



Purification of phycocyanin from *Spirulina platensis* by combination diafiltration/ultrafiltration membrane

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Abstract

Phycocyanin, a blue pigment derived from the cyanobacterium *Spirulina platensis*, is typically purified through either single-step or multi-step processes. The purity of phycocyanin is crucial for its applications as a food colorant, in cosmetics, as a biomarker, and for analytical purposes. In this study, we report the purification of phycocyanin using a multi-step process involving extraction, ammonium sulfate precipitation, and diafiltration/ultrafiltration (DF/UF) with cycle times of 20, 30, and 40 minutes. The aim of this research to determine the effect of DF/UF time on flux, retention rates and purity on phycocyanin purification. The combination of DF/UF allowed the improving the purity of the phycocyanin in the retentate fraction. Sample that have been extracted and ammonium sulphate precipitation, were purified by ultrafiltration membrane using of hydrophilic polyethersulfone (PESH) membrane of 50 kDa MWCO. The DF/UF system was operated at 1.0 ± 0.1 Psi and at $24 \pm 1^\circ\text{C}$. sample were collected during both DF/UF process in order to evaluated flux, retention rates and purity ratio of phycocyanin. The best DF/UF process were operate at 40 min per cycle, with a mean permeate flux $6.89 \text{ L/m}^2\text{h}$ and retention rate 96.99% was found to be optimal. This process resulted in phycocyanin with purity and concentration of 2.54 and 0.30 mg/mL, respectively. Hence, the DF/UF process were operated at 40 min per cycle potential for a biomarker application.

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1. Introduction

The phycocyanin derived from Cyanobacteria (C-PC) market demand in recent years has increased. The global market price of C-PC is \$155.3 million in 2020 and is estimated to reach \$409.8 million by 2030 (1). The price of C-PC depends on the level of purity and its intended use (2). Food grade C-PC purity costs about \$0.13 USD per milligram. While the price of C-PC on reagents or analytical grade can reach up to \$15.00 USD per milligram (3). Phycobiliproteins are a combination of pigments consisting of three types of pigments that can be found in cyanobacteria, rhodophytes and cryptophytes (4). C-PC is a pigment associated with proteins. The pigment is water soluble and has a brilliant blue colour which can accumulate up to 15-20% of the total dry weight of *Spirulina* (1).

There are three types of phycobiliproteins found in cyanobacteria, namely allophycocyanin (APC) which is bluish green, phycocyanin (PC) is blue and phycoerythrin (PE) is red. Phycobiliproteins are water soluble (2). Phycocyanin is a photosynthetic pigment from

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the phycobiliprotein family. Phycobiliproteins consist of polypeptides with a chromophore containing a and b subunits and have a molecular weight of about 20 kDa (5). Phycocyanin are located on phycobilisomes on the outer surface of the thylakoid membranes of cyanobacteria and some eukaryotic algae (6). Commercial phycocyanin is the only blue pigment on the FDA's food coloring-free product list. C-PC can be used in sweets, cold drinks and ready-to-eat cereals (7). In addition, C-PC is also used in the pharmaceutical industry as a functional food because it has antioxidant, neuroprotective, anti-inflammatory and hepatoprotective properties (1). Meanwhile, purer phycocyanin has applications in the pharmaceutical and nutraceutical fields (6). There have been many reports related to phycocyanin purification methods from microalgae (8). The use of purification techniques is carried out in achieving a certain purity to determine the use of C-PC (9).

Phycocyanin purity can be defined as the ratio between the absorbance of phycocyanin biomolecules (620 nm) and the total protein absorbance (280 nm). The value of the purity ratio of 0.7 indicates that C-PC can be used in foods such as dyes. The purity ratio value of 3.9 indicates C-PC is included in the reactive level and the ratio value of 4.0 determines C-PC in the analytical level (10). The purification method by gel filtration chromatography can reach analytical grade phycocyanin (11). In the study of Chaiklahan et al. (12), using a membrane process for the separation and purification of phycocyanin from *Spirulina* sp. Purification methods using ammonium sulphate deposition and the use of ultrafiltration can be used to obtain phycocyanin with a food grade purity level that has a purity ratio ranging from 0.7 to 2.0 (11). In obtaining a product purity it is necessary to apply a sequence of several different techniques. This can increase production costs and can cause significant product loss. One of the purification techniques with low cost and low resolution but can obtain high purity is by precipitation and ultrafiltration (13).

The combination of techniques in purification involves centrifugation, ammonium sulphate precipitation, ion exchange chromatography, gel permeation chromatography, hydroxyapatite chromatography and expanded Bed adsorption chromatography (14). C-PC obtained from *Spirulina platensis* was purified using ammonium sulphate precipitation and followed by different chromatographic methods (15). Ultrafiltration is a membrane separation technique that is commonly used in protein separation. This separation technique has only been used in several studies in the purification of phycobiliproteins from microalgae (16). Membrane technology is easy to implement and can be applied to large-scale production. In addition, this technique is carried out at room temperature which can minimize pigment damage, so that the colour of phycocyanin can be maintained (17). Several studies have used membrane separation techniques. The purification process using ultrafiltration in the research of Herrera et al. (18) used a membrane with a molecular weight cut-off (MWCO) of 50 kDa followed by activated charcoal adsorption and spray drying to produce phycocyanin with a purity ratio of 0.74 (food grade). In addition, in the study of Figueira et al. (19) used an ultrafiltration technique with conventional laboratory cells with a volume of 160 mL stirred with a suspended magnetic rod to simulate tangential flow. While in the study of Amarante et al. (13) used a purification technique by diafiltration/ultrafiltration (DF/UF) with a 50 kDa polyethersulfone membrane 8 diafiltration cycles are required to remove the salt. The ultrafiltration process provides superior phycocyanin extraction results compared to methods such as chromatography or precipitation because it combines size-based separation while maintaining the protein structure. Unlike other methods that require complex stages and specific chemicals, ultrafiltration selectively separates phycocyanin from small impurities

through controlled porous membranes, without the risk of denaturation or loss of biological activity that often occurs in conventional methods (20).

Until now there is no complete information regarding the DF/UF time for each cycle in the purification of phycocyanin from *S. platensis* using an ultrafiltration membrane. Therefore, the aim of this study to determine the effect of DF/UF time on flux, retention rates and purity on phycocyanin purification. Phycocyanin purification was carried out using the membrane ultrafiltration method with a time of 20, 30, and 40 min for each cycle to determine the best flux, retention rate, purity, and concentration of phycocyanin produced from ultrafiltration.

2. Materials and Methods

2.1. Materials

Spirulina platensis was obtained from the Center for Brackish Water Aquaculture Development (BBPBAP) in Jepara, Central Java, Indonesia. The samples were derived from pure phytoplankton isolates grown in liquid seawater with a salinity of 15 ppt. The culture process used sterile Erlenmeyer flasks, with a sample harvest time of 10 days. The samples were prepared in powder form with a moisture content of <10%. Ammonium sulfate and phosphate buffer was purchased from Chem-Mix Pratama, Yogyakarta.

2.2. Extraction Phycocyanin

The methodology of phycocyanin extraction process from *Spirulina platensis* was conducted by modification of Kissoudi et al. (21) method using phosphate buffer solvent pH 7.0. *Spirulina platensis* was dissolved in phosphate buffer solvent in a ratio of 1:20 (w/v). The mixed solution was further macerated using a magnetic stirrer for 2 hours and continued with ultrasonication. The tool used in ultrasonication is a sonicator bath with a frequency of 40 kHz for 20 minutes, a temperature of 25°C. The resulting extract solution was centrifuged at 5,000 rpm for 30 minutes at 4°C. The results obtained in the form of a blue supernatant and a green precipitate. The supernatant was purified by precipitation method used ammonium sulphate.

2.3. Purification of Phycocyanin Using Ammonium Sulphate Precipitation

The supernatant obtained from the extraction process was purified by the ammonium sulfate precipitation method, the methodology presented by Silva et al. (22). The supernatant was added with ammonium sulfate with a saturation level of 0-20% and a saturation level of 20-50%. The precipitation process begins with a saturation level of 0-20%. The process was carried out using a magnetic stirrer for 30 minutes at 4°C. After the 0-20% saturation level was completed, it was then centrifuged at 6,000 rpm for 20 minutes at 4°C. The results of centrifugation obtained a blue and green supernatant. The supernatant was re-precipitated at a saturation level of 20-50%. After the precipitation process was completed, the supernatant was centrifuged again at 6,000 rpm for 20 minutes at 4°C. The results of centrifugation obtained a clear supernatant and a blue surface.

2.4. Ultrafiltration Membrane (DF/UF)

Purification of phycocyanin using membrane process, modification method by Chaiklahan et al. (12) and Figueira et al. (19). This process was conducted at 24°C, and pressure of 1.0 bar. The system was operated in diafiltration/ultrafiltration (DF/UF) process, with evaluated cycle from 1 up to 8 cycles of diafiltration prior to concentration of the extract

in ultrafiltration process. The treatment provided in this study is the duration of the filtration process using ultrafiltration membranes, where the phycocyanin extract was feed into the system, which was operated at 20 min, 30 min and 40 min per cycles. Previous research has shown that extraction time affects the yield of C-PC (23). C-PC increases during the first 5 minutes of extraction, then increases with increasing time (24). Extraction times of 20-60 minutes significantly impact the yield and purity of phycocyanin (25). After the ultrafiltration process, the results obtained are then compared with those before the ultrafiltration process to see the effect of the duration of ultrafiltration provided.

2.5. Phycocyanin Characterization

2.5.1. Phycocyanin concentration

The amount of C-PC in the sample was calculated using the following equation 1, as described by Bennett et al. (26)

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

Where OD_{620} is optical density of the sample at 620 nm and OD_{652} is optical density of the sample at 652 nm.

2.5.2. Purity phycocyanin

Extract purity (equation 2) of phycocyanin was determined for ratio phycocyanin and protein as described by Abalde et al (5).

$$\text{Purity} = \frac{A_{620 \text{ nm}}}{A_{280 \text{ nm}}} \quad (2)$$

Where A_{620} is absorbance of the sample at 620 nm and A_{280} is absorbance of the sample at 280 nm.

2.5.3. Recovery

The recovery (equation 3) was calculated using the ratio of amount of phycocyanin collected to the amount of phycocyanin loaded (19).

$$\text{Recovery (\%)} = \frac{C-PC \left(\frac{\text{mg}}{\text{mL}} \right) \times \text{Collected volume (mL)}}{C-PC \text{ initial extract} \left(\frac{\text{mg}}{\text{mL}} \right) \times \text{Initial volume (mL)}} \times 100 \quad (3)$$

2.5.4. Flux

The permeate flux expressed in L/m^2h , was calculated by measuring the permeate volume collected in a given time, the following equation 4 (27):

$$J_p \text{ (L/m}^2\text{h)} = \frac{V_p}{tA} \quad (4)$$

Where V_p (L) is the permeate volume, A (m^2) is the membrane area, and t (h) is the permeation time.

2.5.5. Retention rate

The retention rate of phycocyanin was determined following equation 5 (28):

$$\text{Retention rate (\%)} = 100 \left[\frac{1 - A_{620} \text{ (permeate)}}{A_{620} \text{ (retentate)}} \right] \quad (5)$$

2.6. Statistical Analysis

The test results were analyzed using ANOVA followed by Tukey's post-test. The results are expressed as mean $\pm$ standard deviation, and the results are considered significant when the p-value is lower than 0.05. Different lowercase letter notations indicate significant differences ($p \leq 0.05$), and the same lowercase letter notations indicate no significant differences ($p > 0.05$). The data was analyzed using SPSS software version 20. The test was repeated three times.

3. Results and Discussion

Phycocyanin is important cyanobacterial accessory pigment having number of a biotechnology, foods and medicine applications (29). Although a number of research are available for the extraction and purification of phycocyanin from *S. platensis* (5,21,30–33,22–29). Extraction and purification of phycocyanin was complete in three major steps: initial step of phycocyanin extraction, ammonium sulphate precipitation, and diafiltration/ultrafiltration (DF/UF) was part modified methods as has been done by Figueira et al. (19).

3.1. Extraction of Phycocyanin

Extraction is the most important requirements for initial step in obtaining large amount of phycocyanin. In the present study, we used a magnetic stirrer method (using 0.1 M phosphate buffer, pH 7.0) and its concentration and purity was assessed (Table 1). In the initial step of phycocyanin extraction showed that the purity of phycocyanin can be categorized as for food grade (25,34,35).

Table 1. Determination of phycocyanin purity and concentration after different steps of purification from *S. platensis*.

Steps of purification	Extract purity (AU)	Phycocyanin (mg/mL)	Recovery (%)
Crude Extract	0.66 ± 0.014	0.35 ± 0.003	100
Ammonium sulphate precipitation	1.85 ± 0.047	0.31 ± 0.006	88
Ultrafiltration (DF/UF)			
20 min/ cycle	2.54 ± 0.1 ^a	0.26 ± 0.01 ^a	74 ^a
30 min/ cycle	2.58 ± 0.17 ^a	0.27 ± 0.01 ^{ab}	77 ^{ab}
40 min/ cycle	2.65 ± 0.1 ^a	0.30 ± 0.01 ^b	85 ^b

values are expressed as mean ± SD of n=3; data followed by the same letters in columns do not differ statistically using Tukey test ($p \leq 0.05$)

3.2. Purification of Phycocyanin

3.2.1. Ammonium sulphate precipitation

Purification of phycocyanin was performed in two stages, ammonium sulphate precipitation and diafiltration/ultrafiltration. The purification step involved fractional precipitation with 20% and 50% ammonium sulphate and its concentration and purity were assessed (Table 1). Precipitation with ammonium sulphate is a technique frequently employed for phycocyanin purification (11,22,36,37). Evaluation of the application of ammonium sulphate precipitation technique at different times was performed by Figueira et al (19). After the ammonium sulphate precipitation steps, the purity of phycocyanin was increased to 1.85. The purity value can be categorized is coloring agents in cosmetic (38). A purity can be reached with the use of ammonium sulphate precipitation between 1.5 and 2.5 (21,39,40).

3.2.2. Diafiltration/Ultrafiltration

Phycocyanin purification using the DF/UF method are showing an increase the purity ratio. To obtain a product with a higher purity, cycle of diafiltration were performed before the concentration by ultrafiltration and compared with test carried our Previously (19). A new

experiment was performed to evaluated different numbers of diafiltration cycle at various time per cycle consisting of 20 min, 30 min, and 40 min (Figure 1).

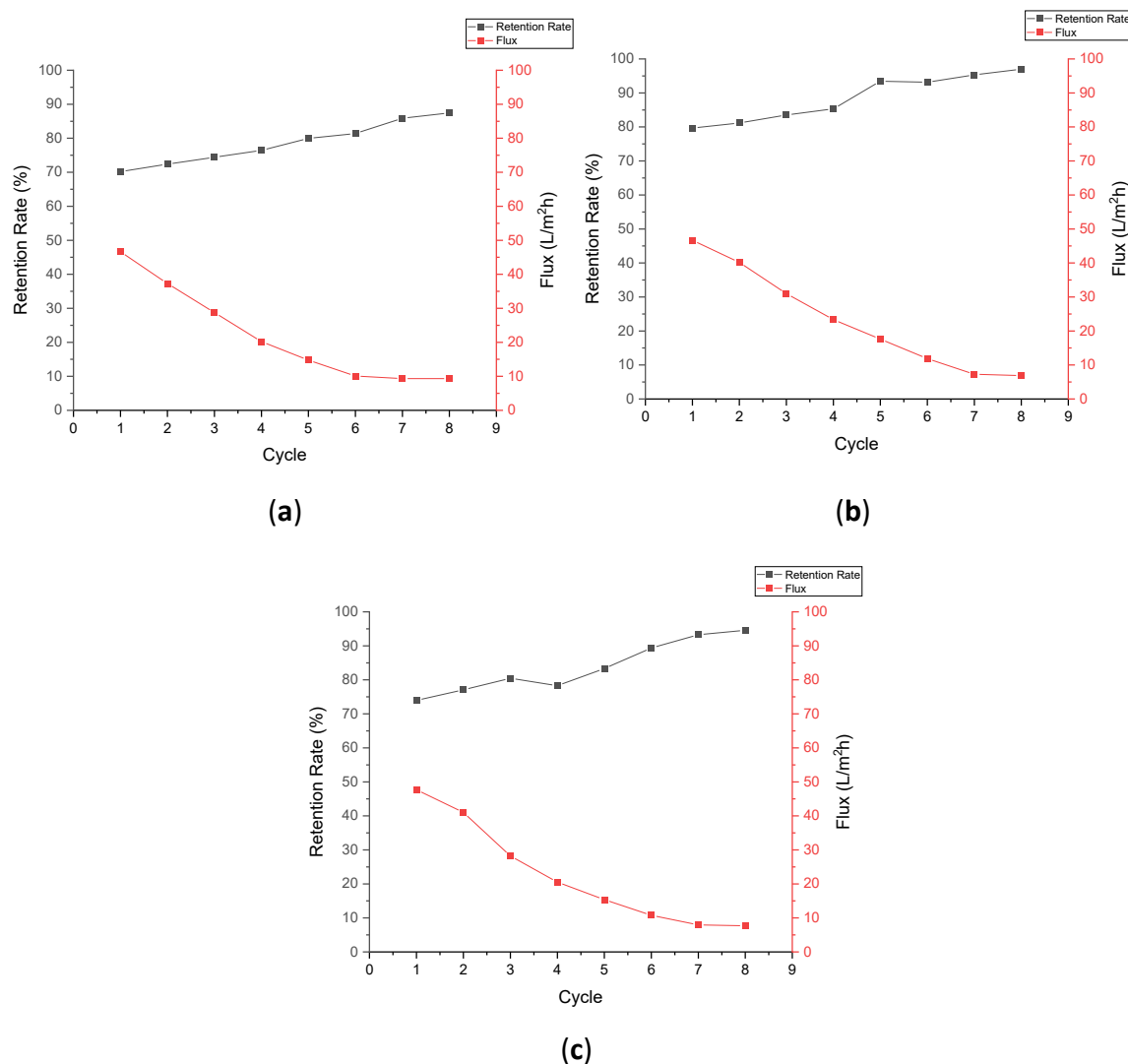


Figure 1. The permeate flux and retention rate of the phycocyanin with different numbers of diafiltration and at various time per cycle. (a) 20 min; (b) 30 min; (c) 40 min.

The result showed the highest purity, and concentration of phycocyanin (Table 1), permeate flux and retention rate which has operated at the time 40 min per cycle. Research in line conducted by Chaiklahan et al. (12), that the ultrafiltration membrane with MWCO at 50 kDa with an average permeate flux of 26.8 L/ h¹ m² and a retention rate of 99% was found to be the best. After the diafiltration/ultrafiltration process was performed on the phycocyanin extraction, the purity level and concentration increased because these two methods selectively separate small-sized impurities from phycocyanin, which has a larger molecular weight. The ultrafiltration membrane is designed to retain macromolecules such as phycocyanin while allowing small molecules like salts, secondary metabolites, or low molecular weight contaminating proteins to flow through (41). Meanwhile, diafiltration enhances purification through buffer exchange, where the remaining solvent along with residual impurities is replaced with a new buffer, thereby reducing interfering substances. This combination can eliminate almost all hydrophilic contaminants and small molecules that

are usually difficult to separate by centrifugation or precipitation. Purification of phycocyanin by using a polyethersulphone (PES) membrane with a molecular weight cut-off (MWCO) of 20 kDa with UF/DF mode can increase the phycocyanin recovery in the retentate (27). The combined ultrafiltration/diafiltration process is suitable for the purification of Measles Virus resulting in the recovery of 50% infectious virus (42).

Although ultrafiltration is effective in purifying phycocyanin, further research is actually needed regarding the degradation of phycocyanin compounds due to prolonged extraction times, considering that phycocyanin is sensitive to environmental factors such as light, temperature, excessive pressure, or mechanical stress during the circulation of the solution in the ultrafiltration system, which can damage the phycocyanin structure (43). The duration of the process can also increase the risks of oxidation and microbial contamination, which can reduce the quality of the final product. Purification using DF/UF are economically viable and simplified for large-scale industrial use for several reason including they are energy-efficient and environment friendly which reduces overall operational costs compared to other purification methods (44) and these membrane-based technologies can reduced chemical use (45).

4. Conclusions

This study demonstrated the potential of phycocyanin purification by DF/UF process can be applied to biomarker. Under these processes, DF/UF process with 8 cycles of diafiltration prior to concentration of the extract in ultrafiltration mode at time 40 min per cycle. The results showed the highest purity with 2.65 of the purity ratios, 0.30 mg/mL of the concentration, permeate flux 6.89 L/m²h and retention rate 96.99 %. This purification processes are an economically produced for industrial scale-up and simplify to handle in large scale.

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Author Contributions

T.W.A, A.D.A, Y conceived and designed the experiments; Y performed the experiments; T.W.A, A.D.A, Y analyzed the data; all authors prepared the original draft; T.W.A, Y reviewed, and editing the final manuscript.

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Data Availability Statement

The data supporting the findings of the article is available within the article.

Conflicts of Interest

All authors declare no conflict of interest.

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