

Research Article

## **Phycocyanin extraction from *Spirulina platensis* and extract stability under various pH and temperature**

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### **Abstract**

Three extraction methods: sonication, repeated freezing and thawing (RFT) and enzymolysis were carried out on two strains of *Spirulina platensis*, IFRPD1183 (Sp1183) and IFRPD1213 (Sp1213). The sonication method using an ultrasonic processor was conducted for 5, 12.5 and 20 s at 70, 85 and 100% amplitude. Results showed that only the extraction time significantly affected ( $p < 0.05$ ) cell disruption. The RFT method was tested at  $-20^{\circ}\text{C}$  for 1-3 h and 1-3 thawing cycles. Results showed that freezing time and thawing cycles significantly affected ( $p < 0.05$ ) cell disruption. Cell disruption from sonication and RFT of Sp1183 was higher than that of Sp1213. Sonication was more effective in breaking the cell envelope compared with RFT. Further extraction of phycocyanin (PC) showed that temperature greatly contributed to extraction efficiency (EE). The enzymolysis method using lysozyme was carried out under various enzyme concentrations, extraction time and temperature. Results showed that %EE was significantly affected ( $p < 0.05$ ) by extraction time and temperature, but not enzyme concentration. Without lysozyme, the maximum EE was obtained at  $44^{\circ}\text{C}$  which was due to ionic strength of buffer solution. Effects of pH and heat on crude PC solution ( $0.123 \mu\text{g/ml}$ ) were determined in citrate-phosphate buffer with an ionic strength of 0.1 M at pH 3.0, 4.0, 4.5, 5.0 and 6.0. Effects of temperature under continuous heating from  $30^{\circ}\text{C}$  to  $68^{\circ}\text{C}$  in 240 s at each pH showed that PC exhibited the highest stability at pH 5.0. At pH 3, PC was very sensitive to heat and underwent rapid changes.

**Keywords:** prokaryotic bacteria, algae, sonication, repeated freezing and thawing, RFT, enzymolysis, lysozyme, Thailand

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## Introduction

Phycocyanin (PC) is used for food colourant, nutraceutical and immuno diagnostic applications and is mainly extracted from *Spirulina* [1, 2, 3]. Cell structure of *Spirulina* is grouped into prokaryotic bacteria. In *Spirulina* cells, carotenoids, chlorophyll, and phycocyanin (PC) are major pigments amounting to 0.4, 1.0 and 14% dry wt, respectively [4]. PC is (blue) pigment protein (biliprotein) located in the thylakoid system or photosynthetic lamellas in the cytoplasmic membrane [1]. When the cell envelope is broken, thylakoid membrane together with PC are released. Typically, cytoplasmic membrane of gram-negative bacteria is enveloped with 4 layers of longitudinal cell wall: Layers I, II, III and IV [5]. Outer membrane (layer IV) is composed of lipopolysaccharides (LPS). Each LPS molecule is linked together with a calcium and magnesium ion. Layer III is composed of protein fibril. Layer II is the strongest layer and is composed of peptidoglycan molecules. There are three main methods for cell envelope disruption: 1) mechanical means such as bead mill, sonication and high-pressure homogenization, 2) physical rupture such as heat, repeated freezing and thawing (RFT), atomization and decompression and 3) disruption with lytic agent such as chemical lysis and enzymatic lysis [6].

PC is a metal free tetrapyrrole, phycocyanobilin (PCB), which is a chromophore, attached to apoprotein by thioether bond. The basic structure of PC consists of two helix subunits, alpha- and beta subunits. These two subunits form a heterodimer. Near the neutral pH, PC usually forms trimer [7], but the hexameric form may be the basic functional unit of this protein [8]. It was found that pH and concentration of PC in solution are main factors of PC aggregation and dissociation to form monomer, trimer, hexamer and other oligomers [9]. The absorption spectra of PC, monomer and all aggregates, exhibit a strong first excited state band at ~ 615 nm and a much weaker second excited state band at ~360 nm [8]. Changes of PC and PCB conformation relate to the absorptivity of PC at both wavelengths.

This work was conducted to study extraction of PC from *Spirulina platensis* and PC stability under various pH and temperature.

## Materials and Methods

Two strains of *Spirulina platensis*, IFRPD1183 (Sp1183) and IFRPD1213 (Sp1213) were studied. The algae were obtained from the algae laboratory of the Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand. They were cultivated in a 380 L outdoor raceway pond in Zarrouk medium [10] at pH 10.0 for 10 d before harvesting for this study.

The optimized extraction of PC production from *Spirulina platensis* was studied with 3 extraction methods; sonication, freezing and thawing (RFT) and enzymolysis. The sonication method was conducted with an Ultrasonic processor, model GE-100 equipped with 1/8 in. stepped microtip, at 100% amplitude giving 20 KHz ultrasonic wave frequency, for 5, 12.5 and 20 s at 70, 85 and 100% amplitude. The RFT method was designed for 1, 2 and 3 h freezing time (Ft) at -20°C and 1, 2 and 3 thawing cycles (FTC). After cell disruption, further extraction of PC was carried out in a shaker at 25 and 37 °C for 4 h. The enzymolysis method with chicken egg white lysozyme, L6876 obtained from Sigma-Aldrich, Co., (Singapore) was

carried out at various enzyme concentration (EC), extraction time (Et) and extraction temperature. Extraction efficiency (EE) was calculated from  $[A_{620} - 0.474 A_{652}] / 5.34$  [11] using a Biocrom UV/VIS Spectrophotometer, model Libra S22.

PC extraction after cell disruption was conducted in 0.1 M phosphate buffer (pH 7.0) solution. Response surface methodology (RSM) with face centered design was used and the data were analyzed with the Design-Expert version 7.1.6 program.

The effect of pH on PC was determined in sodium citrate-phosphate buffer at pH 3.0, 4.0, 4.5, 5.0 and 6.0 with ionic strength of 0.1 M in a quartz cell (1 cm path length) at room temperature. The UV/Vis spectra of PC were recorded between 250 – 700 nm. The absorbance ratio of  $A_{620}/A_{370}$  was used for determining the conformation change of PC [12]. The folding dynamics of PC structure affected by pH was determined from the emission spectra of PC at an excitation lambda 290 nm [13] and 590 nm [12] using a Shimadzu spectrofluorometer, model RF-1501.

The effect of heat on PC was carried out at pH 3.0 to 6.0 in the same buffer as above in a 1 cm path length quartz cell. The sample cell was warmed to 30°C in a water bath prior to heating in the cell holder of the spectrophotometer to 68°C under a circulating water bath. Temperature changes and absorbance readings at 370 nm and 620 nm were recorded every 10 s during 240 s heating.

## Results and Discussion

### *Effects of sonication and RFT on cell disruption*

Cell disruption has been monitored via PC released and expressed as %EE. Figure 1 shows the response surface plot (RSP) of EE affected by sonication time (St) and % amplitude of Sp1183. EE of Sp1183 is higher than that of Sp1213 (Table 1) indicating more cell disruption. In addition, RFT has greater cell rupture on Sp1183 than that on Sp1213. RSP for RFT of Sp 1183 is presented in Figure 2, showing significant effects of FTC and Ft. From our previous microscopic study (not published), Sp1213 and Sp1183 cell wall thicknesses were 64.6 and 35.4 nm, respectively. This may explain why Sp1213 resists cell disruption higher than Sp1183. Cell rupture comparison between sonication and RFT shows that sonication is more effective. Vibration and bubbles generated by ultrasonic wave power inside algae cells are rapid and effective to explode the cells [4] and therefore cell disruption is higher than that treated with RFT.

### *PC extraction after sonication*

After cell disruption with sonication, further extraction of PC was carried out on Sp1183 and Sp1213. The extraction temperature significantly affects ( $p < 0.05$ ) EE. The maximum EE from equation model from RSM of Sp1183 at 20 s sonication and 100% amplitude is 73.8% at 25°C and 8 h whereas 96.8% and 92.1% are obtained for Sp1183 and Sp 1213, respectively at 37°C and 3 h (Table 2.).

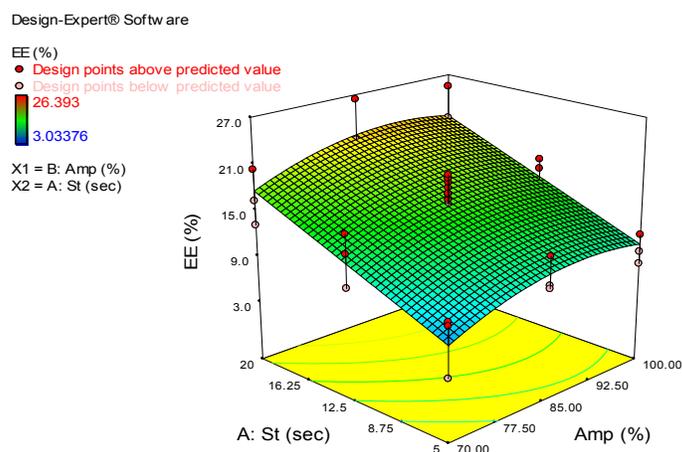


Figure 1. Response surface plot of EE of PC from Sp1183 affected by amplitude and extraction time using sonication.

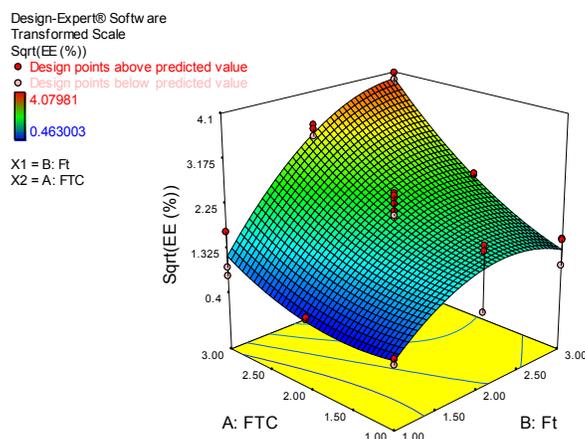


Figure 2. Response surface plot of EE of PC from Sp1183 affected by freeze-thaw cycle and freezing time using RFT.

Table 1. Cell disruption as %EE of *Spirulina platensis* using sonication and RFT.

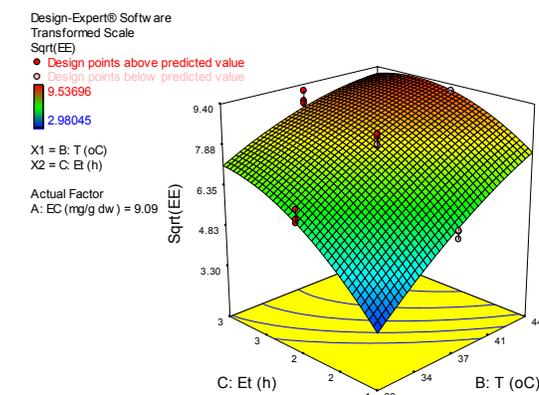
IFRPD No.	Sonication			RFT		
	St (sec)	Amp (%)	EE (%)	FTC (cycle)	Ft (h)	EE (%)
1183	20	91	22.1	3	3	15.6
1213	20	100	14.2	3	3	2.0

Table 2. EE of PC extraction after cell disruption.

IFRPD No.	St (sec)	Amp (%)	Et (h)	Extraction Temp (°C)	EE (%)
1183	20	100	8	25	73.8
1183	20	99	3	37	96.8
1213	20	100	3	37	92.1

**PC extraction with lysozyme**

Cell wall hydrolysis was carried out using lysozyme. EE is significantly affected ( $p < 0.05$ ) by extraction time and extraction temperature (Fig.3), but not enzyme concentration (RSP is not shown). From response surface model, the maximum extraction efficiency is shown in Table 3. Without lysozyme, the maximum EE of Sp1183 at 44 is 87.5% at 2 h extraction while Sp1213 is 103.4% at 2 h extraction. This indicates that extraction at 44°C in buffer solution does not require lysozyme.



**Figure 3. Response surface plot of EE of PC from Sp1183 affected by temperature and extraction time using lysozyme.**

**Table 3. Maximum extraction efficiency of PC with lysozyme.**

IFRPD No.	EC (mg/g dry weight)	Extraction Temp (°C)	Et (h)	EE (%)
1183	0	44	2	87.5
	18.17	44	3	94.5
1213	0	44	2	103.4
	16.12	44	2	108.2

In addition, the effect of ionic concentration was conducted on Sp1213 using only RO water without lysozyme at 37°C. It was found that %EE of PC extracted in buffer is much higher than that in RO water (Table 4.). These results confirm that ionic strength has a greater effect on the EE.

**Table 4. Extraction of PC from Sp 1213 without lysozyme at 37°C.**

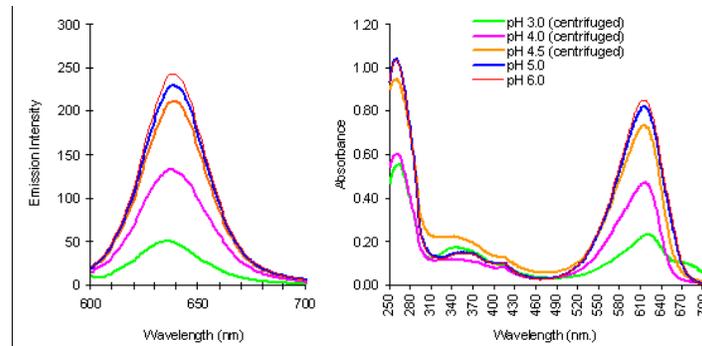
Extraction solution	EE (%)				
	Et (h)	1	2	3	4
RO H <sub>2</sub> O	0.6	2.4	4.4	5.5	
phosphate buffer	46.0	67.0	69.5	71.9	

**Effects of pH and heat on PC**

This study has been conducted under low pH range and continuous heating. Spectra readings are inevitably interfered by protein precipitates. Therefore, interpretation is based on relative spectra readings.

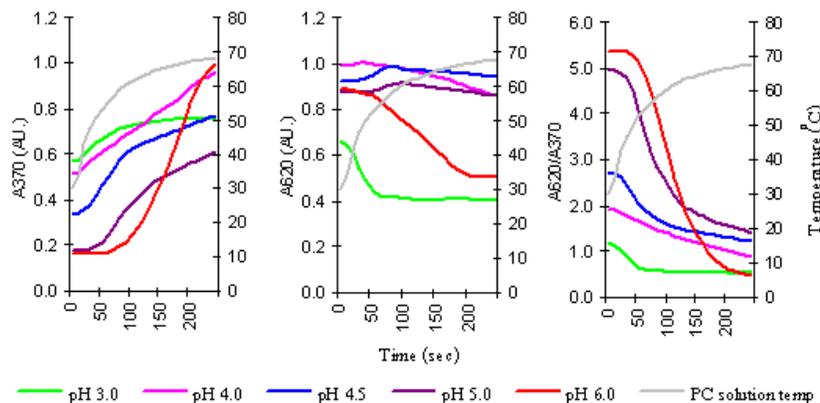
At  $pH \leq 4.5$ , PC structure is unfolded, causing protein precipitates which were removed by centrifugation. As a result, emission intensity and absorbance readings at the same lambda peak are reduced (Fig.4). The lower pH, the higher precipitates occur resulting in inversely reduced absorbance readings of centrifuged samples.

At pH 3, the spectra of centrifuged PC solution noticeably shows a shift in maximum emission lambda and maximum absorbance lambda. This indicates that soluble PC molecules underwent conformation changes due to breaking of salt bridges and hydrogen bonding at low pH [9, 10].



**Figure 4. Absorption and emission spectra of PC at pH 3.0-6.0.**

During heating, the absorbance readings of PC solutions at 370 nm increase at all pH levels (Fig.5). Thus,  $A_{620}/A_{370}$  ratios are decreased with increased temperature. However, the effect of heat, from the absorbance readings at 370 and 620 nm, at pH 5.0 is the least among the other pHs. This suggests that at pH 5 PC has more stable conformation. PC at pH 4 and 4.5 are more stable when compared with those at pH 3.0 and 6.0, indicating that drastic changes of PC conformation, perhaps protein unfolding, has already occurred and reached more stable conformation before measurement. At pH 3, changes rapidly take place at early heating, about 50 s, due to unstable state of PC which is sensitive to temperature changes at very low pH.



**Figure 5. Relative profile changes of A620, A370 and A617/A370 ratio affected by heat and pH.**

From A620/A370 ratios at pH 5-6, PC exhibits continuous changes throughout increasing temperature profile indicating that PC is in its native form and continuously changes during heating.

### Conclusions

The sonication method is very effective in cell wall disruption of *Spirulina* sp., more so than RFT. Sp-1213 has higher resistance to cell disruption by sonication and RFT than Sp-1183. Further extraction is highly affected by temperature.

The effect of ionic strength of an extraction solvent is very high on EE. PC extraction in buffer solution at 44°C for 2 h can be accomplished without lysozyme.

The conformation of PC structure is affected by pH. PC could well retain its native structure at pH>5 and PC forms partial protein unfolding at pH<5.0. Heat strongly exhibits detrimental effect on colour of PC solution at pH>5.0 and pH<3.

The results suggest that in applying sonication for a larger scale, this continuous and short process would be favourable. In the case of RFT method, energy cost is a limiting constraint. Although enzymolysis with lysozyme gives the highest EE, the enzyme is quite expensive and difficult to handle. In addition, the extraction process needs pH and temperature control to obtain a good yield with more stable form of PC.

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