Modification of phycocyanin extraction from dry biomass of *Spirulina* by using ozone water

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Abstract. Phycocyanin is a water-soluble blue-green pigment from cyanobacteria such as *Arthospira plantesis*, widely known as Spirulina. The pigment is widely applied as a natural food colorant or consumed as a supplement due to its antioxidant activity. This study aimed to investigate the potential use of ozone water as an extraction solvent of phycocyanin from dry mass Spirulina. The yield, purity, and antioxidant capacity parameter measured the effectivity of ozone water as an extraction solvent. The phycocyanin was extracted by the cold maceration process preceded by the dissolving of the dry mass Spirulina in ozone water by the ratio 1:100. For the control, the dry mass Spirulina was dissolved in mineral water. The method was followed by centrifugation and spectrophotometer measurement. To check the antioxidant capacity, we measure the phycocyanin inhibition rate to DPPH. The data showed that using ozone water as an extraction solvent successfully resulted in a higher yield and purity of phycocyanin than the control. Besides, there is no negative effect on antioxidant capacity affected by ozone water. Therefore, ozone water is a potential solvent to enhance the extraction of phycocyanin from dry mass Spirulina.

1 Introduction

Arthospira plantesis, known as *Spirulina*, is included in the class of cyanobacteria, which has a habitat in freshwater and marine ecosystems. *Spirulina sp.* has been commercialized and applied to food products, supplements, and cosmetics. *Spirulina* sp. is sold commercially in powder form (dry biomass) and packaged in capsules or tablets as a supplement. The Food and Drug Administration (FDA) classifies *Spirulina sp.* as a GRAS (Generally Recognized as Safe) organism. None of the severe side effects reports from consuming *Spirulina* sp. as much as three grams per day for one month [1]. The results of toxicology studies also show that consuming *Spirulina* sp. does not cause poisoning symptoms [2].

Spirulina has been used as a supplement or functional food ingredient because its nutrients showed health benefits in terms of macronutrients and micronutrients. The protein content in *Spirulina sp.* is around 60-70% of its dry weight. In addition, *Spirulina sp.* contains fiber of

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7.7% of its dry weight and fat content which reaches 5-10% of dry weight and is rich in omega-3 and omega-6 [2]. In addition, Spirulina contains vitamins and minerals. The vitamin content includes biotin, folic acid, cyanocobalamin (vitamin B12), thiamine, riboflavin, niacin, tocopherol, and beta-carotene. *Spirulina sp.* contains high vitamin B12, reaching 162 mcg. In addition, *Spirulina sp.* contains beta carotene reaching 700-1700 mg/kg. The mineral content of Spirulina includes potassium, calcium, phosphorus, magnesium, manganese, and iron. Spirulina also contains pigments, including chlorophyll, phycocyanin, and carotenoids [2, 3, 4]. Phycocyanin is a blue pigment, an antioxidant compound that can scavenge free radicals. In addition, phycocyanin is also an immunomodulator, a compound that can help modulate the body's immune system [4].

Phycobilins in Spirulina capture light on the thylakoid membrane's surface. The protein phycobilins consist of phycoerythrin (C-PE), phycocyanin (C-PC), and allophycocyanin (C-PCA). Phycocyanin is the main pigment in the protein phycobilin, which has a blue color, and its composition can reach 20% of the cell mass [5]. Phycocyanin is a pigment that can dissolve in water or other polar solvents. The stability of phycocyanin is affected by temperature, pH, oxygen, and humidity. Phycocyanin is stable at a pH of 5.5-6.5, where pH affects the intensity and level of the phycocyanin color [5]. Phycocyanin is a natural coloring agent in the food industry. In addition, phycocyanin is used as a nutraceutical ingredient because it has an antioxidant capacity and shows anti-inflammatory and anti-microbial activity. In-vivo test of the antioxidant capacity of phycocyanin in rabbits showed that phycocyanin supplementation of 50 and 100 mg/kg diet led to an increase in glutathione (GSH) and total antioxidant activity (TAC) [6]. Measurement of the antioxidant capacity of phycocyanin can also be conducted using the ABTS (2,2'-azino-bis-(3-ethlbenzthiazoline-6-sulfonic acid)) and DPPH (α,α-Diphenyl-β-pricrylhydrazyl) methods [7].

The extraction process affects the quality of the resulting phycocyanin. Several factors affect the extraction process, including temperature, type of solvent, the ratio of biomass and solvent, pH, and cell disruption method [5, 8]. The non-optimal extraction process causes a decrease in the concentration, yield, and purity of phycocyanin. Cell disruption is an important step in phycocyanin extraction because the unstable cell wall and lysis will make it easier for phycocyanin to exit and dissolve in the supernatant [5, 8]. Several phycocyanin extraction methods include freeze-thaw, maceration, bead-milling, ultrasonication, high-pressure processing, pulse electric fields, and microwaves. Freeze-thaw and maceration methods are conventional methods that do not involve mechanical disruption, so these two methods require a long time to produce high concentrations and yields of phycocyanin. Other mechanical cell destruction methods require a shorter extraction time to produce phycocyanin extracts with higher concentrations and yields [5]. Several previous studies have compared conventional extraction methods with more modern extraction methods. For example, research by [8] reported that in the same solvent, the ultrasonication method produced a significantly higher yield of phycocyanin extract compared to the freeze-thaw method. However, the freeze-thaw method produced phycocyanin extracts with a better purity level than the ultrasonication method.

Meanwhile, the type of solvent affects the yield and purity of the extracted phycocyanin. Phycocyanin extraction uses polar solvents such as water, ethanol, and methanol [9]. In addition, phycocyanin extraction can also use a buffer solution (buffer), such as sodium phosphate buffer solution, to control the pH of the extraction solution (media). Other types of solvents include NaCl solution and $CaCl_2$ solution. Different types of solvents in the extraction process are reported to affect the concentration, yield, and purity of the extracted phycocyanin [5, 10]. Research by [9] compared phycocyanin extracts with water, ethanol, and methanol solvents, where higher concentrations and purity were obtained from phycocyanin extracts with methanol and ethanol solvents compared to water. Several studies reported that using water as a solvent and a phosphate buffer solution with a pH of 6-7

resulted in differences in the concentration and yield of phycocyanin extract but did not affect its purity [8, 10]. Adding salts, such as $CaCl_2$ and NaCl, to the solvent for phycocyanin extraction can increase the phycocyanin concentration produced. For example, 1.5% $CaCl_2$ solution produced phycocyanin extract with a higher concentration than water and sodium phosphate buffer pH 7.4. These results are consistent with several cell disruption methods, such as freeze-thaw, ultrasonication, and microwaves [11].

Ozone water is defined as water that has been ozonized or given the addition of ozone gas. According to the Food and Drugs Administration, ozone is classified as "Generally Recognized as Safe" (GRAS) for contact with food materials [12]. The ozonization treatment as a pre-treatment has carried out microalgae cultures, both cyanobacteria and green algae, as part of the harvesting stage [13-17]. Other studies also reported the combination of ozone as a pre-treatment with the ultrasound method to extract carbohydrates, proteins, and lipids from unicellular green microalgae [13-14]. Ozone is considered a potential pre-treatment method, as indicated by the number of cells disrupted and the efficiency of protein extraction. Ozonation induces cell lysis due to cell pre-oxidation, which facilitates the extraction of molecules and compounds from within the cell [14].

In this study, ozone water was used as a solvent in phycocyanin extraction. Pre-treatment was carried out by dissolving the dry biomass of *Spirulina* sp. with ozonized water, followed by a cold maceration process for 24 hours. Previous studies showed that ozonation causes *Spirulina* sp. lysis, which showed increased phycocyanin yield. However, studies related to the use of ozone water as a solvent for extracting phycocyanin from *Spirulina* sp. have never been done. This study examines the effect of using ozone water as a solvent in phycocyanin extraction on the yield and purity of phycocyanin from dry biomass of *Spirulina* sp. In addition, this study also investigated the use of ozone water as a solvent in the phycocyanin extraction process without a negative effect on the antioxidant activity of phycocyanin from dried biomass of *Spirulina sp.* This research is expected to provide new information regarding the application of ozone water to increase the yield of phycocyanin extraction from *Spirulina* sp. biomass which is expected not to harm the antioxidant activity of phycocyanin.

2 Methodology

2.1 Phycocyanin extraction

Spirulina sp. dry biomass (California Gold Nutrition®) was dissolved in ozone water, as the solvent, with an ozone concentration of 2 mg/L in a ratio of 1:100. As a control, mineral water was used as a solvent. The phycocyanin extraction method uses the cold maceration method by incubating the solution at 4ºC for 24 hours. Each treatment was given three repetitions. After that, all samples were centrifuged for 10 minutes at 4200 rpm at 10ºC. Next, the supernatant and pellet were separated. Phycocyanin is present in the supernatant, which is then continued to measure yield, purity, and antioxidant activity.

2.2 Phycocyanin yield and purity measurement

The yield and purity of phycocyanin were analyzed by measuring the absorbance of the phycobiliprotein protein at 620 nm and 652 nm using spectrophotometry. In addition, the absorbance of total protein was measured spectrophotometrically with a wavelength of 280 nm. The blank used is distilled water. Phycocyanin concentration is calculated by equation (1). In contrast, the yield is calculated by equation (2).

$$C - PC\ (\frac{mg}{mL}) = \frac{OD_{620} - 0.474(OD_{652})}{5.34} \quad (1)$$

$$Yield\ (\frac{mg}{g}) = \frac{C-PC \times V}{biomass} \quad (2)$$

V is the volume of solvent, and biomass is the dry biomass mass of *Spirulina* sp.

The purity of phycocyanin is determined based on the ratio of the absorbance of phycocyanin at a wavelength of 620 nm and the absorbance of total protein at a wavelength of 280 nm [7].

2.3 Measurement of antioxidant activity

Antioxidant activity measurement of phycocyanin extract based on binding activity to 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH). DPPH solution (TCI, Japan) with a concentration of 0.1 mM was prepared by dissolving DPPH in methanol. 1 mL of phycocyanin extract sample was mixed into 9 ml of 0.1 mM DPPH solution and then incubated in the dark at 37oC for 30 minutes. The control used 1 mL of methanol mixed in 9 mL of 0.1 mM DPPH solution. Absorbance measurements were carried out at a wavelength of 517 nm. Equation (3) calculates the percentage of inhibition capacity.

$$\%\ Inhibition = \frac{Abs.control - Abs.sampel}{Abs.control} \quad (3)$$

2.4 Statistic analysis

Data analysis used in this study used a paired sample t-test and one-way ANOVA with GraphPad Prism 9. Analysis was performed at a 95% confidence level.

3 Results and Discussion

Extracting phycocyanin from the dry biomass of Spirulina sp. used different solvents, which were ozone water as the treatment and mineral water as the control. Ozone water was obtained by ozonation of mineral water for 15 minutes. The ozonation process only affects the increase in ozone concentration in water but does not affect temperature, pH, electroconductivity (EC), and total dissolved solids (TDS). Ozonation produces ozone water with an ozone concentration of 1.73 ± 0.31 mg/L.

3.1 The effect of ozone water as a solvent on phycocyanin yield and purity

The effect of ozone water as a solvent was investigated with yield parameters and the level of purity of phycocyanin. The yield is the amount of extract produced, where the higher yield, the more extract is produced. The purity value indicates the quality of phycocyanin, which influences its utilization potential. The criteria for the purity of phycocyanin as a food ingredient is at least 0.7. The number on the purity level describes the ratio between phycocyanin and total protein.

The results of the phycocyanin extraction with ozone water and mineral water are shown in Figure 1. The results show that the phycocyanin extract produced was blue-colored and showed no color difference in the extract results. The measurement results showed that ozone water as a solvent produced a higher yield and purity of phycocyanin compared to phycocyanin extracted with mineral water solvent (Table 1). The use of ozone water produced phycocyanin yields of 30.45 ± 4.28 mg/g with a purity of 0.62 ± 0.07. The yield analysis results and purity values showed significant differences (p-value < 0.05) compared

to the phycocyanin extract using mineral water as solvent. These results indicate the different abilities of ozone and mineral water to extract phycocyanin. In addition, the results indicate the potential for using ozone water as a solvent to increase the yield and purity value of phycocyanin extracted from the dry biomass of *Spirulina* sp.

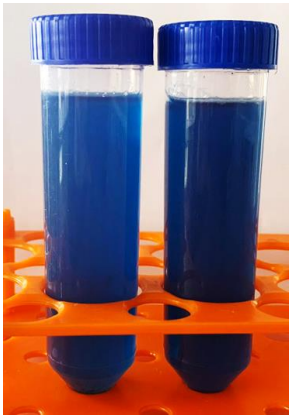

Fig. 1. The result of phycocyanin extract by using (left) mineral water as the control solvent and (right) ozone water as the treatment solvent

Table 1. Yield and purity of phycocyanin from dry biomass of *Spirulina* sp.

Types of solvent	Yield (mg/g)	Purity
Ozone water	30.45 ± 4.28*	0.62 ± 0.07*
Mineral water	27.40 ± 3.88	0.56 ± 0.07

*p-value <0.05 (paired sample t-test; GraphPad Prism 9), showing the significance between two samples in the parameter of yield and purity

The application of ozone has been carried out to increase the efficiency of the microalgae culture harvesting process. A study by [17] showed that direct ozone administration to *Spirulina sp.* causes damage to the cell walls of *Spirulina sp.*, thereby increasing the amount of Spirulina crude extract. The ozonation method in microalgae culture has also been proven to successfully break down and damage the microalgae cell walls, facilitating the process of extracting bioactive components [15, 16]. Ozone causes damage to the cell walls of microalgae and triggers the release of polysaccharides and biopolymers, causing flocculation [15].

In this study, ozone water was used as a solvent in the extraction of phycocyanin, which had never been conducted in previous studies. Ozone water is widely used to inactivate bacteria in washing food such as vegetables and fruits. The ability to inactivate is caused by the interaction of ozone with parts of the bacterial cell wall, which then triggers cell lysis [18]. Research by [19] showed that exposure to ozone causes deformation of the cell structure of *Escherichia coli* and *Pseudomonas aeruginosa* bacteria.

This study found that the use of ozone water in the extraction process increased the yield and purity of phycocyanin. Based on the study of the effect of ozone on bacterial inactivation and harvesting of microalgae cultures, ozone can interact with the cell wall, which then causes deformation and cell lysis. The interaction of ozone in the cell wall is the main hypothesis why ozone water can increase the yield of phycocyanin extract. Ozone interacts

with the cell walls of *Spirulina* sp. and triggers the cell wall lysis so that the phycocyanin extraction process becomes more efficient.

3.2 The effect of ozone water as a solvent on phycocyanin antioxidant activity

The results of the antioxidant activity test were indicated by the percent inhibition value, namely the ability of antioxidants to inhibit DPPH, which was influenced by the concentration of the sample tested. The percent inhibition value of phycocyanin extract with mineral water solvent was 18.86 ± 0.46 %, while the percent inhibition value of phycocyanin extract with ozone water solvent was 19.15 ± 2.55 % (Figure 2). The results showed that the antioxidant activity of phycocyanin extract with ozone water solvent was higher than that of phycocyanin extract with mineral water solvent but insignificant. A higher percentage inhibition value could be due to the higher concentration of phycocyanin in the phycocyanin extract using ozone water as a solvent. Percent inhibition data also showed that ozone water did not have a negative effect on the antioxidant activity of the phycocyanin extract.

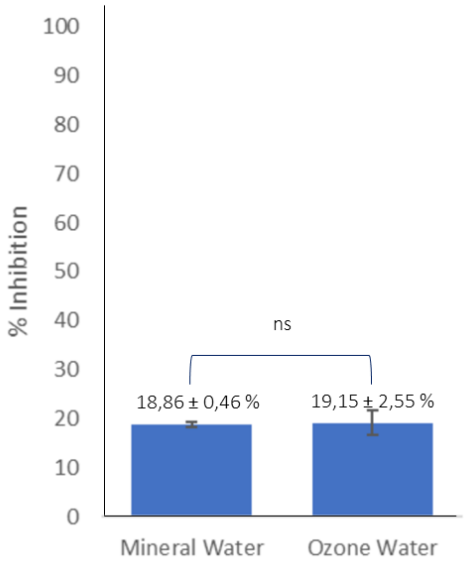

Fig. 2. Inhibition percentage of phycocyanin extract with different solvent types. ns = not significant between two samples (p-value > 0.05)

Previous studies discussed the effect of ozone exposure on the antioxidant activity of fruit and vegetable extracts such as kiwi [20], broccoli [21], and Arugula [22]. An increment of antioxidant activity, measured by the ABTS and CUPRAC methods, occurred when kiwi was exposed to ozone gas at 10 and 100 ppm concentrations for 5, 15, and 30 minutes. The increment in antioxidant activity caused by exposure to 100 ppm ozone for 30 minutes on kiwi with the ABTS and CUPRAC methods reached 19% and 20% [20]. Exposure to ozone for 5 and 10 minutes on broccoli did not damage the antioxidant capacity, vitamin C concentration, total phenolic, and chlorophyll [21]. The same results were obtained in research on Arugula, where exposure to ozone gas showed no decrement in antioxidant capacity [22].

3.3 The comparison of direct ozonation and the ozone water as a solvent on phycocyanin yield and purity

We also investigated the potential utilization of direct ozonation for phycocyanin extraction. We compared the yield of phycocyanin extract of (1) no treatment as control; (2) ozone water as extraction solvent; and (3) direct ozonation. The dry mass *Spirulina* sp. was dissolved in mineral water in the control treatment. While for direct ozonation, the dry mass *Spirulina* sp. was dissolved in mineral water following the ozonation treatment for 10 minutes. All sample solution was stored at 4^{o}C for 24 hours. Later, the phycocyanin extract was separated from the pellet by centrifugation.

The result is shown in Table 2, suggesting that the highest yield and purity were reached by using ozone water as a solvent during the extraction process by maceration. The highest yield of phycocyanin extract was 35.02 ± 2.07 mg/g resulting from using ozone water as a solvent, which also showed significantly higher than control and direct ozonation treatment. The direct ozonation treatment showed a higher yield than the control but was insignificant. The highest purity was 0.68 ± 0.07, resulting from using the ozone water as a solvent during extraction. However, the phycocyanin purity of all treatments was not significantly different.

Table 2. The comparison of yield and purity of phycocyanin from dry biomass of *Spirulina* sp. by different extraction treatments

Treatments	Yield (mg/g)	Purity
Control – Mineral water	26.30 ± 2.53^{a}	0.54 ± 0.04^{a}
Ozone water as solvent	35.02 ± 2.07^{b}	0.68 ± 0.07^{a}
Direct ozonation	27.25 ± 0.84^{a}	0.61 ± 0.06^{a}

*superscript letter showed the p-value <0.05 (one-way ANOVA; GraphPad Prism 9), showing the significance between samples in the parameter of yield and purity

The result suggested that using ozone water as an extraction solvent is more potential to increase phycocyanin yield compared to direct ozonation. This study used the same ratio between biomass and solvent for all treatments. The direct ozonation treatment may need a different optimal ratio of biomass and solvent, which affects ozone production and needs further experiments to investigate the optimal condition. Further experiments need to conduct to explore the potential use of ozone water as a solvent during extraction, for example, by combining it with modern extraction methods.

4 Conclusion

This study showed that ozone water has the potential to be applied as a solvent in the phycocyanin extraction process, which was measured based on the parameters of yield, purity, and antioxidant capacity. Ozone water as a solvent in the extraction process produces a phycocyanin extract with a yield of 30.45 ± 4.28 mg/g and a purity of 0.62 ± 0.07, which is higher than the yield and purity of the phycocyanin extract extracted with mineral water as a solvent. In addition, ozone water did not have a negative effect on the antioxidant capacity of the extracted phycocyanin. The percent inhibition of phycocyanin extract with ozone water solvent was 19.15 ± 2.55%, while the percent inhibition value of phycocyanin extract with mineral water solvent was 18.86 ± 0.46%.

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