

Stability of phycocyanin extracted from *Spirulina maxima* in different pH from indoor and semi-outdoor cultivation

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Abstract

Spirulina maxima is a blue-green microalga that rich in pigment. The pigments in *S. maxima* grouped into primary pigment (Chlorophyll a) and accessory pigments (carotenoid & phycobiliprotein). Phycocyanin is an accessory pigment that belongs to phycobiliprotein, blue colored, and can be used as natural food coloring and drugs. Phycocyanin has activities as antibacterial, antioxidant, anti-inflammatory, antihyperalgesic, and many more. Because of that, phycocyanin usually used in the pharmaceutical industry. However, phycocyanin is a protein that unstable under lights, high temperature, and pH in the storage. This study aims to obtain information about the effect of pH on the stability of phycocyanin extracted from *S. maxima* that cultivated in indoor and semi-outdoor. The steps are cultivation, extraction using different solutions to get blue pigment phycocyanin, and stability test. Phycocyanin was dissolved in a buffer solution at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 for 7 days. Color observations based on visualization and concentration measurements are carried out every day to see changes in phycocyanin. Growth in indoor cultivation with 24 hours light is faster than semi-outdoor a that uses only sunlight. Cultivation gets the optical density value 0,6 at day 20 with indoor cultivation and day 34 with semi-outdoor cultivation. Results show that phycocyanin can be extracted using a buffer phosphate solution. The stability of the pigments can be seen from the color changes and relative concentration using a spectrophotometer. Phycocyanin shows stable in the storage of pH 4 until pH 5.5. The highest relative concentration (C_R) was shown in pH 5.

Keyword: cultivation, microalga, phycocyanin, pigments, *Spirulina maxima*

Introduction

Spirulina is one of the blue-green microalgae that belongs to Cyanobacteria. *Spirulina* sp. is a microalga that is rich in pigments. Pigments contained in *Spirulina* sp. divided into three classes, namely (1) chlorophyll a, (2) carotenoids and xanthophyll, and (3) phycobiliprotein (phycocyanin and allophycocyanin). Phycocyanin pigment is the most dominant pigment in *Spirulina* which consists of 20% cellular protein while chlorophyll a is only 1.7% by cell weight and carotenoids & xanthophyll only around 0.5% by cell weight (Richmond 1988). Phycocyanin can be used as an antioxidant, protect liver function, and prevent stroke. Phycocyanin can also be used as a natural coloring agent. Therefore, it is very

widely used in the fields of food coloring and cosmetics (Arylza 2005). *Spirulina* has been known to be used as food and is noted to have been used in the Aztec era (Dillon, Phuc, & Dubacq 1995).

Microalgae are microscopic size algae, photosynthetic organisms, and can live in a variety of habitats such as sea, pond, lake, and river. Microalgae can be tolerant of various conditions of temperature, salinity, pH, and light intensity. This organism also can grow in symbiosis with other organisms (Khan *et al.*, 2018). Nowadays, microalgae are widely used to source of protein, vitamins, and minerals, and known as functional food. Microalgae have more advantages in the safety aspect compared to other food sources such as yeast or fungi. Microalgae are also superior in the field of efficiency and ease of production compared to single-celled proteins sourced from mammals (Nur 2014). This leads to a growing demand for microalgae for food so that cultivation of microalga is essential to maintain algae stock in the market.

The potential of phycocyanin from *Spirulina maxima* as a source of natural dyes has been widely studied in recent years. Synthetic dye or artificial colorant has been reported more harmful than a natural colorant. Some studies reported the use of these synthetic colorants having a negative effect on behavioral reactions such as hyperactivity in children (Arnold *et al.* 2012; Stevens *et al.* 2013). Currently, natural dyes are generally made from higher plants such as pandan leaves, suji leaves, turmeric, and are only used traditionally. The use of natural dyes on the market still less than synthetic dyes. (Sedjati, Ridlo, & Supriyantini 2015).

Phycocyanin is a pigment-protein complex that is part of the phycobilisomes. It is attached to the surface of the cytoplasmic surface in the thylakoid membrane and serves as the major antenna complex harvesting light for cyanobacteria and microalga (Glazer 1989; Biggins & Bruce, 1989). Phycocyanin is included in an oligomeric protein composed of α and β subunit. In the structure of phycocyanin, attached several open-chain tetrapyrrole to give the typical blue color to phycocyanin. (Stec *et al.* 1999; Padyana *et al.* 2001; Coyler *et al.* 2005). The problem of phycocyanin use for food is its sensitivity to degradation. Parameters that can lead to the degradation process are light, temperature, pH and protein concentration. This parameter makes the protein state of protein disrupted (Berns *et al.* 1989; Sarada *et al.* 1999; Jesperesen *et al.* 2005). This study aims to reveal the effect of pH on the stability of phycocyanin extracted from *S. maxima* that cultivated in indoor and semi-outdoor.

Materials and methods

Material and cultivation preparation

The microalgae *Spirulina maxima* obtained and cultivated from the culture collection of the Bioenergy and Bioprocess Laboratory, Research Center for Biotechnology, Indonesian Institute of Sciences, Indonesia. Materials used in this research are Zarrouk Medium, seawater, solution (n-hexane, ethyl acetate, ethanol, aquadest, and phosphate buffer pH 7 and citrate buffer). Preparation for Cultivation is sterilization of seawater, media, and container for microalgae. The medium used for cultivating *S. maxima* was Zarrouk Medium. Zarrouk Medium is made by mixing all the ingredients into 1 L seawater (Dineshkumar, Narendran, & Sampathkumar, 2016). Zarrouk medium was sterilized by autoclaving at a temperature of 121° C for 15 minutes and then added 0.1 mL A5 solution. This A5 solution was containing trace elements, vitamins and other additional minerals for microalgae growth. (Raoo, Kaushik, & Prasanna 2016). The Composition of Zarrouk Medium showed in Table 1.

Table 1. Composition of Zarrouk Medium

Ingredients	Amounts (g/L)
NaHCO ₃	16.8
NaNO ₃	2.5
NaCl	1.0
K ₂ SO ₄	1.0
K ₂ HPO ₄	0.5
MgSO ₄ · 7H ₂ O	0.2
FeSO ₄ · 7 H ₂ O	0.01
CaCl ₂ · 2 H ₂ O	0.04
Na ₂ EDTA · 2 H ₂ O	0.08
Distilled water	1000 ml
A5 solution*	0.1 ml

*A5 solution consists of Na₂MoO₄, 0.0177; H₃BO₃, 2.86; ZnSO₄·4H₂O, 0.222; MnCl₂·4H₂O, 1.81; CuSO₄·5H₂O, 0.079 (g/L)

Cultivation and extraction

Cells of *S. maxima* were grown in 50 L aquarium with aeration and different illumination condition. Indoor culture used illumination from lamp with 24 hours light and semi-outdoor culture used sunlight for illumination with natural photoperiods. Growth of *S. maxima* counted using optical density (OD) of the culture. The optical density of the culture is determined using a spectrophotometer with a wavelength of 680 nm. (Hadiyanto, Soetrisnanto, & Christwardhana 2014). Wet biomass from cultivation is extracted using many solvents (N-Hexane, Ethyl Acetate, Ethanol, Aquadest, and Phosphate Buffer pH 7) to get blue pigment phycocyanin. One gram of wet biomass was dissolved in a 10 mL solution. Extraction was carried out using the freeze-thawing method reported by Sedjati *et al.* (2015). The effectivity of the extraction solvent is determined by the total amount of Phycobiliprotein concentration. It consists of allophycocyanin (APC), c-phycocyanin (CPC), and phycoerythrin (PE). These were measured as absorbance at 620 and 652 nm and calculated using the following equation (Wu *et al.* 2016).

$$CPC(mg / mL) = \frac{A_{620} - 0.474(A_{652})}{5.34} \quad (1)$$

$$APC(mg / mL) = \frac{A_{652} - 0.208(A_{620})}{5.09} \quad (2)$$

Stability test of phycocyanin

Phycocyanin powder obtained from the extract through freeze-dry process. To evaluate the stability of phycocyanin at pH storage, 20 mg dried phycocyanin was dissolved at 20 ml pH solution with seven pH levels (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0, citrate and buffer phosphate). This method is modified from Chaiklahan *et al.* (2012). Observation carried out every day for seven days to evaluate the pH effect on the phycocyanin.

Analysis

The concentration of phycocyanin was measured using a UV–vis spectrophotometer at the wavelengths of 620 and 652 nm. The phycocyanin concentration (C) was taken as the total amount of CPC and APC. These were measured as absorbance at 620 and 652 nm and

calculated using equation (1) and equation (2). The relative concentration of phycocyanin (C_R) is the remaining concentration of phycocyanin as a percentage of the initial concentration and calculated using the following equation.

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

$$C_R(\%) = \frac{C}{C_0} \times 100\% \quad (4)$$

Results

Growth of *Spirulina maxima*

The growth of *Spirulina maxima* cultivation is shown in Figure 1.

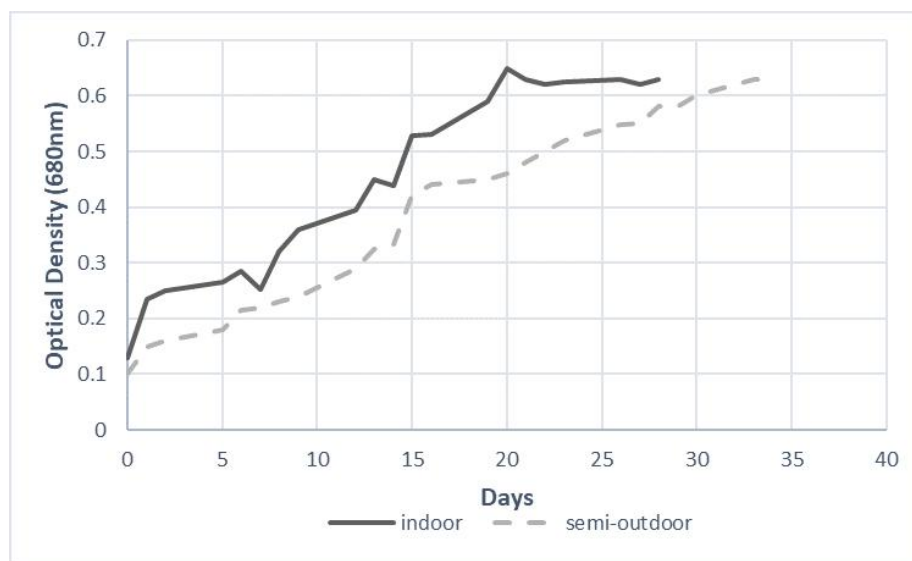


Figure 1. Growth of *S. maxima* culture in indoor and semi-outdoor cultures.

This study showed that the growth of *S. maxima* cultivated with indoor cultivation is faster than cultivated with semi-outdoor cultivation. To reach Optical Density 0.6, growth indoor cultivation takes 20 days while semi-outdoor cultivation takes 34 days.

Phycocyanin extraction

Result of phycocyanin extraction with solvents shown in Table 2.

Table 2. Extract with various solvents

No	Solvent	Phycobiliprotein Conc. (mg/L)		Extract Color
		CPC	APC	
1	N-Hexane	1.31±0.07	0.11±0.06	clear
2	Ethyl Acetate	6.04±0.04	0.12±0.05	yellow
3	Ethanol	0.23±0.03	6.97±0.11	green
4	Aquadest	13.19±0.03	7.02±0.14	green
5	Phosphate Buffer pH 7	73.54±0.08	24.88±0.05	blue

*CPC: C-phycocyanin; APC: allophycocyanin;

In this study, extraction with various solvent shows different extract colors. The extract colors are clear, yellow, green, and blue. The highest phycobiliprotein concentration showed by Phosphate Buffer pH 7 solvent.

Phycocyanin stability test on storage pH

The results of the relative concentration (C_R) of phycocyanin at pH storage for 7 days are shown in Figure 2. Visual observation of phycocyanin color is shown in Figure 3.

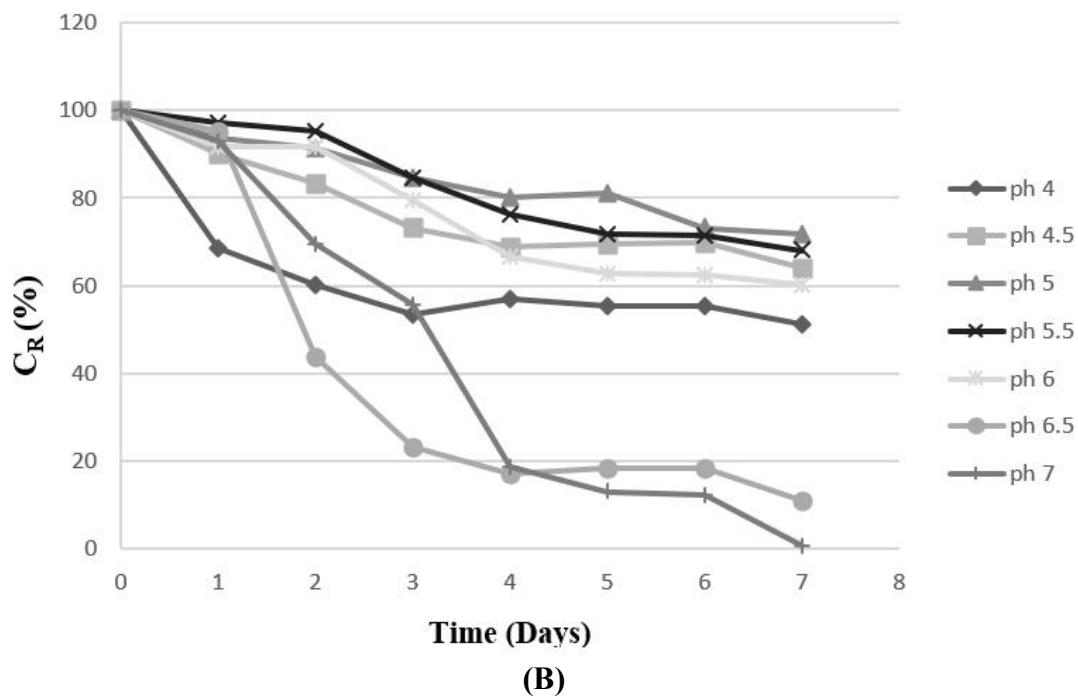
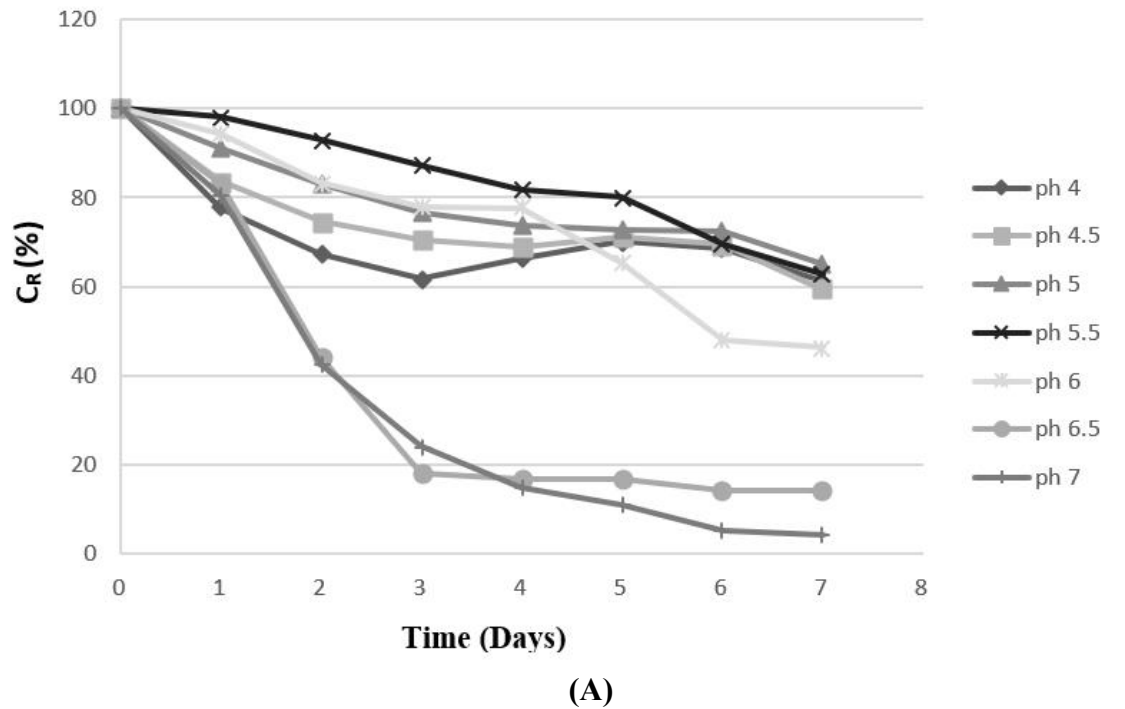


Figure 2. The (C_R) value of phycocyanin from (A) indoor culture and (B) semi-outdoor culture for 7 days.



(A)



(B)



(C)

Figure 3. Phycocyanin color on day 0 (A), Phycocyanin color from indoor cultivation on day 7 (B), Phycocyanin color from semi-outdoor cultivation on day 7 (C)

The results showed a color change of phycocyanin from day 0 until the 7th day of storage. This happened in both phycocyanin from indoor and semi-outdoor. As shown in figure 2, relative concentration (C_R) of phycocyanin in pH 6.0, 6.5, and 7 drastically

decreased. In contrast, pH 4.0, 4.5, 5.0, and 5.5 decreased little. The smallest concentration reduction was the storage of pH 5. The final relative concentration value at pH 5 was $65.1 \pm 3\%$ from the indoor culture phycocyanin and $71.9 \pm 13\%$ from the semi-outdoor culture phycocyanin.

Discussion

This study showed different growth times of *S. maxima* from a different placement of cultivation. Different growth time from the cultivation caused by many factors. This can be caused by different photoperiodic, light intensity, and temperature. According to Becker (1994), the effects of light, such as exposure time and intensity, can affect the growth of microalgae. According to Kawaroe's research (2009), photoperiod has more influence on the growth of microalgae compared to nutrients found in cultivation media.

According to Lavens and Sorgeloos (1996), the minimum exposure time of microalgae cultivation is 18 hours per day. Exposure time in semi-outdoor cultivation is 12 hours according to natural sunlight, while in indoor cultivation is 24 hours. That caused the indoor culture which has sufficient lighting duration can grow faster. The light needed by microalgae in the photosynthesis process has a certain limit or range, the high intensity may cause photo-inhibition of microalgae (Imamoglu *et al.* 2007). This photoinhibition can occur because the organism that experiences it is saturated and inhibits the process of photosynthesis, followed by damage to photosynthetic pigments (Salisbury and Ross 1995). The faster growth makes the phycocyanin production higher because of the higher amount of biomass. According to that, indoor cultivation with 24 hours lighting is good for producing phycocyanin.

The different extract colors caused by the polarity of the solvent. According to Pelczar and Chan (1988), materials and chemical compounds will dissolve easily in solvents that are relatively equal in polarity. Polarity sequences from non-polar to polar from the solvent used are N-Hexane, Ethyl Acetate, Ethanol, and Phosphate Buffer. Refers to Masojidek *et al.* (2004), phycocyanin pigment is a pigment that is associated with protein, water-soluble, and tends to polar so that buffer phosphate can extract phycocyanin from the cell. This phycocyanin result also supported by the high concentration of CPC and APC as part of phycobiliproteins.

The result of this current study indicates that the stability of phycocyanin is better from semi-outdoor cultivation. However, the further study is needed because according to Hadiyanto *et al.* (2015) quality of phycocyanin is affected by many factors such as extraction method, time, and temperature. Another study shows that intensity and quality of light are affecting phycocyanin production (Takano *et al.* 1995).

Based on these figures, all phycocyanin color degraded until day 7. This result assumed phycocyanin undergoing the denaturation process. It causes a decrease in the concentration and discoloration of phycocyanin. According to Bintang (2010), the effect of storage pH can make the protein undergo denaturation. According to Edwards *et al.* (1996), phycocyanin is in the hexameric form at pH 5. The condition is thought to be a factor that can prevent denaturation. Things are different if phycocyanin at pH 7. It becomes a monomeric or trimeric form. This condition causes phycocyanin to become more unstable. According to Duangsee *et al.* (2009), the chemical structure of phycocyanin will open at $\text{pH} < 4.5$ due to damage to hydrogen bonds. Phycocyanin which is stored at a low pH makes the structure of phycocyanin expand caused by denatured protein. Therefore, the structure of phycocyanin is damaged. This causes the color intensity and absorbance readings with a spectrophotometer weak. As reported by Mishra *et al.* (2008), the best storage to maintain the stability of phycocyanin is at pH 5.0 to 6.0. This is also shown in research obtained by Antelo *et al.* (2008) that phycocyanin stable at pH 5.0 to pH 6.0.

Conflict of interest

The authors state no conflict of interest from this manuscript.

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Author contributions

DS and BS designed the study, performed research and collected the data; AD and AT analysed the data; BS wrote the paper. All authors have reviewed the final version of the manuscript and approved it for publication. BS is the main contributor of this manuscript.

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