Continuous extraction of *Spirulina platensis* biopigments using different extraction sequences

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**Abstract.** The use of microalgae *Spirulina* is developing rapidly in the industry. *Spirulina* biomass is widely used in the food, pharmaceutical and cosmeceutical industry because it is rich in biochemicals and antioxidants that are beneficial to human health. This study aimed to determine the biopigment contents in *S. platensis* and examine the sequences of continuous extraction process to obtain the optimal biopigment yields. Biopigments of *S. platensis* extracted in this study were total chlorophyll of 52.28 µg/mL (25.28 µg/mL chlorophyll a and 27 µg/mL chlorophyll b), total carotenoid of 7.9 µg/mL and phycocyanin of 0.020 mg/L. In addition to biopigments, a residue in the form of solid powder was also produced at 20.7 % of biomass. Simultaneous extraction of chlorophyll and total carotenoids was successfully applied in this study, while the optimal phycocyanin extraction should be further developed. The optimal extraction sequence of biopigments was extraction of chlorophyll then carotenoid with low heating and followed by extraction of phycocyanin.

1. Introduction

Nowadays, the use of microalgae biomass is increasingly in demand by various industries [1]. The cosmetics and pharmaceutical industries utilize microalgae biomass and extracts as raw materials in their products because it provides good benefits for human health [2]. One of the essential compounds in microalgae that have benefits for the human health is biopigment [3]. *Spirulina platensis* is a blue-green microscopic autotrophic microalgae that is widely used in food supplements or nutraceuticals, cosmetics, and health products [4-6]. Biopigment of *S. platensis* is one of the promising secondary metabolites that are widely utilized. The biopigments in *S. platensis* include chlorophyll, carotenoids and phycocyanin that act as light harvesting pigments and antioxidants which prevent free radicals [4]. Biopigment has many benefits for the human body including antioxidants, anticancer, and enhancing immunity [5, 6]. Chew et al. [6] reported that β-carotene, astaxanthin and
canthaxanthin decreased mammary tumor volume in mice. These biopigments inhibit the growth of mammary tumors, and their anti-tumor activity is also influenced by the supplemental dose [6].

Microalgal biomass has the potential to be further processed into various products such as food, feed, nutraceuticals, bioenergy and other high value products [2]. However, the development of a large-scale microalgal biorefinery has been limited by obstacles in downstream processing technique, for example, the extraction and purification of biochemicals from microalgal biomass [7]. In its implementation, microalgal biomass is processed mainly as a dry biomass and added to the main product, not processed further in a complex manner. Continuous extraction of biopigments can be applied to optimize the utilization of S. platensis biomass. The determination of the optimal extraction process will improve the biorefinery process of S. platensis to produce multiple products and increase the added value of the biomass. This study aimed to determine the biopigment contents of chlorophyll, total carotenoid and phycocyanin in S. platensis and to examine the sequences of continuous extraction process to obtain the optimal biopigment yields.

2. Materials and Methods

2.1. Equipment and Materials

The equipment used in this study were 20 L plastic containers, measuring cups, jars, large size filters, metal trays, mortars, lid jars, a refrigerator, a balance, measuring cups, pipettes, ovens, transparent plastic jars, freezers, heating mantles, fat boiling flasks, soxhlet extractor, condenser tube circulators, cooling bottles, solvent storage bottles, vial bottles, UV-VIS HITACHI U-2900 spectrophotometer, aerators, funnel, a spray bottle and plastic cups. The materials used were clean water, ethanol 96 %, acetone, filter paper, chlorine, sodium thiosulfate, nylon, and cotton. Spirulina platensis was originally cultivated at the microalgae laboratory, Surfactant and Bioenergy Research Center, IPB University, Bogor, Indonesia.

2.2. Cultivation of Spirulina platensis

Spirulina platensis cultivation was carried out to produce biomass as the raw material for biopigment extraction. Spirulina platensis was cultivated in a 20 L plastic container with sterile freshwater media. Sterile fresh water was obtained by making 60 ppm chlorine solution with tap water, which was aerated for 24 h. After the chlorine odor disappears, water was neutralized with sodium thiosulfate:mass of chlorine (1:1). Culture was made by diluting one part of concentrated S. platensis, with three parts of the culture medium. Walne medium 1:1000 (v/v) was added, and the culture was mixed with an aerator.

Concentrated S. platensis culture was harvested. The thickness of the culture was visually observed and detected by optical density (OD) using a spectrophotometer measured at a wavelength of 680 nm. Culture with a very thick green color and achieved an OD value of ± 1 was harvested. Biomass harvesting was performed by filtering the culture with a filter paper. Biomass was collected in a metal tray and dried in an oven at 45 °C for 16 h.

2.3. Extraction of biopigments

The continuous extraction in this study was applied for three different biopigments from one biomass sample. The first extraction sequence was carried out in the order of chlorophyll, carotenoid then phycocyanin extraction. The second extraction sequence was carried out in the order of phycocyanin, chlorophyll then carotenoid extraction.

1. Chlorophyll extraction

Chlorophyll extraction was performed by the soxhlation method. 150 mL of 96 % ethanol was added to the fat boiling flask then placed in the heating mantle. The packaged extraction sample was inserted into the soxhlet tube then the tube was connected to the boiling flask and the condenser. After the continuous extraction showing the solvent was colorless, the sample was removed from the tube.
and stored in a clean container. The extract was cooled then transferred in a vial. Chlorophyll extract was stored in the refrigerator prior to the quantification.

2. Carotenoid extraction

The extraction package used in chlorophyll extraction was dried. The fat boiling flask and soxhlet tube were washed thoroughly and rinsed with acetone. For the extraction, 150 mL of acetone was added into the fat boiling flask, and the fat flask was placed in the heating mantle. The previously dried extraction package was inserted into the soxhlet tube. Extraction was carried out until the solvent became colorless. Extraction package was removed and stored in a clean container. The extract was dried and concentrated. The extract was then cooled, weighed, then dissolved with 10 mL of 96 % ethanol in a vial. The carotenoid extract was stored in the refrigerator prior to the quantification.

3. Phycocyanin extraction

For phycocyanin extraction, a cold maceration extraction was applied. A transparent plastic jar was cleaned and added with 1 gram of sample. 50 mL of clean water was added. The biomass residue from previous extraction was sedimented then the jar was stored in the freezer overnight. A blue color appeared then the extract was thawed at room temperature. The sample was filtered and the melted phycocyanin extract was collected in a plastic cup and then stored again in the freezer prior to the quantification.

2.4. Quantification of biopigments

Quantification of biopigments in this study was performed using optical measurements by the UV-Vis Spectrophotometer HITACHI, type U-2900 with equations as follows.

Chlorophyll was quantified at absorbance (A) 665 nm and 652 nm wavelengths [8].

\[
\text{Chlorophyl a (}\mu g/\text{mL}) : (16.29 \times A_{665}) - (8.54 \times A_{652}) \quad \text{……..(1)}
\]

\[
\text{Chlorophyl b (}\mu g/\text{mL}) : (30.66 \times A_{652}) - (13.58 \times A_{665}) \quad \text{……..(2)}
\]

\[
\text{Total Chlorophyl (}\mu g/\text{mL}) : \text{Chlorophyl a + Chlorophyl b } \quad \text{……..(3)}
\]

Total carotenoid was quantified at a wavelength of 450 nm [9, 10].

\[
\text{Total carotenoid (}\mu g/\text{mL}) : A_{450} \times 25.2 \quad \text{……..(4)}
\]

Phycocyanin was quantified at 615 nm and 625 nm wavelengths [11].

\[
\text{Phycocyanin (}\mu g/\text{mL}) : \frac{(A_{615}) - (0.474 \times A_{625})}{5.34} \quad \text{……..(5)}
\]

3. Results and Discussions

In this study, two different extraction sequences of the three biopigments were applied. The first continuous extraction sequence was chlorophyll extraction followed by carotenoid extraction and ended by phycocyanin extraction. Using this sequence, chlorophyll extract was good, carotenoid and phycocyanin were extracted, but not optimal. The second extraction sequence was phycocyanin extraction followed by chlorophyll extraction and ended by carotenoid extraction. Using this sequence,
Phycocyanin and chlorophyll extracts were good, but carotenoid was not extracted. The results of the first continuous extraction sequence are presented as follows.

3.1. Chlorophyll content
The chlorophyll content of S. platensis is shown in Table 1.

| Sample | Absorbance | Content (µg/mL) | Yield (%) |
|--------|------------|-----------------|-----------|
|        | 652 nm     | 665 nm          | Chl-a     | Chl-b | Total Chl | Chl-a | Chl-b | Total Chl |
| 1      | 2.044      | 2.618           | 25.19     | 27.12 | 52.31     | 37.79 | 40.67 | 78.46   |
| 2      | 2.039      | 2.626           | 25.36     | 26.85 | 52.22     | 38.05 | 40.28 | 78.33   |
| 3      | 2.043      | 2.623           | 25.28     | 27.02 | 52.30     | 37.92 | 40.53 | 78.45   |
| **Mean** | **25.28** | **27.00**      | **52.28** | **37.92** | **40.49** | **78.41** |

Biopigments of S. platensis extracted in this study were total chlorophyll with a concentration of 52.28 µg/mL of 25.28 µg/mL chlorophyll a and 27 µg/mL chlorophyll b, total carotenoid of 7.90 µg/mL and phycocyanin of 0.020 mg/L. As an autotrophic microorganism that performs photosynthesis, chlorophyll content is the most dominant among other biopigments in S. platensis [4].

3.2. Total carotenoid content
The total carotenoid content of S. platensis is shown in Table 2.

| Sample | Absorbance 450 nm | Content (µg/mL) | Yield (%) |
|--------|-------------------|-----------------|-----------|
| 1      | 0.311             | 7.84            | 0.78      |
| 2      | 0.314             | 7.91            | 0.79      |
| 3      | 0.315             | 7.94            | 0.79      |
| **Mean** | **7.90**         | **0.79**        |           |

In accordance with this finding, the results of S. platensis biomass were dominated by the chlorophyll content, followed by carotenoid and finally phycocyanin. Chlorophyll content obtained from the sample was considerable, but for the other two biopigments, the yields were very low. The total carotenoid content of S. platensis was 7.90 µg/mL.

3.3. Phycocyanin content
The phycocyanin content of S. platensis is shown in Table 3. The phycocyanin content of S. platensis after continuous extraction sequence was 0.020 mg/mL. The yield decreased significantly when compared to a single extraction of direct phycocyanin from the biomass, for instance 0.4-0.8 mg/mL, of extracted phycocyanin from S. platensis using different extraction methods [11]. However, the yield could be optimized, especially for the phycocyanin. Therefore, it is necessary to separate or to improve one of the biopigment extractions, i.e., phycocyanin, in the extraction chain of microalgae biopigments.
The suboptimal yield of biopigments was caused by the damage to the compound due to the heat applied, especially during the extraction process. With heating, many compounds in *S. platensis* will experience damage, so that the levels will decrease, including biopigment. Carotenoids and phycocyanin have heat-sensitive properties, in which the carotenoids will be degraded and phycocyanin will experience denaturation [12]. In soxhlation extraction, heat damage is caused by several factors. First, the heat that propagates with the glass medium from the heating mantle to the extraction chamber. Second, heat comes from solvent vapor which does not condense due to ineffective cooling, then fills the extraction chamber.

### 3.4. Composition of *S. platensis* biomass

Figure 1 shows the composition of *S. platensis* biomass extracted in this study.

![Figure 1. Biopigments and the residual biomass composition of *S. platensis*.](image)

From one gram sample of *S. platensis*, 79.3 % was the extracted biopigments, i.e., chlorophyll a of 37.9 %, chlorophyll b of 40.5 %, total carotenoid of 0.8 % and phycocyanin of 0.1 %. This extraction process produced a residual of 20.7 % or 0.21 g, in the form of a fine gray powder. *S. platensis* fine powder is composed of fibers and other chemical components such as minerals [13]. Fiber is a lignin complex compound with polysaccharides that is resistant to the digestive process. Therefore, the fiber and some minerals are not destroyed or dissolved in the extraction process. The *S. platensis* residue is potential to be used as an additive to animal feed to increase the nutrient contents in feed products [14].

### Table 3. Phycocyanin content of *S. platensis*.

| Sample | Absorbance 615 nm | Absorbance 625 nm | Content (mg/mL) | Yield (%) |
|--------|-------------------|-------------------|----------------|----------|
| 1      | 0.193             | 0.189             | 0.019          | 0.097    |
| 2      | 0.196             | 0.181             | 0.021          | 0.103    |
| 3      | 0.190             | 0.178             | 0.020          | 0.099    |
| Mean   |                   |                   | 0.020          | 0.100    |
4. Conclusions

Biopigments of *Spirulina platensis* in this study were total chlorophyll with a concentration of 52.28 μg/mL of 25.28 μg/mL chlorophyll a and 27 μg/mL chlorophyll b, total carotenoid of 7.90 μg/mL and phycocyanin of 0.020 mg/L. Continuous extraction of the three biopigments using a simple extraction method could not produce optimal yields. Simultaneous extraction of chlorophyll and carotenoid was successfully applied in this study, while the optimal phycocyanin extraction should be further developed. The optimal extraction sequence was extraction of chlorophyll then carotenoid with low heating and followed by extraction of phycocyanin. This extraction process produced three biopigment fractions and a solid residual fraction which is potential for animal feed application.

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