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Research Article

Production of high-antioxidant yoghurt using phycocyanin from microalgae *Spirulina* sp

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Abstract. Yoghurt is a functional food product widely used to improve the digestive system in the body. Yoghurt fermentation usually uses lactic acid bacteria from the *Lactobacillus bulgaricus, Streptococcus thermophilus, and Lactobacillus casei*. Phycocyanin has been used as a natural dye for food, cosmetics, and medicine. Phycocyanin is a complex protein that can be an anticancer, antioxidant, and immunity booster. In this study, the effect of the number of additions of phycocyanin, storage time, and time of addition of phycocyanin to the antioxidant activity, protein, and organoleptic properties of the yoghurt produced. In this study, cow milk was pasteurized and fermented to become a yoghurt. Afterwards, phycocyanine was added to the yoghurt before being stored and analyzed. Spectrophotometric analysis was utilized to determine the antioxidant activity and protein content. An organoleptic test using a Likert scale was conducted to determine the product's suitability to consumer tastes. Increased phycocyanin concentrations were added (0, 0.25, 0.5, and 0.75 wt. %), resulting in a decrease in IC50 value of 8855.53, 5843.371, and 4147.548 ppm, which shows an increase in antioxidant activity. At the level of consumer preference in terms of taste, the most preferred is a concentration of 0.5 wt. %, while in terms of colour and aroma, at a concentration of 0.25 wt. %. While the longer the storage time (1, 3, and 5 days), the increase in IC50 value of 2777.111, 5179.547, and 5916.884 ppm, and there was a decrease in protein content in the sample. For the level of consumer preferences in terms of taste and aroma, the most preferred is variable with a storage time of 1 day, and in terms of colour, the most preferred at five days storage. The IC50 value in the variable with the addition of phycocyanin after fermentation is less than the addition after fermentation, which is 5351.865 ± 1606 and 3897.162 ± 1678 ppm. The protein content is higher when adding phycocyanin before fermentation. While yoghurt with the addition phycocyanin before fermentation showed higer consumer preference in terms of taste and aroma. In contrast, the addition of phycocyanin after fermentation is preferred in terms of colour.

Keywords: Yoghurt, Phycocyanin, Antioxidants, Protein, Organoleptic

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1. Introduction

Yoghurt is a functional food product widely used to improve the digestive system in the body. Yoghurt fermentation usually uses lactic acid bacteria from *the Lactobacillus bulgaricus, Streptococcus thermophilus*, and *Lactobacillus casei*. The main raw material for making yoghurt is fresh milk. Yoghurt consumption continues to increase yearly, as indicated by the increase in yoghurt production. According to data from Badan Pusat Statistik (2008), yoghurt production in Indonesia from 2002 to 2005 increased with an average annual growth of 56.97%. Yoghurt can supply all essential amino acids and other nutrients. However, yoghurt does not contain enough vitamin C and B complex substances. If yoghurt is consumed regularly, it can balance the intestinal microflora where harmful bacteria can be reduced in number and beneficial bacteria will dominate and vice versa the intestine (Yuguchi et al., 1992).

Spirulina platensis (Arthospira) is a microalgae with the highest protein content compared to other sources. Spirulina has been developed in the food industry because it contains many nutrients. Many food products use spirulina, including yoghurt. *Spirulina sp.* contains phycocyanin blue pigments about 20% of their dry weight (Ridlo et al., 2015). According to Richmond (2004), phycocyanin is a polar and water-soluble pigment associated with proteins. Phycocyanin can be extracted using water solvents or buffers. Phycocyanin is a complex protein that can serve as an anticancer, antioxidant and immunity booster (Kozlenko and Henson, 1998) and antioxidants. Due to its benefits, phycocyanin has been used widely in the food, cosmetics, and medicine industries.

Antioxidants are electron-giving compounds that can inactivate the development of oxidation reactions by preventing the formation of free radicals. Nowadays, antioxidants that are widely used in food are generally synthetic antioxidants. However, the use of synthetic antioxidants as food is not recommended by the Ministry of Health because it is thought to cause cancer (carcinogenic) (Barus, 2009).d

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2. Materials and Methods

2.1. Material

The materials used in this study were cow milk, a starter of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, aquadest, DPPH reagent, methanol, ovalbumin and phycocyanin. Furthermore, the equipment used in the study was a beaker glass, electric stove, aluminium foil, measuring cup, weighing scale, glass stirrer, test tube, measuring flask, pan, glass jar, thermometer, measuring pipette, cuvet, and UV-Vis spectrophotometer.

2.2. Experiment variable

The controlled variables in this study were the volume of cow milk per variable 200 ml, milk pasteurization temperature 70°C, yoghurt fermentation temperature 42°C, and addition of bacterial starter per variable 0.2 gr. The independent variables are phycocyanin concentration (0, 0.25, 0.50, 0.75 wt. %), yoghurt storage time (1, 3, 5 days), and time of addition of phycocyanin (before and after fermentation). The flowchart of this study is shown in Figure 1.

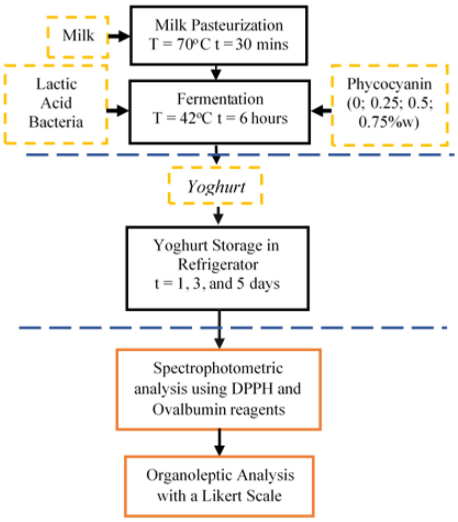

Figure 1. Flow Chart of Phycocyanin Yoghurt Making

2.3. Preparation of cow milk

Cow milk was put into a pan, pasteurized at 70°C for 30 minutes, and then cooled to 40°C (Aswal et al., 2012).

2.4. Yoghurt Fermentation and Addition of Phycocyanin

Pasteurized cow milk was put into a glass jar according to the variable. Then, 0.2 gr of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* starter was added to cow milk. The amount of phycocyanin added to yoghurt depends on the density of the yoghurt. Phycocyanine was added according to the calculation of the needs of each variable. Afterwards, the yoghurt was homogenized and fermented for 6 hours (anaerobic fermentation) at an incubator temperature of 42°C (Aswal et al., 2012; Smith, 2015).

2.1. Yoghurt Storage

The yoghurt is then stored for 1, 3, and 5 days in the refrigerator to evaluate the effect of storage time on the quality of the yoghurt.

2.2. Antioxidant Activity Analysis

The antioxidant activity analysis was conducted by making a DPPH solution by dissolving 2 mg in p.a methanol to 100 ml. Furthermore, the sample was covered with aluminium foil. The sample solution is concentrated to 625, 1250, 2500, 5000, and 10000 ppm. Afterwards, the absorbance of the DPPH control solution was measured using a 2 ml mixture of DPPH solution and 3 ml of methanol, which was added into the test tube. Then, the mixture is vortexed for 30 seconds, and the absorbance was measured with a spectrophotometer at a wavelength of 517 nm. The inhibition percentage was calculated using equation 1, where A_0 was blank absorbance, and A_1 was sample absorbance.

$$\%\ Inhibition = \frac{A_0 - A_1}{A_0} x\ 100\% \quad (1)$$

Then, using the calculation of the IC_{50} value of %inhibition data (as the y-axis) and concentration (as the x-axis) plotted in the graph, find the regression equation in the form y = mx + c. In this calculation method, the value of y is substituted with 50 so that the x value was obtained, which is the IC_{50} value in units of ppm (Filbert et al., 2014). This value is inversely proportional to antioxidant activity. The lower the IC_{50} value, the stronger the antioxidant activity (Ridlo et al., 2015).

2.3. Protein Content Analysis

The ovalbumin standard curve was made with a concentration of 0.25, 0.125, 0.0625, and 0.03125% and phosphoric acid blank in methanol in a UV-Vis spectrophotometer with a wavelength of 595 nm. The x-axis is the concentration, and the y-axis is absorbance. The linear regression equation was calculated in the form y = mx + c. Then, each 1 ml and add 4 ml blank. Then, the sample was incubated at room temperature for 5 minutes.

Furthermore, the absorbance was analyzed with a spectrophotometer and then inputted into the y value in the standard curve regression formula. There is an x value, then multiplied by the sample dilution factor. Protein content in yoghurt is obtained in units of a per cent (Bio-Rad Laboratories, 2018).

2.4. Organoleptic Analysis on the Likert Scale

The Likert scale organoleptic analysis is used to find product suitability with consumer tastes. The organoleptic analysis of the Likert scale was carried out by taking 5 ml of each variable and testing it on 26 respondents, who assessed the yoghurt in terms of taste, aroma, and colour. Then, the data obtained is calculated as the average value (mean) to indicate the middle value that can represent the entire data and the standard deviation to indicate the heterogeneity of the data and also indicates the representation level of the mean value of the overall data (Sopiah, 2014).

3. Result and Discussion

3.1. Effect of Phycocyanin Addition Concentration on Antioxidant Activity in Yoghurt

This study found that IC_{50} levels were getting lower along with the higher concentration of phycocyanin addition. IC_{50} values will be inversely proportional to antioxidant activity. Therefore, If the IC_{50} decreases, the antioxidant activity will be stronger (Ridlo et al., 2015). The effect of phycocyanin addition concentration on antioxidant activity in yoghurt is shown in Table 1.

Table 1. Analysis Results of IC_{50} on Yoghurt with Independent Variable Phycocyanin Addition Concentration

Phycocyanin Concentration (%w)	IC_{50} (ppm)
0.00	9358.917
0.25	8855.53
0.50	5843.371
0.75	4147.548

Table 1 shows that phycocyanin addition concentration will be directly proportional to the increase in antioxidant activity. In contrast, for variables with phycocyanin concentrations of 0 wt. % and 0.25 wt. %, the value of $p > 0.05$ showed that the antioxidant activity did not change significantly. Whereas in comparing the variables added by phycocyanin (0.25, 0.5, and 0.75 wt. %), p-value <0.05 was obtained. This value indicates that the increase in phycocyanin addition concentration will significantly affect the antioxidant activity in yoghurt.

Antioxidant activity of a compound exists when the compound can donate hydrogen atoms to free radicals DPPH. Phycocyanin is a natural antioxidant from phytochemical groups (Hamid et al., 2010). Phycocyanin addition will increase antioxidant activity in the yoghurt. Then, along with the increase in the phycocyanin addition concentration, the antioxidant activity in the yoghurt will be higher. This happens because the more antioxidants added to the yoghurt, the more donor hydrogen atoms will be given to free radicals DPPH.

3.2. Effect of Phycocyanin Addition Concentration on Organoleptic Result in Yoghurt

This study added phycocyanin with various concentrations (0, 0.25, 0.5, and 0.75 wt. %). Figure 2 shows the result of the organoleptic test using the Likert scale—the sample with the addition of phycocyanin 0.5 wt. % has the most similar flavour to the control sample, whereas the sample with 0.75 wt % phycocyanin addition has the most distant flavour. In the study conducted by Malik (2013), which used spirulina powder as an additive in yoghurt, the pH of yoghurt during storage decreased in line with the concentration of spirulina powder addition. Phycocyanin used in this study was extracted from *Spirulina plantesis*. Therefore, it still carries its chemical properties and causes the flavour of yoghurt to become more acidic along with the concentration of phycocyanin addition. The value of $p > 0.05$ indicates that changes in the concentration of phycocyanin addition have no significant effect on the flavour of yoghurt.

In terms of colour, the control variables have an average value of 3.61, while the variables with various phycocyanin concentrations (0.25, 0.5, and 0.75 wt. %) have an average value of 3.37, 2.75, and 2.5. The data shows that the phycocyanin concentration of 0.25 wt. % has colour with good criteria, which is the most preferred compared to other variables. In the comparison of variables with the phycocyanin concentration of 0 wt. % and 0.25 wt. % obtained value of $p < 0.05$. This value indicates that the addition of phycocyanin causes the colour of yoghurt to change significantly. In comparison, the comparison of variables that were added by phycocyanin was obtained with a p-value$>$ 0.05. This value indicates that the change in the phycocyanin concentration does not significantly influence the colour of yoghurt. This is because of the phycocyanin addition of 0.25 wt. % gives a brighter blue colour than the phycocyanin addition of 0.5 wt. % and 0.75 wt. %. According to Monica and Luzar (2011), the colour blue in food is rarely used because it damages the appetite. This results in the control variable having the highest value of 3.61, and with the increase of phycocyanin addition, which consumers least prefer.

While based on aroma, the variable with phycocyanin concentration of 0.25 wt. % has the closest value to the control variable. The aroma of the control variable is 3.18, while the variable with phycocyanin concentrations of 0.25, 0.5, and 0.75 wt. % has an average value of 3.15, 3.08, and 2.9. Increased phycocyanin concentration caused a decrease in the level of consumer preference for aroma. This is because the aroma of phycocyanin will be more dominant with increasing phycocyanin concentration. Phycocyanin has a distinctive aroma and is slightly rancid, so adding too much will increasingly reduce the distinctive aroma of yoghurt. The results of the organoleptic test show a value of $p > 0.05$, which indicates that changes in the concentration of phycocyanin addition did not significantly influence the aroma of yoghurt.

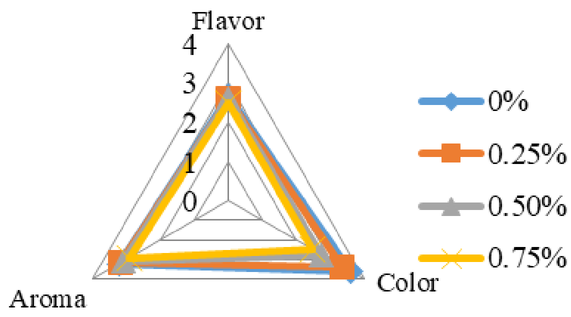

Figure 2. Concentration of Phycocyanin Addition VS Consumer's Response to Flavor, Color and Aroma of Yoghurt

3.3. Effect of Storage Time on Antioxidant Activity in Yoghurt

The effect of storage time on antioxidant activity in yoghurt can be seen in Figure 3. Samples with a storage time of 1 day have an average IC_{50} of 2777.111 ppm. This shows that at a concentration of 2777.111 ppm, the sample can inhibit 50% free radical activity. Whereas for the longer storage time, 3 and 5 days, the IC_{50} value is increasing with an average of 5179.547 and 5916.884 ppm, respectively. It can be concluded that the longer the storage time, the more concentration of samples needed to inhibit 50 % of free radical activity. In other words, the length of storage time will be inversely proportional to the increase in antioxidant activity.

During storage, antioxidant activity will tend to be stable or slightly decreased (Rahmawati, 2016). Storage at refrigerator temperature does not affect antimicrobial activity because cold temperatures can maintain the antimicrobial activity of the product. In this study, yoghurt stored at the refrigerator's temperature is not perfectly packed, which allows contaminants during storage and causes the antioxidant activity to decrease. From the results of the analysis obtained p value> 0.05, this value indicates that storage time does not significantly influence the antioxidant activity in yoghurt.

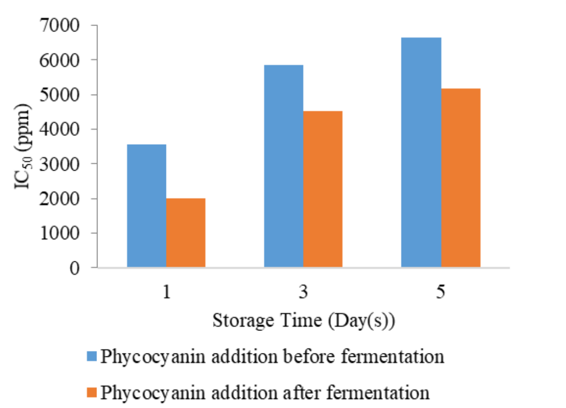

Figure 3. Effect of Storage Time on Antioxidant Activities in Yoghurt

3.4. Effect of Storage Time on Protein Content in Yoghurt

Figure 4 shows the effect of storage time on yoghurt protein content, which shows that protein content after stored for 1,3 and 5 days was 0.7609 ± 0.0133, 0.6404 ± 0.0154, and 0.6397 ± 0.0369 g / 100 g yoghurt, respectively. On the other hand, protein content in the control variable is 0.3116 ± 0.0308 g / 100 g yoghurt. The p-value obtained from the data was <0.05 from days 1-3, which means that there are significant changes in the protein content of yoghurt, whereas, on days 3-5, the obtained value was p> 0.05, which means that there are no significant changes. It means the change in protein content on day 3-5 was not as significant as the change from day 1-3.

According to Spolaore et al. (2006), phycocyanin is a complex phycobiliprotein produced by cyanobacteria, so its addition to yoghurt will increase protein content besides giving colour. According to the data from the analysis, the longer the storage time of yoghurt, the lower the protein content. This is because yoghurt contains lactic acid, which decreases pH, while proteins can be denatured at acidic pH (Dissnayake, 2013). Therefore, the protein content will be reduced in line with the storage time because it is denatured.

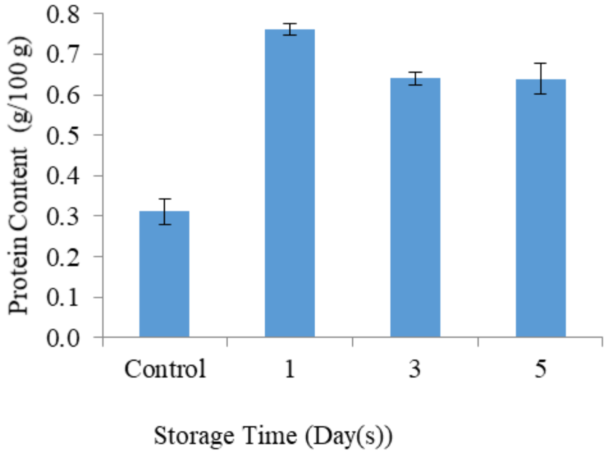

Figure 4. Storage Time VS Yoghurt Protein Content

3.5. Effect of Storage Time on Organoleptic Result in Yoghurt

The effect of storage time on respondent's preference is shown in Figure 4. Samples with a taste with the closest value to the control variable are samples with a storage time of 1 day. The longer the storage time, the level of consumer preference for flavour will decrease. This is because the longer the storage time, the pH will decrease due to the metabolic activity of bacteria that produce lactic acid (Izadi et al., 2015). From the analysis result, the value of $p > 0.05$ is obtained. This shows that the length of storage time does not significantly influence the taste of yoghurt after being stored.

In terms of colour, the variable with a 5-day storage time has the highest value compared with other variables, with 3.04 points, which falls under good criteria. Meanwhile, the variables with 1 and 3 days storage times have values of 2.86 and 2.72, respectively. The p-value obtained from the result was > 0.05. This value indicates that the storage time does not significantly influence the colour of yoghurt. The consumer value at the 5-day storage time is highest because phycocyanin protein will be denatured longer by lactic acid, so phycocyanin, a phycobiliprotein, will lose its colour to be brighter. According to Monica and Luzar (2011), the blue colour in food is rarely used because it damages the appetite. So that brighter colours will be preferred by consumers. One day of storage gets a higher score than three days because one day of storage makes yoghurt still very fresh and attractive even though it is still thick blue, so the value is not greater than five days of storage.

Based on aroma, the variable with one day storage time has the highest value compared to other variables, which is 3.27 and belongs to the good category. The longer storage time causes a decrease in the level of consumer preference for aroma. This is because the variable contaminated during storage, besides the decreasing pH, causes a change in chemical properties that affect the aroma of yoghurt. The p-value obtained from the data results was $p > 0.05$. Thus, the length of storage time does not significantly influence the aroma of yoghurt.

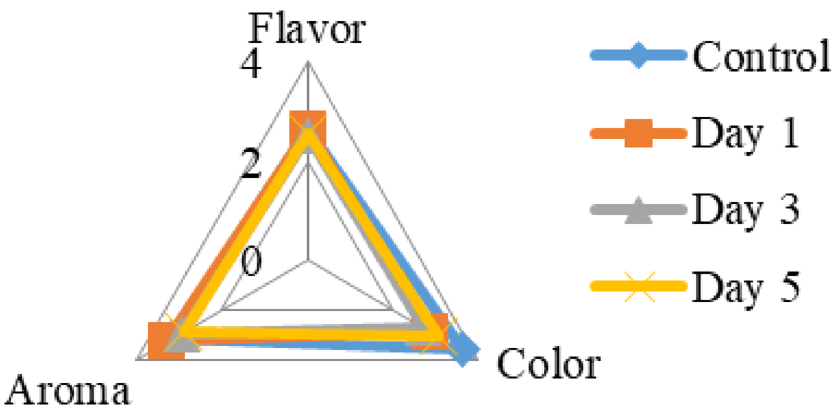

Figure 5. Storage Time VS Consumer's Response to Flavor, Color and Aroma of Yoghurt

3.6. Effect of Time for Phycocyanin Addition on Antioxidant Activity in Yoghurt

The addition of phycocyanin after fermentation resulted in a smaller IC_{50} value than phycocyanin before fermentation, as shown in Table 2. The sample with the phycocyanin addition after fermentation has an average IC_{50} value higher than the sample with the phycocyanin addition before fermentation. It can be concluded that with the addition of phycocyanin after fermentation, the concentration of samples needed to inhibit 50% of free radical activity is less. In other words, antioxidant activity will be better when the phycocyanin is added to the sample after fermentation. If phycocyanin is added before fermentation, bacterial activity during fermentation will degrade the chemical properties of phycocyanin. Antioxidant activity can be influenced by fermentation because protein bonds will be released and open groups of thiol compounds that can act as hydrogen donors (Rahmawati, 2016). Nonetheless, adding phycocyanin after fermentation can prevent phycocyanin's chemical properties and antioxidant content from being affected by bacterial activity. Therefore, the antioxidant content in yoghurt will be higher. From the data of the analysis obtained p value> 0.05, this value indicates that the addition of phycocyanin before and after fermentation does not give a significant difference to the antioxidant activity in yoghurt.

Table 2. Results of IC50 Analysis on Yoghurt with Independent Variable Time for Phycocyanin Addition

Time for Phycocyanin Addition	IC_{50} average (ppm)
Before fermentation	5351,865±1606
After fermentation	3897,162±1678

3.7. Effect of Time for Phycocyanin Addition on Protein Content in Yoghurt

Figure 3 shows the protein content analysis results with a difference in the time of phycocyanin addition. This study found that adding phycocyanin before fermentation had the highest protein content of 0.7609 ± 0.0133 g / 100 g of yoghurt. At the same time, the variables of phycocyanin addition after fermentation had a protein content of 0.5519 ± 0.0031 g / 100 g of yoghurt. The control variable's protein content was 0.3116 ± 0.0308 g / 100 g of yoghurt. According to Wahyudi (2006) and Putri (2016), protein content in fresh cow's milk in Indonesia ranged from 2.63-3.50 g / 100 g milk. However, the protein content results in this study were lower than the literature due to protein denaturation during fermentation 6 hours at 42 ° C (Patel et al., 2004). From these data, a value of $p < 0.05$ was obtained, which means that the treatment of the phycocyanin addition significantly affects the protein content of yoghurt.

The highest protein content was obtained in yoghurt with phycocyanin addition before fermentation. This result is linear with a study conducted by Christaki (2015), which found that all Phycobiliproteins can be dissolved in water so that if they participate in the fermentation process of milk, according to Buckle et al. (1987), the majority consists of water into yoghurt at a temperature of 42°C, phycocyanin which has been mixed at the beginning will be dissolved in milk more perfectly because the temperature treatment factor is higher than if it added after fermentation. Moreover, temperature can affect the solubility of proteins in water, and solubility will be greater at higher temperatures (Pace et al., 2004).

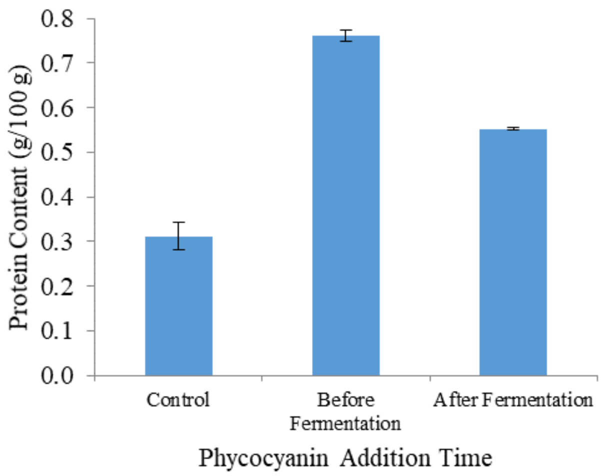

Figure 6. Time for Phycocyanin Addition VS Yoghurt Protein Content

3.8. Effect of Time for Phycocyanin Addition on Organoleptic Result in Yoghurt

This study added phycocyanin to the sample before and after fermentation. Based on the test results of respondent's preferences using the Likert scale, the results are shown in Figure 7. The graph shows that the sample with flavour with the closest value to the control variable is the sample with the addition of phycocyanin before fermentation. Phycocyanin is a pigment associated with protein (Richmond, 2004), so it will become a nutrient for bacterial growth when added before fermentation. Bacterial activity during fermentation will neutralize the original taste of phycocyanin so that it approaches the taste of yoghurt in general. Whereas if it is added after fermentation, the chemical properties of phycocyanin are still powerful because it does not go through fermentation so that it will affect the flavour of the yoghurt. From the results, the value of p> 0.05 is obtained. This shows that the addition of phycocyanin before and after fermentation does not give a significant difference to the flavour of yoghurt.

In terms of colour, the variable with the phycocyanin addition after fermentation has the highest value compared to other variables. In addition, from the results, the value of p> 0.05 was obtained. This value showed that adding phycocyanin before and after fermentation did not give a significant difference to the colour of the yoghurt. This is because, according to Christaki (2015), it is known that all Phycobiliproteins can be dissolved in water so that if they participate in the fermentation process of milk, which according to Buckle et al. (1987), the majority consists of water into yoghurt at a temperature of 42oC, phycocyanin which has been mixed at the beginning will be dissolved in milk more perfectly so the colour is thicker than if it is added after fermentation. According to Monica and Luzar (2011), the colour blue in food is rarely used because it damages the appetite. So that brighter colours will increase the level of consumer preference for yoghurt.

While based on aroma, the variable with the addition of phycocyanin before fermentation has a higher value compared to other variables. Consumers prefer variable with the phycocyanin addition before fermentation. This is because phycocyanin has a distinctive and slightly rancid aroma. If the addition is done after fermentation, the aroma of phycocyanin will dominate because it has not been too affected by bacterial activity. Whereas if the addition of phycocyanin before fermentation, the properties of phycocyanin will be neutralized so that the variable will be closer to the typical aroma of yoghurt. From the results, a value of p> 0.05. Thus, the addition of phycocyanin before and after fermentation does not provide a significant difference in the aroma of yoghurt.

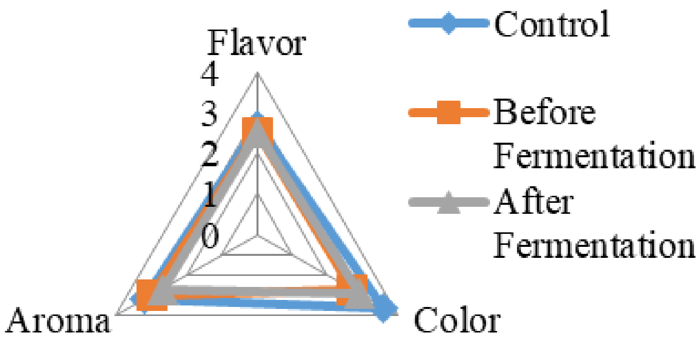

Figure 7. Time for Phycocyanin Addition VS Consumer's Response to Flavor, Color and Aroma of Yoghurt

4. Conclusion

Phycocyanin addition in the process of making yoghurt can increase the nutrient content of yoghurt. Increasing the concentration of phycocyanin addition will increase the antioxidant activity and protein content in yoghurt. The longer storage time causes the content of antioxidants and protein in yoghurt to decrease. Besides that, the addition of phycocyanin before fermentation as a whole gives a better yoghurt than the addition of phycocyanin after fermentation. In contrast, the organoleptic test gives different results for each variable.

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