The effect of using different polar solvents on the stability of thermal extraction phycocyanin from *Spirulina platensis*

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Abstract. *Spirulina platensis* is one type of blue-green autotrophic microalgae which is included in the cyanobacteria class. *Spirulina platensis* microscopically, it looks like a thin thread (filament) in the form of a spiral. Phycocyanin, and phycobiliprotein are used in food, biotechnology, and the cosmetics industry because of their color and antioxidants. This goal of research is to provide information of the best polar solvents on the thermal stability of phycocyanin extract from *Spirulina platensis*. The research method used was the complete random design (CRD) method. The results of the concentration and purity of phycocyanin showed that the heating process using 80°C for 60 minutes produced phycocyanin. The best polar solvent shown in ethanol.

1. Introduction

Microscopically *Spirulina platensis* appears as a thin thread (filament) in the form of a spiral [1]. Filaments are mobile cell colonies. These microalgae are 200-300 µm in length and 5-70 µm wide, 1-12 µm in diameter, and do not have a cell nucleus [2].

Phycocyanin is a blue pigment in the main light pigment of the organisms. Phycocyanin is an extracted and purified form of *Spirulina platensis*, *S. fusiformis*, *S. maxima*, *Synechococcus sp.*, Oscillator quadripunctulata, Aphanizomenon flos-aquae, Phormidium and commercial products [3].

The degradation of phycocyanin proteins depends on pH, light, protein concentration, and temperature. The application of phycocyanin to food products and other products is strictly limited due to their sensitivity to heat treatment which results in blue pigments [4]. The use of phycocyanin in food products requires high temperatures in process such as cooking and sterilization. Phycocyanin could also be used as an efficient preservative such as sodium azide (NaN3) and dithiothreitol (DTT) which are commonly used as preservatives. Phycocyanin is not to be used to analyze poisons during food grade process, therefore two extraction methods to obtain high-quality extracts and thermal stability of phycocyanin must be considered.

Preservatives are needed to ensure that food or additives produced remain safe, untouched and stable for longer periods of time. This study investigates the optimization of the chemical process of phycocyanin extraction from *Spirulina platensis* and the effect of methanol, ethanol, and aquadest on its thermal stability.

2. Materials and methods
This study was conducted in Chemistry and Analysis laboratory, Faculty of Fisheries and Marine, Universitas Airlangga.

2.1. Materials
The materials used in this study were *Spirulina platensis* microalgae obtained from Green Gold Spirulina in the form of dry (powder/rituration), aquadest, ethanol 96%, and methanol 96%. The tools used during this study were analytic balance (Mettler Toledo), Erlenmeyer, spatula, measuring cup, test tube, micropipette, centrifuge (Hettich EBA 20, Germany), water bath, cuvette and UV–VIS spectrophotometer (X-ma 1200, China).

2.2. Procedure
2.2.1. Phycocyanin extraction from *Spirulina platensis*
This study used *Spirulina platensis* microalgae in dry form (powder/rituration). The method used in extraction is maceration, one of the simple methods that are widely used both for small and industrial scale [5]. The maceration process started with weighing spirulina powder and dissolving it into tubes which each contain aquadest solvent, 96% ethanol, and 96% methanol and then incubated using room temperature for 12 hours. The addition of *Spirulina platensis* microalgae extract concentration refers [6] in which there are three formulations available.

A1: *Spirulina platensis* of aquadest 6%
A2: *Spirulina platensis* of ethanol 6%
A3: *Spirulina platensis* of methanol 6%

2.2.2. Thermal stability test
A. Analysis of phycocyanin concentration and yield
A 0.6 g dry biomass *Spirulina platensis* was extracted and separated using centrifugation at 6000 rpm for 15 minutes. The centrifugation supernatant heated at 80°C for 60 minutes, then the concentration was measured using a UV–VIS spectrophotometer at a wavelength of 615 nm and 652 nm. The concentration of phycocyanin (Cpc) was calculated by the following equation:

\[
C_{pc} = \frac{(OD_{615} - 0.474OD_{652})}{5.43}
\]

Description:
- \(C_{pc}\) : Phycocyanin concentration (mg mL\(^{-1}\))
- \(OD_{615}\) : Density wavelength 615 nm
- \(OD_{652}\) : Density wavelength 652 nm

B. Calculating phycocyanin purity
The purity of fractionation in phycocyanin was measured by a UV–VIS spectrophotometer in the absorbance ratio at 615 divided by 280 nm [7]. This ratio indicated extract purity (EP) to most forms of contaminating proteins. Absorbance at 615 nm indicated phycocyanin concentration, whereas absorbance at 280 nm reflected the total concentration of protein in the solution [8]:

\[
EP = \frac{OD_{615}}{OD_{280}}
\]

Description:
- \(EP\) : Extract purity
- \(OD_{615}\) : Density wavelength 615 nm
- \(OD_{280}\) : Density wavelength 280 nm

C. Yield
The yield of phycocyanin was calculated according to [9]

\[ \text{Yield} = \frac{C_{PC} \times V}{DB} \]

Description:
- Yield : mg g\(^{-1}\)
- \(C_{PC}\) : Phycocyanin concentration (mg mL\(^{-1}\))
- \(V\) : Solvent volume (ml)
- \(DB\) : Dry biomass (g)

D. Relative concentration of phycocyanin

The thermal stability of polar solvents as fractionation of phycocyanin from \(Spirulina platensis\) microalgae by adding each different polar solvent which are 96% methanol, 96% ethanol, and aquadest [10]. The phycocyanin sample was incubated using 80°C in a water bath. Determination of the level of phycocyanin samples was taken at 60 minutes. The relative concentration of phycocyanin (CR) was determined by the remaining phycocyanin concentration and the initial concentration using the equation:

\[ C_R = \frac{C}{C_0} \times 100 \]

Description:
- \(C_{r}\) : Relative concentration of phycocyanin (%)
- \(C\) : Remaining phycocyanin concentration (mL\(^{-1}\))
- \(C_0\) : Initial phycocyanin concentration (mL\(^{-1}\))

2.3. Data analysis

Data obtained from the results of this study was analyzed using analysis of variance (ANOVA) to determine the differences in the results of each treatment according to complete random design (CRD), then proceed with Duncan's multiple range test whether it is reacted to the influence of the results of the study [11].

3. Results and discussion

3.1. Concentration and purity of phycocyanin

| Table 1. The result of concentration and purity of phycocyanin. |
|---------------------------------------------------------------|
| Type of solvent | Concentration of phycocyanin (mg mL\(^{-1}\)) ± SD | Purity of phycocyanin (mg/mL) ± SD |
|-----------------|---------------------------------------------------|----------------------------------|
| A1              | 0.516±0.184                                      | 0.516±0.184                      |
| A2              | 0.877±0.151                                      | 0.878±0.150                      |
| A3              | 0.834±0.052                                      | 0.834±0.052                      |

Note: A1 (\(Spirulina platensis\) of aquadest), A2 (\(Spirulina platensis\) of ethanol) and A3 (\(Spirulina platensis\) of methanol). Letter notations superscripts different in the same column show a comparison between treatment with very significant differences (p<0.005).

The result of the ANOVA test shows that there are significant differences in the phycocyanin concentration (P<0.05). The test was using multiple tests and showed that treatment A1 was significantly different from A2 and A3, whereas in treatments A2 and A3 showed no significant difference. Thus it could be seen in Table 1 that the highest treatment for phycocyanin concentration is treatment A2 of 0.877 mg mL\(^{-1}\) which produces the highest concentration of phycocyanin, while treatment A3 is 0.834 mg mL\(^{-1}\) and treatment A1 of 0.516 mg mL\(^{-1}\) produces the lowest concentration of phycocyanin.
The result of the ANOVA test shows that there are significant differences in the phycocyanin concentration (P<0.05). The average result of phycocyanin purity could be seen in Table 1. The test was using multiple tests and showed that treatment A1 was significantly different from A2 and A3, while treatment A2 and A3 showed no significant difference. Thus, it could be seen that the highest treatment for phycocyanin purity is that A2 treatment of 0.878 mg mL⁻¹ produces the highest phycocyanin purity, while A3 treatment is 0.834 mg/mL and A1 treatment of 0.516 mg mL⁻¹ produces the lowest phycocyanin concentration.

3.2. Yield and relative purity of phycocyanin

| Type of solvent | Yield (mg/g) ± SD | Relative purity of phycocyanin (%) ± SD |
|-----------------|------------------|---------------------------------------|
| A1              | 3,094±1,512      | 0,600±0,394                           |
| A2              | 3,867±1,529      | 0,617±0,406                           |
| A3              | 4,520±1,384      | 0,642±0,421                           |

Note: A1 (Spirulina platensis of aquadest), A2 (Spirulina platensis of ethanol) and A3 (Spirulina platensis of methanol). Letter notations superscripts different in the same column show a comparison between treatment with very significant differences (p<0.005).

Table 2 shows that the average yield of extraction in three different types of polar solvents shows that there are no significant differences. The test was using multiple tests and showed that treatment A1 was not significantly different from A2 and A3, whereas A2 and A3 showed no significant differences. Thus it could be seen that the best treatment for phycocyanin concentration is A3 treatment of 4.520 mg/g, and the lowest recovery is A2 of 3.867 mg/g, and A1 of 3.094 mg/g.

The highest relative rate of phycocyanin indicates that there is no real difference. The test was using multiple tests that treatment A1 was not significantly different from A2 and A3, while treatment A2 and A3 showed no significant difference. Thus it could be seen that the highest treatment of relative concentration of phycocyanin is to show A3 treatment of 0.642%, the lowest treatment is A2 of 0.617%, and A1 of 0.600%.

3.3. Discussion

Spirulina platensis is a microalga that does not have heterosis, thus this species is unable to fix nitrogen from the air. Fulfilling its nitrogen requirements is highly reliable to its availability in the medium. In addition, according to [12], microalgae also need organic micronutrients in the form of vitamin elements that support their growth. In the field of aquaculture, Spirulina is used as natural food for fish, shrimp and abalone cultivation. Microalgae cultivation activities have become an independent field of aquaculture because the development of aquaculture products is extremely rapid in fulfilling its function as food for human needs [13].

Polar pigment extraction which produced extracts in blue by using aquadest, ethanol and methanol solvents is a polar solvent. Chlorophyll a pigment are bluish green, orange for carotenoids, blue for phycocyanin, allophycocyanin are turquoise and phycoerythrin are red. Extraction with polar solvents will extracts polar compounds in dry cells of Spirulina platensis. Polar compounds that could be taken by aquadest, ethanol and methanol solvents consist of phycobiliprotein or phycobilin and watersoluble proteins. These substances will respond to or absorb light with a wavelength range of 500-730 nm[14].

Measurements of absorption or absorbance in a certain range of wavelengths in each extract will produce different spectra and further detailed process of identification of the pigments contained in Spirulina platensis. Each of these compounds has different spectroscopic properties which have maximum absorption at certain wavelengths. The maximum uptake of phycocyanin is at a wavelength
of 610 - 620 nm, the maximum uptake of allophycocyanin at 650 - 652 nm and the maximum uptake of phycoerythrin 540 - 570 nm [15].

The phycocyanin concentration of *Spirulina platensis* extract with ethanol solvent resulted in higher phycocyanin compared to distilled water or aquadest and methanol (Table 1). This is most likely the phycocyanin pigment that could dissolve perfectly and is more polar because aquadest is a polar solvent which is good enough to dissolve phycocyanin. The polarity of a solvent is proportional to the dielectric constant it has. Aquadest contains a dielectric constant of +0.80 \( \varepsilon^\circ \) while ethanol +0.68 \( \varepsilon^\circ \) and methanol +0.73 \( \varepsilon^\circ \) [16].

The results of data analysis showed that there was a significant difference in the treatment of phycocyanin purity measured using a UV-VIS spectrophotometer based on the ratio \( A_{615}/A_{280} \), measurement of \( A_{615} \) was the absorbance value of phycocyanin and \( A_{280} \) is the protein absorbance value. In Table 1 the results of yield had the highest average phycocyanin purity with ethanol solvent of 0.854 mg/mL, significantly different compared to other types of solvent treatments. According to [17] the criteria for phycocyanin purity for food (Food grade) is to have a minimum limit of 0.7 mg/mL.

The average yield showed significant results with the highest methanol solvent of 4.520 mg/g and the lowest was aquadest solvent 3.094 mg/g. This occurs due to the different types of solvents affecting the amount of extract produced. Methanol solvents produce higher yields compared to other solvents which have boiling points of 67ºC, ethanol 78ºC while aquadest has a boiling point of 100ºC [18].

Analysis of thermal stability data on phycocyanin extract to temperature was carried out by heating phycocyanin extract at 80 ºC for 60 minutes, indicating that the average phycocyanin was stable (Table 2). Heating at 80ºC does not result in the decomposition of phycocyanin. According to [19] Phycocyanin tends to be resisted to heat and light, and does not resist acid. Phycocyanin will fade (denatured) at temperatures above 45ºC or at pH below 4 (stable at pH 4-9). Although the stability of the pigment is relatively lower, phycocyanin has advantages, and that is to produce the brightest and brilliant blue.

A lower average concentration value over a long period of heating indicates that there is the highest antioxidant activity [20]. This could be caused by the heating which damage the pigment. The main components of the polar pigment (phycobilin) *S. platensis* consist of 3 compounds: (1) phycocyanin, (2) allophycocyanin and (3) phycoerythrin. [6] states that the pigments contained in *S. platensis* are phycocyanin.

High temperatures caused damage to the proteins in phycocyanin and leaded to degradation of the inhibitory activity of peroxide. Proteins could undergo a process known as denaturation. Denaturation could change the nature of proteins to be difficult to dissolve and become thicker (coagulation) and will cause damage to the nutrients of spirulina [21]. Coagulation could occur due to heating [20]. [6] states that other factors that affect phycocyanin extract are antioxidant concentrations, extraction medium, temperature, medium pH, chemical structure, and position in molecules.

Phycocyanin extract at a short time in the heating process provides better activity compared to longer heating times. Along with time of heating and temperature degrees, the phycocyanin substance decreases. The decrease in phycocyanin substances could be seen by the decreasing extract that has been heated.

4. Conclusion

Based on the results of the research that has been carried out, it can be concluded that the solvent that can produce extracts of phycocyanin with the best thermal stability in *Spirulina platensis* is ethanol as the solvent.

5. References

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