The effect of different concentrations of ammonium sulfate and pH extraction on the production of phycocyanin from Galdieria sp.

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Abstract. The alternative blue pigment of phycocyanin sources other than Spirulina is Galdieria from Cyanidiales order. Galdieria sp. is unicellular red microalgae which naturally found in volcanic area with a high temperature and low pH (0.5–3). In this study, Galdieria sp. 009 has grown autotrophically in Allen medium and on different concentrations of ammonium sulfate. The growth and in vivo phycocyanin content were quantified in the cultures. Phycocyanin was extracted using a physical method with different extraction pH (3, 5, and 7). Highest specific phycocyanin content up to 100 mg g\(^{-1}\) was observed in cell grown on Allen medium with two times ammonium sulfate concentration and extracted with pH 7. The different concentrations of ammonium sulfate used in the Allen medium impacted the yield of phycocyanin. Although extraction at pH 7 caused high phycocyanin content, the phycocyanin tends to have high thermostability (>60°C) and purity index at pH 5. The phycocyanin extracted from Galdieria sp.009 may potentially be an alternative blue food colorant beside Spirulina, as it has higher thermostability that Spirulina phycocyanin (>47°C).

Keywords: Galdieria sp., microalgae cultivation, pH extraction, phycocyanin, thermostability

1. Introduction
The colorant agent is one of the essential materials in the industrial product, especially in the food and beverage industry [1]. Colorant agents provide an attraction effect on the consumers [1]. Recently, consumers have more concern about uses colorant agents, particularly synthetic food colorant [2]. The food and beverage industry wants dyes that are easily controlled, reproduced, and can be modified [3]. The aroma of food is challenging to convey to other media, but color can be a powerful communication tool for consumers [3]. The industry is starting to use color as a form of quality for food [3, 4]. Therefore, standardized color is a crucial component in improving food quality [5]. Many synthetic colorants are particularly correlated to behavioral problem of children [6, 7]. This issue leads to a growing demand of natural colorant agents in food and beverages industry [2]. Natural colorant agents are found in nature, such as from animals or plants [8]. The use of natural dyes for food has several benefits including non-toxic, renewable, easily degraded, and environmentally friendly [8].
Nature serves us with various natural colorants, but the blue colorant is one of the colors that hard to find naturally [9]. In nature, there are three varieties of blue colors based on the source, i.e., gardenia blue, indigo, and phycocyanin [9]. In some countries, indigo is no longer used because of its low stability, while gardenia blue produced undesirable turquoise colors [10]. Phycocyanin is a pigment-protein complex that is part of the phycobiliprotein family found in cyanobacteria and microalgae [11]. Phycocyanin is composed of two components, a chromophore, and a protein [12]. The chromophore is a tetrapyrole known as bilin, which gave the typical blue color to the phycocyanin [12, 13]. This chromophore is attached to the cysteine amino acid residue in the apoprotein by a thioether linkage [12, 13]. Generally, commercial phycocyanin produced by Spirulina sp. [14]. The first commercial phycocyanin is used for food and cosmetic products in Japan [15]. Phycocyanin also used in confectionery, ice cream, and several other food products [15]. Besides use in food as a colorant, phycocyanin is also used as a fluorescent agent in immunological assays, as a label in cell sorting, gel electrophoresis, and gel exclusion chromatography [16, 17].

In search of other alternatives for more stable phycocyanin, the species from red microalgae were observed. Red microalgae contain phycocyanin which functioning as a light-harvesting antenna in photosynthesis, as found in cyanobacteria [18, 19]. Galdieria sulphuraria is one of the known species of red microalgae which also have phycocyanin [18]. Galdieria sulphuraria belongs to Cyanidiophyceae which is dominant species in sulfuric acid hot springs [20]. This species can grow in low pH (0.05 – 5.0) and moderate temperature ranging from 35 – 56°C, in the lower temperature, they can grow slowly [20, 21]. Galdieria may be a suitable strain for phycocyanin source alternatives [19]. This paper characterizes the growth and phycocyanin production in Galdieria sp. 009 grown in different concentration of ammonium sulfate and determine the best extraction condition to obtain a high yield of phycocyanin.

2. Material and methods

2.1. Microbial culture and growth medium

Galdieria sp. 009 was isolated from Rengganis crater, West Java. Stock cultures were maintained by sub-cultivation in Allen medium under constant light (800 lux) on a shaker at 150 rpm and room temperatures (25 ± 2°C). Allen’s medium per liter [22] consist of 1.32 g (NH₄)₂SO₄, 0.28 g KH₂PO₄, 0.25 g MgSO₄.7H₂O, 0.074 g CaCl₂.2H₂O, 11 mg FeCl₃, 2.8 mg H₂BO₄, 1.8 mg MnCl₂, 0.218 mg ZnSO₄.7H₂O, 0.05 mg CuSO₄, 0.023 mg NH₄VO₃ and 0.024 mg Na₂MoO₄.2H₂O. The pH of the medium was adjusted to 2.0 with 4.0 M H₂SO₄ before to autoclaving at 120°C for 20 mins. All chemical were reagent grade and were obtained from Merck.

2.2. Growth conditions

Three different concentrations of ammonium sulfate were used (0.66 g L⁻¹; 1.32 g L⁻¹; and 1.62 g L⁻¹) to observe its effect on the phycocyanin accumulation, while the other medium components were constant. The cultivations were conducted in a one-liter photobioreactor with a temperature-controlled condition (40°C, 150 rpm, and continuous constant light). Each culture was inoculated with autotrophically grown microalgae cells an initial OD (optical density) at 800 nm of about 0.1 and cultivated for up to 21 days.

The cultures were analyzed for growth and phycocyanin in vivo production almost daily for 21 days spectrophotometrically. The growth was monitored at 800 nm and in vivo phycocyanin monitored at 620 nm and 652 nm.

2.3. Preparation of phycocyanin

The culture was harvested after 21 days of cultivation by centrifugation at 8000 rpm for 10 min. The cell pellets were washed with distilled water. Washed cell mass was lyophilized and weighted as dry biomass. 50 mg of dry biomass from each cultivation was suspended in 10 ml buffer. In this study, three different buffer (pH 3, 5, and 7) were used to analyze whether extraction pH impacted the quality
and quantity of phycocyanin. The cell mixtures were extracted using a sonicator with 30 mm probe and 30 Hz. After sonication, the mixture was kept for 24 hours. The cell debris was removed through centrifugation at 10,000 rpm for 15 min at 4°C, and blue colored supernatant containing phycocyanin was collected in a fresh tube.

2.4. Spectroscopic estimation of phycocyanin
The phycocyanin content of each buffer was measured spectrophotometrically. Phycocyanin and allophycocyanin showed the maximum absorption at 620 nm and 652 nm, respectively. The concentration of phycocyanin in the solution calculated using eq.1 [23]:

$$\text{Phycocyanin (mg mL}^{-1}\) = \frac{A_{620} - 0.474 \times A_{652}}{5.34}. $$

The purity of phycocyanin was assessed by calculating the ratio of absorption at 620 nm to 280 nm, wherein 620 nm is the maximum absorbance of phycocyanin, and 280 nm is the absorbance of total protein.

2.5. Effect of temperatures on phycocyanin stability
To investigate the effect of different pH on the stability of phycocyanin, 1 mL phycocyanin solution in a microtube was incubated in the water bath for 30 min at different temperatures (25, 30, 40, 50, 60 and 70°C). After 30 min, the samples were analyzed by spectrophotometer [24]. The remaining concentration of phycocyanin (C_r, %) relative to initial concentration calculating using the following equation C_r (%) = C/C_0 x 100, the relative concentration of phycocyanin is the remaining concentration of phycocyanin as the percentage of its initial concentration (C_0).

3. Result

3.1. Microalga strain
The strain used for this experiment is Galdieria sp. 009 isolated from Rengganis Crater, West Jawa. The preliminary in-vivo microscopic overview (figure 1) showed the cell was a spherically shaped, solitary cell, and devoid of flagella. The cell diameter varied between 3 to 5 µm. Microalgae cell size and shape can change during different stages of their life cycle [25]; therefore, the size of the cell during the cultivation is not constant.

![Microphotograph of Galdieria sp. 009 in autotroph condition. Scale bar: 5 µm.](image)

3.2. Growth of Galdieria sp. in different Ammonium sulfate concentration
In order to know whether the ammonium sulfate concentration affected the growth of Galdieria sp. 009, the cells were cultivated in the batch system at 1 L photobioreactor with three different concentrations of ammonium sulfate: 1.32 g L^{-1} (defined Allen medium), 0.66 g L^{-1} and 2.64 g L^{-1}. All medium were adjusted into pH 2.0 by adding 4 M sulfuric acid. The growth curves of Galdieria sp.
009 were carried out by measured the optical density at 800 nm almost every day for 21 days (figure 2). The culture grown with 1.36 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) has a more prolonged lag phase compared to the other cultures. Its start doubled at day five and continuously increasing until harvesting day (day 21).

![Figure 2](image)

**Figure 2.** The growth curve of *Galdieria* sp. 009 in different concentrations of Ammonium sulfate.

The culture grew with 0.66 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) have a slower growth rate compared with the other. The specific growth rate of each ammonium sulfate concentration showed in table 1. The *Galdieria* sp. 009 grew with 2.64 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) has the highest biomass production and specific growth rate of about 0.943 ± 0.055 g L\(^{-1}\) and 0.216 day\(^{-1}\), respectively. The higher ammonium sulfate concentration added into the medium, the higher biomass obtained during cultivation.

| [(NH\(_4\))\(_2\)SO\(_4\)] (g L\(^{-1}\)) | Specific growth rate (day\(^{-1}\)) | Biomass productivity (g L\(^{-1}\)) |
|--------------------------------|----------------------------------|----------------------------------|
| 1.32                          | 0.185                             | 0.601 ± 0.031                    |
| 0.66                          | 0.135                             | 0.572 ± 0.024                    |
| 2.64                          | 0.216                             | 0.943 ± 0.055                    |

3.3. *Phycocyanin production in vivo*

The daily phycocyanin content *in vivo* was estimated using eq. 1. The cultures were measured by spectrophotometer at 620 nm and 652 nm, as the maximum wavelength of phycocyanin and allophycocyanin [23]. The result showed that phycocyanin extracted from *Galdieria* sp. 009 grew with 2.64 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) have the highest phycocyanin content (figure 3A). The highest phycocyanin
production per cell basis (phycocyanin content per cell) was the culture grown with 2.64 g L⁻¹ (NH₄)₂SO₄ about 9 pg cell⁻¹.

![Figure 3. Daily phycocyanin content in vivo of Galdieria sp. 009 grown in different ammonium sulfate concentration (A). Insert graph: phycocyanin production in cell basis (B).](image)

### 3.4. Phycocyanin production in different extraction pH

The cultures were harvested by centrifugation after 21 days cultivation and extracted by a physical method to obtain phycocyanin. Three different pH were used to extract phycocyanin from Galdieria sp. 009 (pH 3, 5, and 7). Table 2 showed the phycocyanin production and purity index of phycocyanin from Galdieria sp. 009. The highest phycocyanin was obtained from Galdieria sp. 009 with 2.64 g L⁻¹ (NH₄)₂SO₄ and extracted by pH 7 (98.8 ± 4.3 mg g⁻¹). The highest purity index occurred on phycocyanin extracted with pH 5, for all cultures grew with 2.64 g L⁻¹ (NH₄)₂SO₄.

The visual observation of all the phycocyanin extract, the phycocyanin extracted using pH 5 from three different ammonium sulfate concentration have a brighter blue color (figure 4).

### Table 2. Phycocyanin production and purity index of Galdieria sp. 009 in different ammonium sulfate concentration and varied extraction pH.

| [(NH₄)₂SO₄] (g L⁻¹) | pH 3 | pH 5 | pH 7 |
|---------------------|------|------|------|
| Phycocyanin production (mg g⁻¹) | Purity index (A₆₂₀/A₂₈₀) | Phycocyanin production (mg g⁻¹) | Purity index (A₆₂₀/A₂₈₀) | Phycocyanin production (mg g⁻¹) | Purity index (A₆₂₀/A₂₈₀) |
|---------------------|------|------|------|
| 1.32 | 38.2 ± 8.4 | 0.7 ± 0.01 | 40.6 ± 0.9 | 1.7 ± 0.1 | 80.4 ± 0.3 | 0.7 ± 0.01 |
| 0.66 | 10.5 ± 0.6 | 0.5 ± 0.10 | 22.2 ± 4.3 | 0.8 ± 0.1 | 51.5 ± 1.2 | 0.5 ± 0.01 |
| 2.64 | 35.6 ± 0.2 | 0.9 ± 0.10 | 51.4 ± 5.8 | 1.9 ± 0.1 | 98.8 ± 4.3 | 0.6 ± 0.10 |

### 3.5. Thermostability of phycocyanin extracted with different pH.

The stability of phycocyanin in varied temperatures was showed in figure 5. Based on the result showed in figure 5, the phycocyanin extracted by pH 5 was more stable at the higher temperature. In figure 5A, the phycocyanin from Galdieria sp. 009 grew with 1.34 g L⁻¹ (NH₄)₂SO₄ z more stable when extracted with pH 5. The concentration remained about 80% at 60°C, while at the same
temperature, the phycocyanin extracted by pH 3 and pH 7 were less than 60%. The same result showed for phycocyanin from Galdieria sp. 009 grown with 0.66 g L\(^{-1}\) (figure 5B) and 2.64 g L\(^{-1}\) \((\text{NH}_4\text{)}_2\text{SO}_4\) (figure 5C). This result was the same as found in Cyanidioschyzon merolae phycocyanin, which more stable at pH 5 than at pH 7 [26].

**Figure 4.** Phycocyanin extract from Galdieria sp. 009 with different ammonium sulfate concentration (A) 1.32 g L\(^{-1}\); (B) 0.66 g L\(^{-1}\); (C) 2.64 g L\(^{-1}\).

4. **Discussion**

Galdieria sp. is unicellular red microalgae inhabit sulfuric hot spring with low pH (0.05 – 5.0) and high temperature (up to 56°C). This species is known as one of the red microalgae that also have phycocyanin as their light-harvesting antenna in the photosynthesis process [19]. In this study, Galdieria sp. 009 cultivated in three different concentrations of ammonium sulfate (1.32; 0.66; and 1.64 g L\(^{-1}\)). Ammonium sulfate is the major component of Allen medium [22]. In the define Allen medium, the concentration of ammonium sulfate is 1.32 g L\(^{-1}\). The result showed that the growth rate of Galdieria sp. 009 was increasing in the high concentration of ammonium sulfate (figure 2).

The phycocyanin production *in vivo* of Galdieria sp. 009 was determined to evaluate whether the amount of phycocyanin in the cell affected by the different concentrations of ammonium sulphate. Figure 3A showed the productivity of phycocyanin *in vivo*, while the production of phycocyanin per cell basis shown in figure 3B. Microorganisms generally need nitrogen as building blocks. The nitrogen deficiency can result in phycobilisome degradation as the main store of cellular nitrogen and shrinkage of chloroplasts, both in eukaryotic microalgae and cyanobacteria [27, 28].

Dried biomass of each culture condition was extracted using a buffer with different pH. In the last few years, phycocyanin from Galdieria sp. extracted by the freeze-thaw method and using buffer pH 7 [19, 29, 30]. In this study, different extraction pH was used. The highest phycocyanin content indeed occurred in phycocyanin extracted in pH 7, but the highest purity index was found in phycocyanin extracted in pH 5 (0.8 to 1.9). The purity index is the ratio of phycocyanin absorption at 620 nm and 280 nm. Phycocyanin solution with a purity index of at least 0.7 is considered to be food-grade phycocyanin. Meanwhile, a purity index of at least 4 is considered as analytical grade [31].
Phycocyanin is a light-harvesting protein complex, which is sensitive to light and temperature. To be used in the food industry, the pigment should pass some criteria such as stable at high pH due to pasteurization and cooking process. When the phycocyanin solution extracted with pH 5 was incubated for 30 min in a temperature ranging from 25 to 70°C, the absorption of the spectra slowly decreases until the temperature reaches 60°C (20% decreasing). Phycocyanin degradation was increased after 60°C. Phycocyanin commonly exists as hexamer at pH 5, and according to Edwards et al. [32], the hexameric form gives some protection against denaturation. Meanwhile, phycocyanin predominantly in the monomeric or trimeric form in buffer solution pH 7, resulting in lower thermostability.

**Conclusion**
The concentration of ammonium sulfate in the liquid medium affected the growth rate and the production of phycocyanin in *Galdieria* sp. 009. In 2.64 g L\(^{-1}\) ammonium sulfate, the phycocyanin production reaches 9 pg cell\(^{-1}\) and total biomass of 0.943 g L\(^{-1}\). The phycocyanin production of *Galdieria* sp. 009 reached 98.8 mg g\(^{-1}\) when grown in 2.64 g L\(^{-1}\) ammonium sulfate and extracted with pH 7. Although the phycocyanin amount was higher when extracted with pH 7, the visualization and the purity index were better in phycocyanin extracted with pH 5. The phycocyanin extracted with pH 5 also has higher thermostability, up to 60°C with C\(_R\) about 80%.
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