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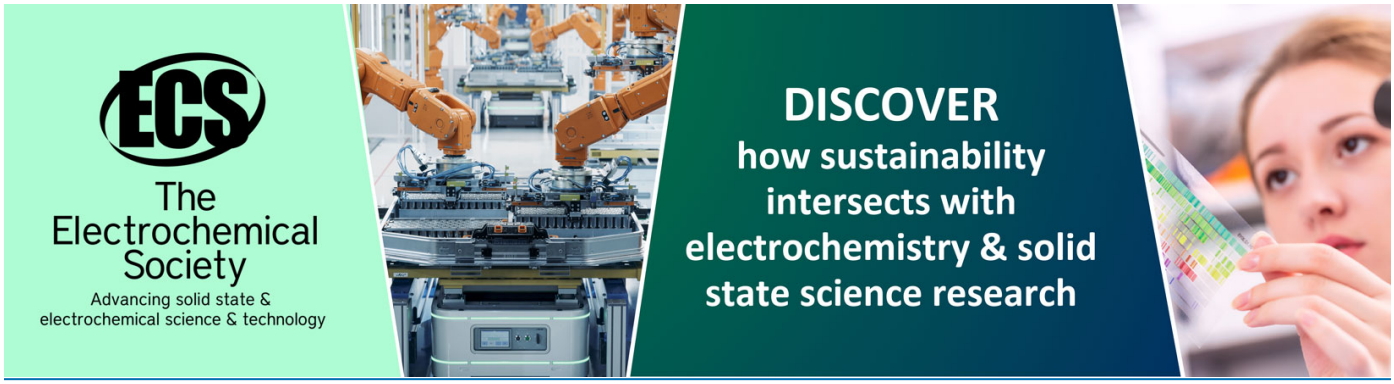

Phycocyanin extraction in Spirulina produced using agricultural waste

N Taufiqurrahmi[1], P Religia[2], G Mulyani[1], D Suryana[4], Ichsan[4], F A Tanjung[5], Y Arifin[3],[5]
[1]Chemical Engineering Department, Surya University, Boulevard Gading Serpong, Tangerang 15810, Indonesia
[2]Department of Food Technology, International University Liaison Indonesia, IULI – Eco Campus, The Breeze BSD City, Tangerang Selatan 15345, Indonesia
[3]Department of Food Business Technology, Prasetiya Mulya University, BSD Raya Utama, BSD City, Kabupaten Tangerang 15339, Indonesia
[4] PT Maris Sustainable Indonesia, Jl Tipar Cakung, Pool PPD 5, Cakung, Jakarta Timur 13910, Indonesia
[5]Department of Chemical Engineering, Curtin University Sarawak Campus, CDT 250, Miri 98009, Sarawak, Malaysia

Email: yalun.arifin@prasetiyamulya.ac.id

Abstract. Phycocyanin is a pigment-protein complex synthesized by blue-green microalgae such as Arthrospira (Spirulina) platensis. This pigment is used mainly as natural colouring in food industry. Previous studies have demonstrated the potential health benefits of this natural pigment. The price of phycocyanin is a vital factor that dictates its marketability. The cost of culturing the algae, particularly from the substrate used for growth, is one of the main factors that determine the price of phycocyanin. Another important factor is the growth yield of the algae. In our research, agricultural waste such as charcoal produced from rice husk was utilized for the algae cultivation to replace the synthetic chemicals such as urea and triple superphosphate used the mineral medium. The use of this low cost substrate increases the cell concentration by 60 % during 8 days' cultivation to reach 0.39 g/l. The phycocyanin extraction was performed using water at the different biomass-to-solvent ratio and shaking rates. The phycocyanin concentration and purity (A_{615}/A_{280}) obtained were 1.2 g/l and 0.3. These values are 40 % and 20 % lower than the value obtained from the algae produced using the synthetic chemicals. Further purification produced the extract purity required for food grade. The biomass-solvent ratio does not significantly affect the extract purity; however, the higher shaking rate during extraction reduces the purity. This finding demonstrates the potential of using rice husk as an alternative substrate to cultivate algae for phycocyanin extraction.

1. Introduction

Arthrospira platensis, also known as Spirulina, has received considerable attention as food supplement, feed, and medicinal properties due to its high protein content (up to 70%) and nutritional values [1-3]. This cyanobacterium has a potential for inhibiting cancer in animal cells [4]. Other studies show that Spirulina has demonstrated antiviral effect [5], and stimulated immune system [6].

Spirulina contains a pigment called phycocyanin. It is a protein-pigment complex from the phycobiliprotein (PBP) family characterized by its intense blue color. PBPs build a peripheral accessory light-harvesting complex called phycobilisome (PBS), which is assembled on the surface of the thylacoid membrane. Phycocyanin is a hydrophilic protein that has a fluorescent pigment that divided into three groups: phycocyanin (dark blue), phycoerythrin (red) and allophycocyanin. Its main function is to transfer the excitation energy to the center of reaction where the maximum wavelength of absorption is near to 620 nm. Recently, phycocyanin is used as food coloring that is approved by the Federal Drug Administration (FDA) in the US. This natural pigment has shown antioxidant acitivities both in vivo and in vitro [7]; therefore, it can be considered a nutraceutical compound.

Phycocyanin harvesting from Spirulina biomass is achieved by extraction. There many factors that affect the result of the extraction, such as the biomass content of phycocyanin, size distribution, solvent, extraction time, temperature, mixing rate, and ratio of biomass to solvent. Previous study shows water is a good solvent for phycocyanin extraction [8].

The cost of phycocyanin production depends on the cost of Spirulina cultivation. The mineral medium used is the largest cost component in the cultivation. Our research is trying to produce phycocyanin from the Spirulina that is cultivated using the medium from agricultural waste. Rice husk biocharcoal (RHB) was selected as one of the main components in the growth medium. Rice husk, which is 20 % of the rice biomass, is one of the major agricultural wastes. Several studies have been done in utilizing RHB, a product of partial combustion of rice husk, for improving terrestrial farming [9-10], but so far no application in cultivating aquatic biomass such as Spirulina.

In our research, the phycocyanin productivity is compared with the result from Spirulina cultivated with a standard mineral medium. The biomass to solvent ratio and shaking speed were varied to obtain the optimum values and to study the effect to the phycocyanin concentration and purity.

2. Materials and methods

2.1. Culture condition

Spirulina was cultivated in a 1-liter medium using 2-liter flask. The standard medium MSI used is developed by Maris Indonesia, which consists of 0.5 g/l $NaHCO_3$, 0.5 g/l NaCl, 0.05 g/l urea, 0.02 g/l triple superphosphate (TSP), and 0.01 g/l cobalamin (vitamin B12).

The rice husk biocharcoal (RHB) medium consists of 0.4 g/l $NaHCO_3$, 0.5 g/l NaCl, 0.01 g/l cobalamin, 0.025 g/l urea and 20 ml/l of RHC extract. The extract was obtained by batch extraction using 8 gram of rice husk charcoal (supplied by Maris Sustainable Indonesia) mixed with 400 ml of water. The extraction was performed at room temperature for 12 hours, followed by a filtration to remove the solid.

Mixing was done by sparging the air using a pump to make sure the cells and nutrients are mixed uniformly throughout the cultivation system. After 8 days' cultivation, the biomasswas harvested using 40-mesh filter paper. The harvested biomass is covered with 10 mm transparent glass, sun-dried for 4 hours and stored at -18°C for cell disruption. Dried biomass was crushed and sieved (160 mesh) to make the size distribution same so the contacts between solvent and biomass will be uniform. The biomass dry weight was measured.

2.2. Phycocyanin Extraction

The extraction method is developed based on the previous publication [8]. Two gram of biomass was suspended in 30, 50, 75, or 100 ml of water in a 250-ml baffled shake flask The flask was placed in incubator shaker (Heidolph Incubator 1000, Schwabach, Germany) that run at 25°C (Figure 1) for 30 hours. Two speeds were used for the shaking: 150 and 200 rpm. Samples were collected and followed by centrifugation at 4°C and 6000 rpm for 10 min. After centrifugation, the supernatant was collected for analysis using UV-Vis spectrophotometer (Biochrom Libra S12, Cambridge, UK) at 652nm, 615nm, and 280nm. Phycocyanin concentration (PC) was defined as:

$$PC = \frac{(OD_{615} - 0.474(OD_{652}))}{5.34} \quad (1)$$

where PC is phycocyanin concentration (mg/ml), OD_{615} is optical density of sample at 615nm, and OD_{652} is optical density of sample at 652nm.The purity of phycocyanin was determined by the A_{615}/A_{280} ratio. Absorbance at 615 indicates the phycocyanin concentration and 280 indicates total protein concentration in the solution:

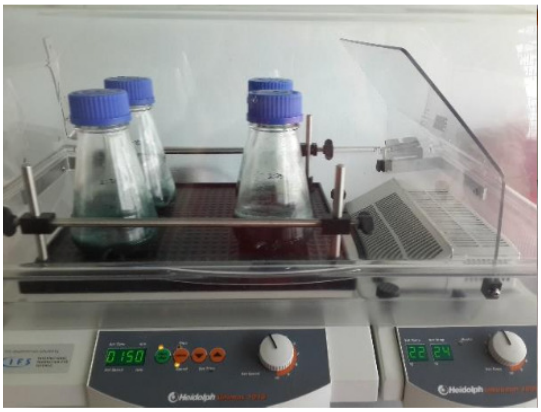

Figure 1. Extraction apparatus

$$EP = \frac{OD_{615}}{OD_{280}} \quad (2)$$

where EP is extract purity, OD_{615} is optical density of sample at 615nm, and OD_{280} is optical density of sample at 280nm.

2.3. Phycocyanin Purification

Phycocyanin was purified using a standard ammonium sulfate precipitation method in fractionation of 0-20%/20-50%. Sample was centrifuged 6000 rpm and 4°C for 10 minutes. The supernatant was poured into a beaker glass under cold condition. Ammonium sulfate was added slowly with stirring at 200 rpm to prevent clumps. After all powder was added, the stirring continued for an hour, followed by centrifugation at 6000 rpm and 4°C for 20 minutes. The pellet was resuspended with 10 mM sodium phosphate buffer pH 7.00.

3. Results and discussion

3.1. Improved biomass production in cultivation using rice husk biocharcoal (RHB) medium

The biomass production of Spirulina in rice husk biocharcoal (RHB) medium is 60 % higher than the result from the mineral medium (Table 1). Rice husk charcoal extract was intended to replace urea and TSP as the source of nitrogen and phosphorus. Rice husk biocharcoal typically contains significant amount of silica, potassium, calcium, and magnesium at 100 - 200 mg/kg and some trace metals [9]. Previous study shows the utilization of RHB increases the growth of water spinach [9] and paddy [10]. The addition of these elements is expected to improve the growth yield and maintain the alkalinity of the medium. Spirulina grows optimally at slightly alkaline pH at 8 – 9. The improvement in the growth yield is beneficial for future biorefinery from this biomass.

Although the biomass concentration is higher, the PC and EP values obtained from the Spirulina grow in RHB are lower than the values obtain when the cyanobacterium grow in mineral medium MSI. In other words, the use of RHB increases the biomass production but not the phycocyanin level. When the biomass to solvent ratio is increase to 2 g: 30 ml, the PC and EP values are similar to the data from MSI medium cultivation. The lower PC and EP values seen in the extract from the cell growing in RHB medium may indicate lower phycocyanin content that normally seen in the cells growing heterotrophically (lower photosynthetic activity) [11].

Table 1. The Spirulina cell biomass concentration in the culture, phycocyanin concentration (PC), and phycocyanin extract purity (EP) from the cultivation using mineral medium (MSI) and rice husk biocharcoal medium (RHB). The data is from the experiment with the shaking speed of 200 rpm after 10-hour extraction.

	Medium (biomass to solvent ratio)		
Parameters	**MSI (2 g: 50 ml)**	**RHB (2 g: 50 ml)**	**RHB (2 g: 30 ml)**
Cell concentration (g/l)	0.24	0.39	0.39
PC (mg/ml)	2.03	1.20	2.25
EP	0.36	0.32	0.31

3.2. Effect of Biomass to Solvent ratio, Extraction Time and Shaking Rate

The phycocyanin concentration (PC) and purity (EP) from the Spirulina grow on RHB medium are shown in Figure 2 to 3. The highest ratio of biomass to solvent (2 g: 30 ml) produced the highest content of phycocyanin extracted as seen in Figure 2. However, the change in the ratio did not affect the extract purity (Figure 3). The same results also observed in a previous research [8] when the mineral Zarrouk medium was used. Still, the higher biomass to solvent ratio has an economic advantage in the downstream processing. The lower amount of solvent used that resulting in a higher PC will reduce the cost for bioseparation.

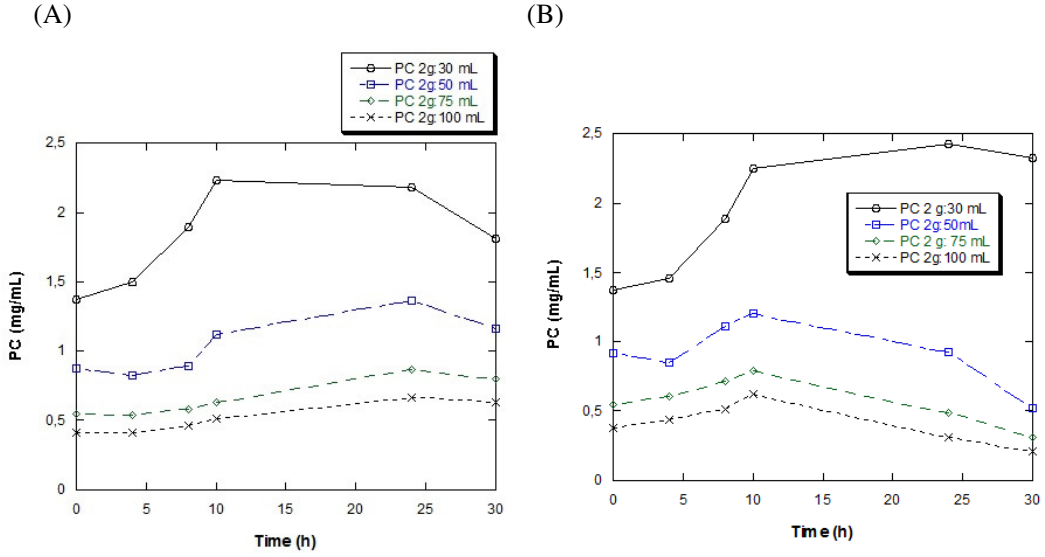

Figure 2. (A) Phycocyanin concentration (PC) from different biomass to solvent ratio on shaking rate 150 rpm (A) and 200 rpm (B)

The PC from all experiments increased for the early hours (<10 hours), but the decreases were generally observed at the final hours of the extraction process. EP values were relatively constant at 0.3 ± 0.06, except when the stirring speed was at 200 rpm. Longer extraction period did not increase PC and EP. Several factors could cause the decrease of PC and EP. The temperature of the suspension was

maintained constant; therefore, it is unlikely that the decrease is due to the increase in temperature that may degrade the phycobiliproteins. Previous study shows that phycocyanin is stable at up to 40 °C [12]. We predict that the mechanical impact may be the cause of PC and EP reduction. This needs to be further investigated.

The possibility that the mechanical factor may damage phycocyanin can be further observed when the stirring speed is increased. The significant drops in the PC and EP values were seen at 200 rpm after 10-hour extraction, whereas the values were relatively constant when the extraction was at 150 rpm. Although the higher shaking rate is expected to assist the phycocyanin extraction by improving cell disruption, this strategy is only useful at the early hours of extraction (<10 hours).

Table 2. The phycocyanin concentration (PC), and phycocyanin extract purity (EP) from the cultivation using mineral medium (MSI) and rice husk biocharcoal medium (RHB) after purification using ammonium sulfate. The data is from the experiment with the shaking speed of 200 rpm after 10-hour extraction.

	Medium (biomass to solvent ratio)		
Parameters	**MSI (2 g: 50 ml)**	**RHB (2 g: 50 ml)**	**RHB (2 g: 30 ml)**
PC (mg/ml)	2.42	1.50	2.62
EP	0.71	0.72	0.81

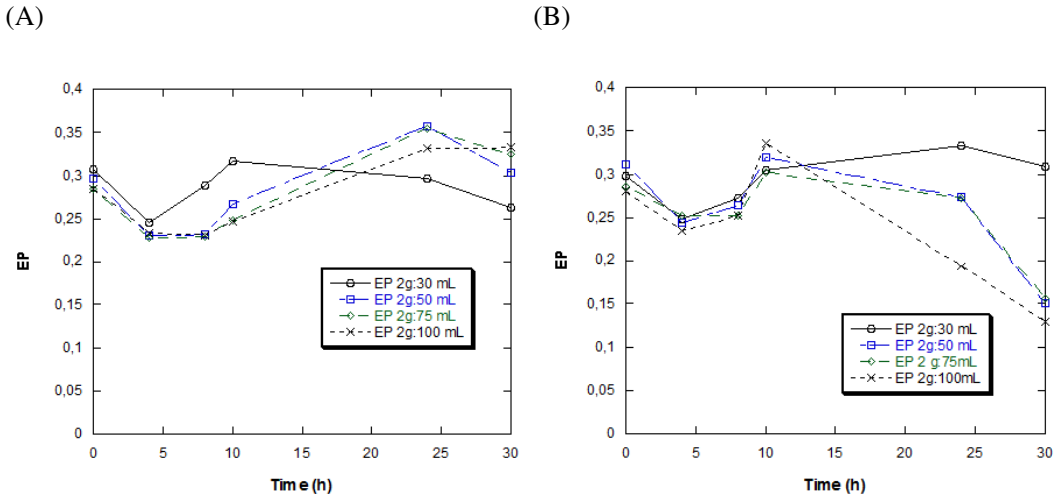

Figure 3. Extract Purity (EP) from different biomass:solvent ratio at shaking rate 150 rpm (A) and 200 rpm (B)

The PC values increase slightly (about 20%) after the purification (Table 2). The EP values increase more than double reaching the value for required for food grade phycocyanin (EP > 0.7) [13]. This result shows that the phycocyanin can be produced from *Spirulina* grow in the medium from agricultural waste such as rice husk.

4. Conclusion

In this research, we have demonstrated that an agricultural waste such as rice husk can be used to cultivate Spirulina for its phycocyanin extraction. The replacement of urea and triple superphosphate in the mineral medium with rice husk biocharcoal increases the biomass concentration but not the phycocyanin level. The extraction should be performed at high biomass to solvent ratio, high shaking rpm, and at the period of less than ten hours. Longer extraction period will reduce the phycocyanin concentration and purity.

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