

Where β is the intercept, β_1 and β_2 is the coefficient of linear, $\beta_{1,1}$ and $\beta_{2,2}$ is quadratic coefficient, $\beta_{1,2}$ is the interaction coefficient, and error is the error variable. Y_i is the respond of Yield or EC_{50} at frequency of 28 kHz and 42 kHz. The regression parameter of β, β_1, β_2, $\beta_{1,1}$ and $\beta_{2,2}$ were determined by using parameter estimation method in Matlab software and suitability of model was evaluated for its coefficient determination (R^2).

The linear regression model obtained from Equation 4 was used to determine optimum values of temperature and extraction time by using *fmincon* function in Matlab software. The objective function (J) in this optimization was formulated to minimize simultaneously the EC_{50} as well as to maximize the Yield of phycocyanin extract with subject to temperature range (X_1) of 20-60°C and extraction time (X_2) of 20-60 min.

$$\min J = -Y_{yield} + Y_{EC50}$$

$$Y = f(X_1, X_2)$$

$$\text{s.t.} \quad 20 \le X_1 \le 60^o C$$

$$20 \le X_2 \le 60 \text{ min}$$

From this optimization procedure, the optimum temperature ($X_{1,opt}$) and extraction time($X_{2,opt}$) will be obtained. The yield and EC_{50} optimum also can be recalculated by using Equation 4. The experiment of UAE method at the optimum process condition was also conducted to confirm the prediction.

3. Results and Discussion

3.1 Effect of extraction time

Figure 2 shows the changes of extract phycocyanin from biomass *Spirulina platensis* at various extraction time of 20, 35, 50 minutes. It is shown that the extraction yield increases exponentially by increasing the extraction time. The yield gradually increases after 20 min and remains constant after 35 minutes. The increase of yield showed that the ultrasound power has influenced the amount of cell disruptions during extraction. The formation of bubbles due to cavitation process of ultrasound gives a large energy around it causes disruption of the cell wall. The cell disruptions will release the phycocyanin from the cell. A longer extraction time will increase the rate of mass transfer and it is also improved by continuous exposure of ultrasound until the solvent reaches the saturation and then the mass transfer rate is negligible (Dey and Rathod, 2013).

Herrero (2005) showed a comparison sonication times of the yield of phycocyanin extraction. It was observed that a sonication time of 9 minutes gave a yield of 19.7%, while a sonication of 3 minutes resulted in a yield of 19.62%. At the optimum condition, phycocyanin separated in the cell structure which affecting antioxidant activity. The extraction time also affects to EC_{50} values. A longer extraction time will decrease the EC_{50} value which means that more antioxidant have captured the free radicals from DPPH.

As compared to conventional soxhlet extraction method, the UAE gives significant improvements in yield and processing time. It shows that the conventional extraction method only gained yield of 11.13% within 4 hrs of extraction, while the ultrasound resulted yield of 15.97% within 50 minutes (Figure 2). By extending the extraction time the antioxidant activity in the extraction can be reduced, which is shown by the increase of EC_{50} values.

3.2 Effect of extraction temperature

The variation of temperature between 30 to 60°C was studied to evaluate its effect to extraction yield (Figure 3). The irradiation generated by ultrasound wave significantly affect the optimum yield at 45°C. The irradiation could generate cavitation which break the cell wall of cell. Therefore, this can cause an increase in the mass transfer of phycocyanin from cell into the solvent. Sarada *et al.* (1999) stated that yield of phycocyanin will increase by increasing temperature until it reaches an optimum level.

However, we also observed that the yield has decreased after the temperature rise to 60°C. This is due to protein denaturation after the temperature reached more than 55°C (Sarada *et al.*, 1999). The native or functional structure of phycocyanin (Figure 1) has strong correlation to protein which held together by intricate balance of covalent (ion dipole and hydrogen bonds) and non-covalent interactions (hydrophobic and van der Wall interactions) (Creighton, 1984). High temperature of extraction will cause thermal vibration leading to a non-proteolytic modification of the unique in the native structure of phycocyanin, giving rise to definite

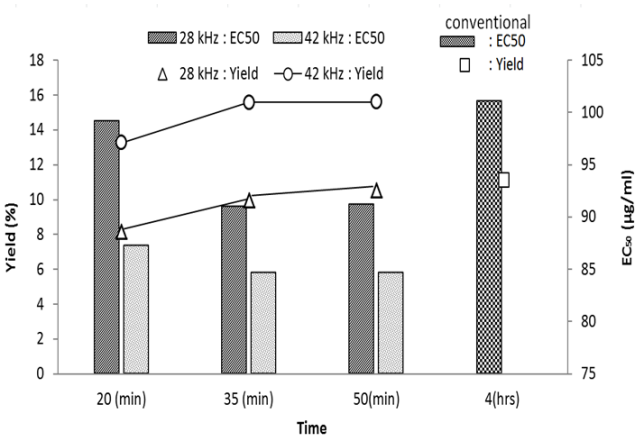

Figure 2. Effect of time on the yield and EC_{50} values in the UAE methods and compared to the conventional method. Both experiments were conducted at constant temperature of 45°C (Hadiyanto *et al.*, 2016).

changes in chemical, physical and biological properties (Volkin and Middaugh, 1992).

Moreover, the cavitation intensity was also significantly reduced by the increase of temperature (Dey and Rathod, 2013). Therefore, increasing temperature higher than protein denaturation point will decrease the yield of phycocyanin. At temperature higher than 60°C the phycocyanin pigment has been reduced during 4 hours extraction and the antioxidant activity was also decreased due to coagulation.

3.3 Effect of ultrasonic frequency

Figure 4 shows the effects of ultrasound frequency in phycocyanin extraction at frequency of 28 kHz and 42 kHz. The yield of phycocyanin extract significantly increased at frequency of 42 kHz than at 28 kHz. The increase of ultrasonic frequency can collapse material due to the cavitation effects. The cavitation will increase the diffusion and also mass transfer at the solid-liquid surfaces. At the ultrasound frequency of 28 kHz the rate of diffusion between solvent into biomass is insufficient because the emitted frequency was only partially able to disrupt or break the cell wall from biomass. The extraction mechanism involves two types of physical phenomena: diffusion through the cell walls and washing out (rising) the cell contents once the wall are broken. Both phenomena are significantly affected by ultrasound irradiation (Vinatoru, 2001). At the higher ultrasound frequency (42 kHz), the extraction can result in high antioxidant activity (lower EC_{50}) and high yield as compared to the low frequency (28 Hz).

3.4 Activity of antioxidant (EC_{50})

The higher temperature is one of the factors that causing damage of phycocyanin. The deactivation of antioxidants was indicated by a high value of EC_{50}, while at higher temperatures the antioxidant will be unstable. As shown in Figure 3, it is observed that the activity of antioxidants will decrease while the EC_{50} value increase and it is influenced by temperature. The optimum temperature for highest antioxidant activity was found at the experimental setting of 45°C.

The phycocyanin pigment can be considered as an active antioxidant if the EC_{50} value < 200 µg/mL (Winarni *et al.*, 2015). At this value, the antioxidant will radically reduce AAPH (2,2'-azobis-2-amidinopropane dihyroxy chloride). Moreover, Romay *et al.* (2003) also stated that at active condition the structure of phycocyanin contain open tetrapirol chain which may have the ability to capture oxygen radicals. Besides, the phycocyanin can also capture free radicals and prevent the radical chain reaction (Agustini, 2007).

The experiment results show that conventional extraction method has lower DPPH inhibition against free radical DPPH. This is shown by a high EC_{50} value = 101.1 µg/mL. The ultrasound irradiation could generate cavitation which favor cell disruption, thinning the cellular membrane and favor the free radicals to be produced and therefore increase

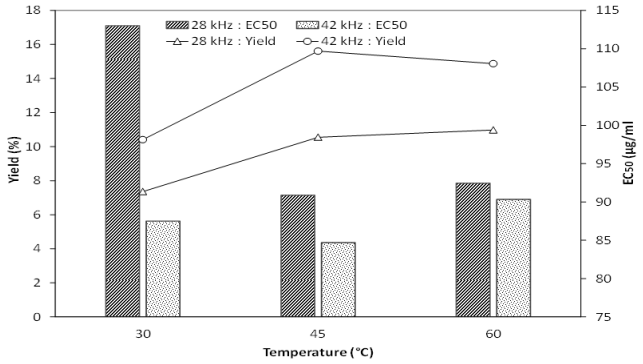

Figure 3. Effect of temperature on the yield and EC50 values in the UAE methods.

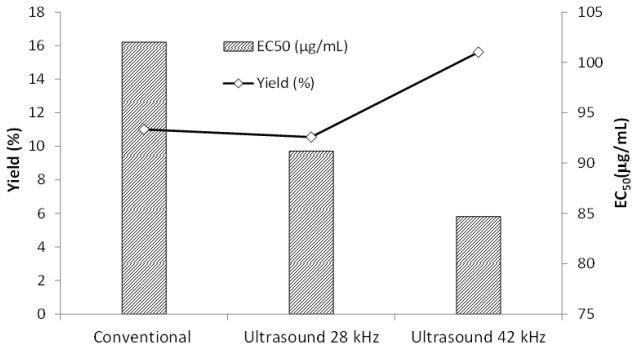

Figure 4. Comparison of UAE with two different frequencies and conventional method.

the antioxidant activity (Khosravi *et al.*, 2013). It is also proven that by using UAE method, the EC_{50} values was lower than the conventional extraction, which were 91.03 µg/mL and 84.7 µg/mL, for frequency 28 and 42 kHz, respectively (Figure 4).

3.5 Optimization using central composite design (CCD)

Table 2 shows the result of regression coefficient of Equation 4 obtained for ultrasound frequency of 28 kHz and 42 kHz. The response was evaluated for its yield and EC_{50} values based on the obtained coefficients.

The coefficients were estimated by using multiple linear regressions (MLR). The regression coefficients were evaluated by the coefficients of determination (R^2), and their residual standard deviation (RSD). The suitability of the model Y_1, Y_2, Y_3 and Y_4 was determined by high value of R^2 and lower value of RSD. The respond obtained by using model (Equation 4) was shown in Table 3. It depicts that the predicted responds are significantly closer to the experimental data both for frequency 28 kHz and 42 kHz.

The optimum process conditions were estimated by searching the optimum values for temperature and time using optimization method of Equation 4. The result presented in Table 4 shows that the increase of ultrasound frequency will increase the yield as well as the antioxidant activities (reduc-

Table 2. Experimental matrix design and results obtained from multiple linear regression.

Parameters	Regression coefficients			
	Frequency of 28 kHz		Frequency of 42 kHz	
	Yield (Y1)	EC_{50} (Y2)	Yield (Y3)	EC_{50} (Y4)
β_0	-17,97	300,43	-31,633	176,365
β_1	0,750	-6,765	1,566	-2,783
β_2	0,352	-1,755	0,349	-1,364
$\beta_{1,1}$	-0,005	0,057	-0,0136	0,0217
$\beta_{2,2}$	-0,0018	0,0098	-0,002	
$\beta_{1,2}$	-0,0035	0,0182	-0,0035	0,0196
Statistics for goodness of fit of the model				
R^2	0,986	0,995	0,975	0,945
R^2_{adj}	0,975	0,991	0,956	0,900
RSD	0,113	1,583	0,394	1,999
F_{test}	88,826	247,97	20,96	62,01

Table 3. Experimental and predicted responds using central composite design.

Run	Temperature (°C)	Time (min)	Respond							
			Frequency 28 kHz				Frequency 42 kHz			
			Exp		Predicted		Exp		Predicted	
	(X_1)	(X_2)	Yield	EC_{50}	Yield	EC_{50}	Yield	EC_{50}	Yield	EC_{50}
1	-1 (30)	-1 (20)	4.18	128.09	4.11	128.94	7.71	100.2	7.26	98.62
2	-1 (30)	0 (35)	6.38	120.15	6.25	118.94	8.3	90.13	8.81	92.55
3	-1 (30)	+1 (50)	7.36	113.01	7.56	113.38	10.41	87.51	10.35	86.49
4	0 (45)	-1 (20)	8.16	99.21	8.55	97.53	13.27	87.25	14.45	86.78
5	0 (45)	0 (35)	10.13	91.23	9.89	91.63	15.57	85.15	15.22	85.12
6	0 (45)	+1 (50)	10.55	90.89	10.4	90.17	15.58	84.68	15.97	83.45
7	+1 (60)	-1 (20)	11.02	91.14	10.7	91.97	15.31	85.22	15.18	85.5
8	+1 (60)	0 (35)	10.87	91.36	11.24	90.17	15.3	89.18	15.16	88.24
9	+1 (60)	+1 (60)	10.98	92.45	10.93	92.81	14.87	90.33	15.14	90.99
10	0 (45)	0 (35)	10.01	90.70	9.89	91.63	15.63	84.29	15.22	85.12
11	0 (45)	0 (35)	10.08	91.02	9.89	91.63	15.56	83.49	15.22	85.12
12	0 (45)	0 (35)	9.58	91.18	9.89	91.63	15.68	85.81	15.22	85.12

ing the value of EC_{50}). It also shows that at higher ultrasound frequencies (42 kHz), the extraction time could be shortened (from 41 to 20 min), however to accelerate the extraction, the higher temperature was still used. From this optimization, the maximum yield could be obtained was 15.66% and EC_{50} was 86.97 mg/mL at ultrasound frequency of 42 kHz, temperature of 55°C and extraction time of 20 min.

4. Conclusions

An ultrasound-assisted extraction (UAE) method has been utilized for the extraction of phycocyanin from microalgae *Spirulina platensis* by using ethanol solvent. Effects of several experimental parameters such as extraction time, temperature and ultrasound frequency on the extracting yields and antioxidant activity of phycocyanin have been evaluated. Ultrasonic wave has been used as a powerful tool, which efficiently improved the extracting performance of phycocyanin. The optimal extraction conditions were achieved at biomass/ethanol solvent ratio of 15 gr/250 mL and extraction for 20 min at 55°C under ultrasonic irradiation of 42 kHz. Under these optimal conditions, the yield of phycocyanin was 15.66 % and the EC_{50} was 88.61 mg/mL. The use of UAE method could improve the extraction process of phycocyanin

Table 4. Optimum value of Yield and EC_{50} obtained by optimization of Equation 4. The predicted values were compared with the experiment data which was obtained from the experiment at the optimum process conditions.

		Optimum conditions (X_{opt})		Value of Respond	
		Temperature (°C)	Time (min)	Predicted	Experiment
Frequency 28 kHz	Yield (%)			11,53	10.72
	EC_{50}	52.8	41.4	89.2	90.45
Frequency 42 kHz	Yield (%)			15.66	15.42
	EC_{50}	55.05	20.1	86.97	88.61

as compared to the conventional extraction (without sonication). The conventional extraction gains yield of 11.13% within 4 hrs extraction time.

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