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Enhancement of Phycocyanin Extraction from Dry *Spirulina platensis* Powder by Freezing-Thawing Pre-treatment

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Abstract. Phycocyanin (PC) is a bioactive compound that can function as an antioxidant, anti-inflammatory, immunomodulatory, and anti-cancer agent. It can act as a potential material in preventing COVID-19 and curing those suffering from it. *Spirulina platensis* (SP) is one of the microalgae rich in proteins and PC. This study aimed to determine the optimum PC extraction from SP, using distilled water as solvent through freezing-thawing pre-treatment. The variables set in the investigation were water content in SP before freezing (24.7-84.9 % wet basis), soaking time (0.25, 1, 2, and 6 hours), raw materials' storage period (1-13 months), freezing time (1-141 days), and the (solvent/biomass) ratio (20-440 mL/g). *Spirulina platensis* powder was soaked, frozen, thawed, and extracted in batch operation. The residue was extracted with the same solvent. The PC concentration in the filtrate was determined by measuring its absorbance using a spectrophotometer at wavelengths 615 and 652 nm. The experiment gave the optimum yield at a water content of 81.9% (wet basis), soaking time of 6 hours, freezing time of 1 day, and a solvent-to-biomass ratio of 100 mL/g. The optimum storage period of the raw material was one month. The phycocyanin IC₅₀ value of 1.485 mg/L.

Keywords: Freezing-thawing pre-treatment; Phycocyanin; *Spirulina platensis*

1. Introduction

The COVID-19 pandemic has encouraged researchers to prevent its spread and treat patients suffering from it. People with comorbidities, namely degenerative diseases, are highly vulnerable to severe symptoms. Before this pandemic, some degenerative diseases, such as heart disease, stroke, and cancer, were the leading causes of death (Ministry of Health RI, 2019). The number of people dealing with cancer increases yearly (Sung *et al.*, 2021; Bray *et al.*, 2018). Phycocyanin is one of the phycobiliproteins and bioactive components in microalgae that functions as an antioxidant (Renugadevi *et al.*, 2018; Dejsungkranont, Chen, and Sirisansaneeyakul, 2017), an immunomodulator (Grover *et al.*, 2021), and an anti-cancer agent (Czerwonka *et al.*, 2018; Hernandez, Khandual, and Lopez, 2017; Pan *et al.*, 2015). It can inhibit inflammation that causes damage to lung tissues (Li *et*

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al., 2020). It can also significantly reduce inflammatory levels (Grover *et al.*, 2021; Fernández-Rojas, Hernández-Juárez, and Pedraza-Chaverri, 2014). C-phycocyanin strengthens immunity and is safe to consume since it does not trigger acute diseases and sub-chronic toxicities (Grover *et al.*, 2021).

Microalgae are photosynthetic microorganisms that convert solar energy into chemical energy through photosynthesis in their chlorophyll. Microalgae can grow in fresh water and seawater. Microalgae have diverse nutritional content, especially protein, carbohydrates, and fats (Rosmahadi *et al.*, 2021). Various microalgae that can function as a food source or energy include *Botryococcus braunii*, *Chlorella vulgaris*, *Dunaliella tertiolecta*, *Spirulina platensis*, and *Tetraselmis suecica* (Rosmahadi *et al.*, 2021; Rosli *et al.*, 2020). *Spirulina platensis* is one of the microalgae that can be a source of protein (Sela, Budhijanto, and Budiman, 2021; Vernes *et al.*, 2019; Soni, Sudhakar, and Rana, 2017). It is preferable due to its easiness of being cultivated in fresh water. The content of PC in SP varies from 5 to 20% (Garcia and Mejia, 2021). Consuming *Spirulina* or phycocyanobilin-enriched *Spirulina* extracts may potentially boost type 1 interferon response in the circumstances of RNA viral infection (McCarty and DiNicolantonio, 2020). Phycocyanin isolation begins with the cell wall breaking. The bioactive substances inside the cell can get out more quickly through the broken cell wall so that the extraction of PC becomes fast. If the cell wall remains intact, the extraction will be prolonged because the molecules have to diffuse through it. In general, microalgae cell walls are pretty strong, thus requiring an extraordinary method to break them down. The success of PC extraction significantly hinges on this initial step (Chia *et al.*, 2019).

Various ways of cell wall breaking have been carried out, including sonication (Dianursanti *et al.*, 2020; Pratiwi, Utama, and Arbianti, 2020; Pan-utai and Iamtham, 2019; Ilter *et al.*, 2018; Rodrigues *et al.*, 2018; Tavanandi *et al.*, 2018), microwave (Wang, Zhang, and Fang, 2019; Ilter *et al.*, 2018), homogenization with a stirrer (Rodrigues *et al.*, 2019; Ilter *et al.*, 2018; Tavanandi *et al.*, 2018; Silveira *et al.*, 2007), freeze-thawing (Chia *et al.*, 2019; Ilter *et al.*, 2018; Tavanandi *et al.*, 2018), pulsed electric field (Jaeschke *et al.*, 2019; Martínez *et al.*, 2017), and high-pressure homogenization of up to 350 bars (Deniz, Ozen, and Yesil-Celiktas, 2016). Phycocyanin is very sensitive to temperatures above 60°C (Su *et al.*, 2014; Chaiklahan, Chirasuwan, and Bunnag, 2012; Antelo, Costa, and Kalia, 2008), so a proper method is needed to extract it from SP. The disadvantages of conventional methods include the relatively long stirring time (Rodrigues *et al.*, 2018; Silveira *et al.*, 2007). The agitation process usually comes into contact with the ambient air, so the PC's quality is not good if not immediately stored at low temperatures.

This study used the freezing method to break the cell wall. This method is considerable because it can maintain the quality of the product gained, given that PC is easily damaged if left at room temperature (or higher) or exposed to ambient air. However, freezing also causes ice expansion which can break the cell wall due to volume changes (Dombrovsky *et al.*, 2015). Therefore, the water amount in *Spirulina* must be precise to ensure a successful extraction. A small amount of water in the cell makes the ice expansion insufficient, thus preventing the cell wall from breaking. Several researchers have extracted phycocyanin from *Spirulina platensis* by the freezing-thawing method at a freezing temperature of -20°C (Prabakaran *et al.*, 2020; Chentir *et al.*, 2018) or -40°C (Tavanandi *et al.*, 2018). Phycocyanin yielded 52.82%-62.76% with 4-6 freezing-thawing cycles (Prabakaran *et al.*, 2020; Tavanandi *et al.*, 2018). However, repeating freezing-thawing cycles are time and energy-consuming and only suitable for laboratory scales (Jaeschke *et al.*, 2021). Therefore, it is necessary to study the freezing-thawing method with only one cycle using a freezing temperature slightly below 0°C to save energy and obtain satisfactory extraction results.

In this research, distilled water used as a solvent and the appropriate water content used in *Spirulina platensis* will determine the success of the freezing process. If the cell lacks water, the expansion of ice inside the cell is not enough to break down the cell wall. Conversely, excess water will cause it to be outside the cell. It will freeze both inside the cell and outside the cell. The ice outside the cell will prevent the cell wall from breaking, thus decreasing the number of cells broken. As a result, the phycocyanin content will also decrease. This phenomenon indicates that an appropriate water content allowing the cell wall to break during freezing is necessary. In this case, the freezing-thawing method is superior, as it can damage the cell walls and obtain a better quality of PC produced. Therefore, this research aimed to determine the optimum water content in freezing SP to get a good extract. The variables studied were soaking time, the storage period of raw materials, freezing time, and the solvent-to-biomass ratio.

2. Methods

2.1. Materials

Spirulina platensis powder was purchased from Nogotirto Algae Park, Yogyakarta, Indonesia. The content of water, protein, fat, ash, and carbohydrates was determined based on the proximate analysis (AOAC, 2010). The solvent used was distilled water.

2.2. Freezing-thawing pre-treatment and extraction

The researchers prepared several specimens, each of which contained one gram of SP powder added with various amounts of distilled water to get different water contents. Each of them was soaked for 15 minutes, 1, 2, and 6 hours, and then let to freeze. After 24 hours of freezing, they were thawed and then added with distilled water for extraction using a vacuum filter. The absorbance was measured using a spectrophotometer. Figure 1 depicts the experimental procedures (where t_1 was soaking time, and t_2 was freezing time).

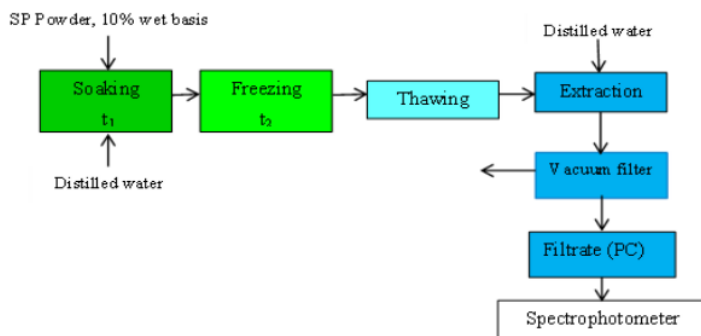


Figure 1 Experimental procedure of PC extraction from SP powder by the freezing-thawing method

2.3. The equilibrium of solid-liquid extraction

Experiments on solid-liquid extraction equilibrium were carried out in batches, in which the residue from the first extraction was added with pure solvent (distilled water). After filtering, the second residue was added with distilled water and then filtered. The extraction was complete when no phycocyanin was found in the extract, as indicated by the absorbance at a wavelength of 652 nm near zero.

2.4. Phycocyanin determination

The concentration of phycocyanin in the filtrate was determined using a spectrophotometer at 615 nm and 652 nm, with the following equations (1) to (4) (Rodrigues *et al.*, 2019; Pan-utai and lamtham, 2019; Rodrigues *et al.*, 2018; Deniz Ozen, and Yesil-Celiktas, 2016; Silveira *et al.*, 2007).

$$CPC = \frac{(OD_{615} - 0.474(OD_{652}))}{5.34} \quad (1)$$

$$APC = \frac{(OD_{652} - 0.208(OD_{615}))}{5.09} \quad (2)$$

$$PCt = CPC + APC \quad (3)$$

where *CPC* was the concentration of chloro-phycocyanin (g/L), *APC* was the concentration of allophycocyanin (g/L), *PCt* was total phycocyanin (g/L), *OD₆₁₅* was the filtrate's optical density at 615 nm, and *OD₆₅₂* was the filtrate's optical density at 652 nm from a spectrophotometer.

The yield of phycocyanin (mg/g) was:

$$Yield = PCt \times V/DB \quad (4)$$

where *V* was the solvent volume (mL), and *DB* (dry basis) was the mass of SP powder (g).

2.5. Antioxidant activity

Antioxidant activity was investigated by DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity. *Spirulina platensis* (water content 80%) 100 mg dissolved in 5 mL of ethanol p.a. The sample was extracted using sonication for 15 minutes, then filtered. Put the filtrate in a 10 mL volumetric flask, add ethanol to 10 mL, and mix homogeneously. We prepared various solutions concentrations of ethanolic extract. One mL of the sample was mixed with 1 mL of 0.15 mM DPPH in absolute ethanol. The mixtures were then incubated at room temperature for 30 mins in the dark. A spectrophotometer measured the absorbance at 517 nm to monitor the DPPH radical decrease. The *IC₅₀* was defined as the concentration of ethanolic extract of phycocyanin to scavenge 50% initial DPPH radical, and it was reflected by a 50% reduction of absorbance (Abdullah *et al.*, 2020; Pan-utai and lamtham, 2019).

3. Results and Discussion

Based on the proximate analysis, *Spirulina platensis* contained 9.57% water, 39.77% protein, 0.8% fat, 7.12% ash, and 42.76% carbohydrates (by difference). Phycocyanin can function as an immunomodulator, reduce inflammatory level, and strengthens immunity. It does not trigger acute diseases and sub-chronic toxicity (Grover *et al.*, 2021). According to D'Alessandro and Filho (2016), the structure of phycocyanin is shown in Figure 2.

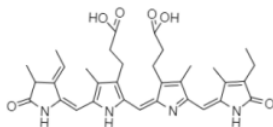


Figure 2 The structure of phycocyanin

3.1. Effect of water content and soaking time

Figure 3 shows the effects of water content and soaking time on the extraction yield. Having the same water contents, the longer the soaking time, the higher the PC content.

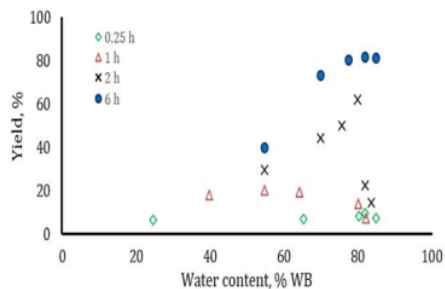
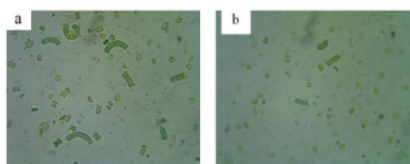


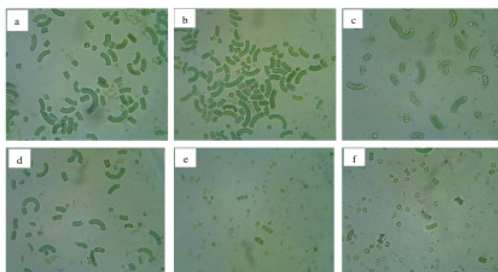
Figure 3 Effects of water content and soaking time on the extraction yield

The longer the soaking time, the more sufficient the duration taken by the distilled water added to SP powder to diffuse into the biomass pores. If the water is already inside the cell before freezing, when it freezes, its phase inside the cell will change, its volume will expand, and the formed ice will break down the cell wall. Consequently, PC will quickly come out during the thawing and extraction stages. The more the cell walls are broken, the higher the PC content. When the soaking time was 15 minutes, the yield would be below 10%. For 15 minutes, not all of the water added to the biomass could enter the cell. At a water content of 81.9 %, the yield obtained was below those when the soaking times were 1, 2, and 6 hours. Of all the experiments, the highest PC content of 81.65% was obtained when after soaked for 6 hours before being frozen. Microscopic visualization of the cells after freezing (with soaking times of 1 and 6 hours before freezing) is present in Figure 4.



Figures 4 Microscopic images of the cells after freezing at a water content of 81.8 % wet basis: a) soaked for 1 hour; b) soaked for 6 hours

Figures 4 show that the six-hour soaking made the number of cells broken more than the one-hour one did. Figure 4b indicates that in the six-hour soaking, the amount of water diffusing into the cells was more than that in the one-hour one (Figure 4a), increasing the number of cells broken significantly. The microscopic images of the effect of water content on the number of cells broken are shown in Figure 5.



Figures 5 Images of SP cell breaking after freezing: a) Initial (non-freezing), b) X=54.8 %, c) X=69.9 %, d) X=77.4 %, e) X=81.9 %, f) X=84.9 % wet basis

Figure 5a shows the initial SP powder condition before freezing. Figures 5b, 5c, and 5d show that after freezing at a water content of less than 80%, the cells' states were relatively the same as their initial state. These data show that water content below 80% is insignificant for breaking down cell walls. Figures 5e and 5f indicate higher numbers of cells broken.

3.2. Effect of raw materials storage period

The raw materials storage period also affects the extraction yield. It can be seen in Figure 6 that the longer the storage period of raw materials, the lower the yield. For the same soaking time (6 hours), at the one-month storage period (T6-S1), the highest yield was around 80%, while at the four-month storage period (T6-S4), the yield was 59%.

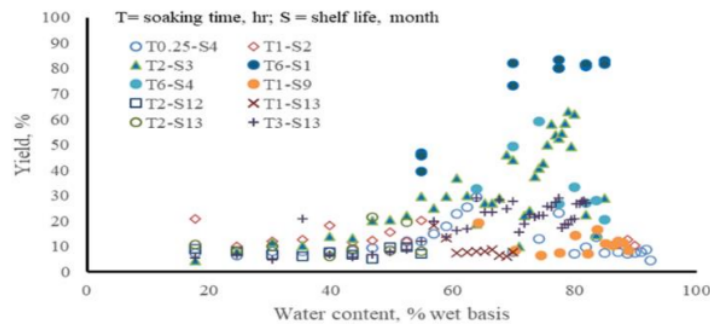


Figure 6 Effect of soaking time and storage period of raw SP on yield PC

For a storage period of 12-13 months, the maximum yield was 30%. It was probably due to the decrease in PC content in the raw material (possibly due to oxidation), so the extracted product's PC content also decreased.

3.3. Effect of freezing time

The effect of freezing on the total PC is described in Figure 7. Theoretically, the freezing time would not affect the resulting extract for the same water content. At the water content of 80%, freezing time of 1 day and 13 days resulting in the optimum of total phycocyanin.

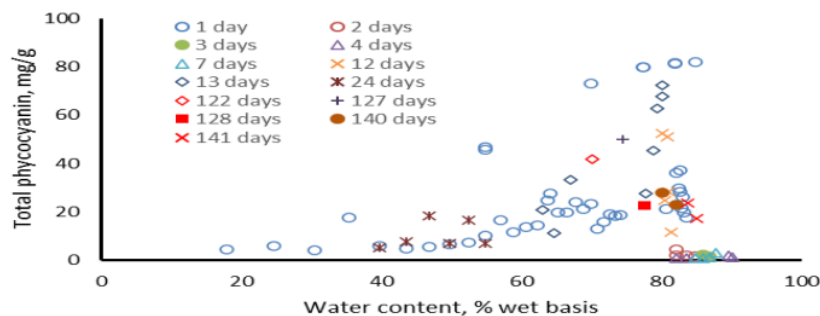


Figure 7 Effect of freezing time on total phycocyanin extracted

Figure 7 shows that the water contents, ranging from 40% to 60%, resulted in the relatively same total phycocyanin for freezing times 1 and 24 days. Likewise, at water contents above 80%, there were no significant differences between those after freezing for 140 and 141 days. It seemed that the freezing time did not affect the phycocyanin content if the frozen state had been reached, but it could be explored more to find the optimum freezing time.

3.4. Effect of (Solvent/L₇omass) ratio

The solvent-to-SP biomass ratio was also studied in the extraction step, as presented in Figure 8.

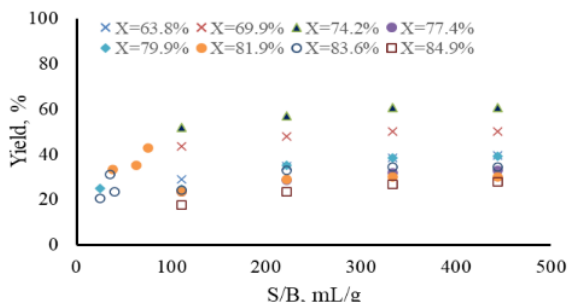


Figure 8 Effect of the ratio of solvent to biomass on the yield

The higher the solvent-to-biomass ratio, the higher the yield (PC produced). After the S/B ratio = 200, the yields were relatively fixed, meaning the extraction was close to equilibrium. The higher the S/B ratio, the lower the PC concentration in the extract because the raw biomass content did not change. The previous researcher obtained a maximum yield of 74.51 mg/g (dry biomass) with a purity of 0.56 at a ratio of S/B of 10 after four cycles (Tavanandi *et al.*, 2018). In this study, the ratio of S/B of 100 with one freezing-thawing cycle yielded a maximum PC of 84.69 mg/g (dry SP). Compared to Tavanandi *et al.* (2018), this study used one freezing-thawing cycle, requiring less energy than four cycles (and a freezing temperature of -40 °C). This method is more applicable for scaling up (using the freezing temperature of 0 to -2 °C). Another previous researcher operating the ultrasonic extraction method found the highest concentration of phycobiliprotein with a mixed solvent containing N-methyl-2-hydroxyethyl ammonium acetate and N-methyl-2-hydroxyethyl ammonium format (2-HEAA+2-HEAF) at pH 6.5 with (S/B) 7.93 mL/g. The extract contained CPC and APC of 5.95 mg/g and 6.34 mg/g, respectively (Rodrigues *et al.*, 2018). The complete comparison of research results is presented in Table 1.

Table 1 Comparison of the PC extraction results from SP with those in other studies

The extraction method and Solvent	S/B, mL/g	Yield, mg/g	References
Four freezing-thawing cycles, distilled water	10.00	74.51	Tavanandi <i>et al.</i> , 2018
Freezing, CaCl ₂ 1.5%	100.00	55.33	ilter <i>et al.</i> , 2018
Ultrasonic, 2-HEAA+2-HEAF	7.93	12.29	Rodrigues <i>et al.</i> , 2018
Mechanical agitation, 2-HEAA+2-HEAF	6.59	22.07	Rodrigues <i>et al.</i> , 2019
Freeze-dried and homogenization, 0.01M Sodium phosphate buffer (pH 7.0)	25.00	78.17	Pan-utai and lamtham, 2019
One freezing-thawing cycle, distilled water	100.00	84.69	This study

Remarks: 2-HEAA+2-HEAF = N-methyl-2-hydroxyethyl ammonium acetate and N-methyl-2-hydroxyethyl ammonium format.

Phycocyanin extraction from SP occurs rapidly. Assumably, the extraction reaches equilibrium in the same manner. Phycocyanin is very soluble in water. After thawing, the extraction stage takes place quickly. In this study, the experiments were carried out in batches, in which the residue from the first extraction was added with pure solvent (distilled water) and filtered again until completed. The extraction was complete if there was zero phycocyanin content in the extract. The extract was initially blue and became brighter after the next process, as shown in Figure 9.

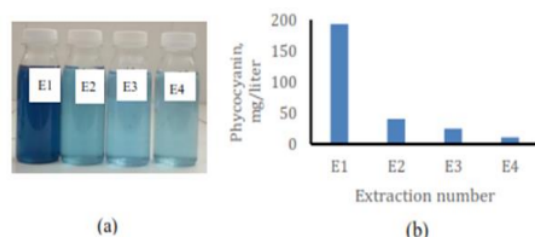


Figure 9 Phycocyanin from SP: a) images of extracts, b) PC concentrations

3.5. Antioxidant activity

Antioxidant activity is represented by the IC_{50} value, namely the concentration of the solution sample required to inhibit 50% of DPPH free radicals. The IC_{50} value of CPC from *Oscillatoria tenuis* was 1.75 mg/mL, while Ascorbic acid was 0.015 mg/mL (Thangam *et al.*, 2013). The concentration of CPC extracted from 0.01 g/mL of SP was 2.418 mg/L, achieving an IC_{50} value of 1.485 mg/L. The previous researcher achieved the optimum scavenging activity (52.13 % inhibition) of freeze-dried SP under homogenization of 0.02 g/mL biomass concentration (0.01 M solvent concentration). The concentration of CPC was 1.67 mg/mL (Pan-utai and lamtham, 2019).

4. Conclusions

The water content in the biomass affected PC extraction from SP by freezing-thawing pre-treatment. The addition of distilled water to dry SP until the optimum water content of 81.9 % (wet basis) with soaking time for 6 hours resulted in a yield with a percentage of 81.65 %. If the water contents (before freezing) were the same, the freezing time did not affect the extraction results. The greater the solvent-to-biomass ratio, the greater the yield obtained until equilibrium. The optimum freezing time was 81.9 % (wet basis), the raw material storage period was one month, and the solvent-to-biomass ratio was 100 mL/g, and the IC_{50} of 1.485 mg/L. We recommend measuring the temperature changing from room to freezing temperature using a data logger and modeling the freezing zone during phase changes.

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