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6 The Application of Red Pigments from *Streptomyces* K-4B and Dayak Onions (*Eleutherine palmifolia* (L.) Merr.) In Colouring Glycerine Soap

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Abstract. Glycerin soap has been colored with red pigment from *Streptomyces* K-4 B and Dayak onion (*Eleutherine palmifolia* (L.) Merr). Both red pigments from *Streptomyces* K-4B and Dayak onion were extracted with ethanol by maceration method, followed with soxhlet extraction. The concentration of red pigment added was varied (0, 200, 300, 400 μ L) to evaluate the best product. The resulted glycerine soaps were characterized and analyzed based on SNI 06-3532-1994. The research results indicated that the glycerine soap has water content ranged from 0.36% to 12.56%; the amount of fatty acid ranged from 14% to 36.75%; the amount of free fatty acids ranged from 0% to 0.37%; the non-saponifiable fat ranged from 0.001 to 0.019%; the pH ranged from 10.33 to 11.06; the foam stability ranged from 0.61% to 89.09%. The results of analysis of variance showed that the effect between treatments significantly different at 95% confidence level ($\alpha = 0.05$) on the characteristics of glycerine soap. The results of an organoleptic test with parameters observed were color, aroma, texture, foam, rough impression upon usage and rough impression after usage, gave "like to very like soap" with a maximum score of 4.67 (1 to 5 scale). Based on the color assessment, the organoleptic panelists preferred the glycerine soap of SK-4B3 (red pigment from *Streptomyces* K-4B, 200 μ L) with the score of 4.30 (like to very like).

Keywords: Streptomyces, Dayak onion, glycerine soap, pigment,

1. Introduction

Glycerine soap is a translucent soap which has shiny appearance when compared with other regular soaps. Colouring glycerine soap becomes a necessity to increase consumer's interest for the product. The color itself can be originated from synthetic or natural dyes. Natural dyes are natural colors or pigments obtained naturally from microbes, plants, animals, or mineral sources. Asnani and Ryandini [1] have isolated 26 marine actinomycetes from the mangrove area in Segara Anakan Cilacap, Central Java. *Streptomyces* K-4B, as one of the isolated indigenous marine actinomycetes, appears to express red pigment produced by its mycelium. Furthermore, the ethanol extract of mycelium from *Streptomyces* K-4B recently has been reported to have antibacterial activity [2]. Interestingly, the ethanol extracts from Dayak onions (*Eleutherine palmifolia* (L.) Merr.), which is also an indigenous plant from Kalimantan, also contain beautiful red pigment. Harlita [3] has reported the various



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antibacterial activities² from hexane, ethyl acetate, and ethanol extracts of Dayak onions. Based on those findings, both red pigments from indigenous *Streptomyces* K-4B and Dayak onions can be considered as potential natural pigments with antibacterial activities.

Due to the fact that glycerine² soap needs colouring to attract consumers, thus the aims of the research were (1) to apply both red pigments from *Streptomyces* K-4B and Dayak⁵ onions to color glycerine soap, (2) to characterize the quality of glycerine soap colored with red pigments from *Streptomyces* K-4B and Dayak onions based on SNI² 6-3532-1994; and (3) to evaluate the consumers preference towards the color of glycerine soap using red pigments from *Streptomyces* K-4B and Dayak onions. Characterizations of glycerine product were water content, a number of fatty acids, a number of free fatty acids, the nonsaponifiable fat, the pH, and the foam stability. The consumer preference was evaluated using organoleptic tests based on color, aroma, texture, and the impression of the glycerine soap before and after used application.

2. Materials and Methods²

The main materials used in this study were *Streptomyces* K-4B, Dayak onions (*Eleutherine palmifolia* (L.) Merr.), and coconut oil (Barco). The cultivation of *Streptomyces* K-4B used Starch-casein nitrate media. The extraction of red pigments used 96% ethanol. The chemicals used for making glycerine soap were NaOH, white sugar (*Gulaku*), stearate acid, 70% ethanol, and glycerine. The chemicals used for soap characterizations were 20% H₂SO₄ (v/v), n-hexane, anhydrous sodium sulfate, KOH, 0.5N HCl, methyl red, and phenolphthalein indicators.

The research was conducted using Factorial Completely Randomized Design. Factors to be tested were two type of red pigments (W_1 = red pigment from *Streptomyces* K-4B; W_2 = red pigment from Dayak onions), and three concentrations of pigments (K_1 = 400 mL; K_2 = 300 mL; K_3 = 200 mL). The control group was treated without any addition of pigment. Each treatment was repeated three times, so there were 18 experimental units. The independent variables were types and concentrations of pigments. The dependent variable was the glycerine soaps produced using the pigments. The parameters measured were (1) water content, (2) a number of fatty acids, (3) a number of free fatty acids, (4) the nonsaponifiable fat, (5) pH value, (6) the stability of the foam, and (7) organoleptic preferences.

2.1. Pigment production from *Streptomyces* K-4B

The pigment production from *Streptomyces* K-4B was carried out following the procedure reported by Asnani, *et.al.* [4]. The pure culture of *Streptomyces* K-4B was cultivated in Starch-casein nitrate (SCN) medium for eight days which then served as an inoculum. As many as 10% of inoculum was subsequently cultivated in 100 mL of SCN, then incubated for 21 to 28 days. After incubation has completed, the pH was lowered to pH 2 with 2 N HCl, and the mycelium was separated using cold-centrifuge. The mycelium obtained was soxlet extracted with 96% ethanol, and then the red filtrate obtained was evaporated to yield concentrated red pigment.

2.2. Extraction of red pigment from Dayak onions

Extraction of red pigment from Dayak onions was performed using known maceration procedure⁴ [3]. A total of 100 grams of powder Dayak onions was macerated in 1.000 mL of 96% ethanol (1:10 w/v) for 3 x 24 hours with occasional stirring. After three days, the mixture was filtered, and the red filtrate obtained was evaporated to give concentrated red pigment from Dayak onions.

2.3. The making of glycerine soap

Production of glycerine soap was based on modified transparent soap formula from Tokosh and Mercer [5]. 60 grams of coconut oil was mixed with 20 grams of stearic acid then heated at a temperature of 70 °C until stearic acid completely dissolved. Saponification reaction was carried out by adding NaOH solution (13 g NaOH/ 27 g distilled water) into the solution of the oil-stearate acid. The mixture was stirred until saponification has completed as indicated by soap-trace formation.

After completion of saponification, a solution of sucrose (30 g sucrose/30 mL distilled water) was added with continuing heating until the mixture became homogeneous. Translucent appearance was obtained by adding around 6 grams of glycerin and 20 grams of ethanol with slow stirring. After that, the two types of red pigment with various concentrations were added to research treatments. The soap mixture was then poured into soap molds, covered with linen fabrics, and let them dried at room temperature.

2.4. Characterization of glycerine soap

The glycerine soaps produced were characterized using standard methods [6]. Water content was determined by weighing approximately 4 grams of soap (W) and placed in a weighed cup. The soap sample along with the cup (W₁) was heated in an oven with temperature of 100-105 °C until the weight remained constant (W₂). The calculation of water content was determined by equation (1).

$$\text{Water content} = \frac{(W_1 - W_2)}{W} \times 100\% \quad (1)$$

A number of fatty acids were determined by extraction method [6]. 10 grams of soap sample was diluted with 50 mL of distilled water, added three drops of methyl red indicator. 20% of H₂SO₄ solution was added until all fatty acids freed from sodium. The solution was then extracted with n-hexane. The extraction process was repeated until the solvent amounts reached approximately 100 mL. The n-hexane filtrates were combined, dried over anhydrous sodium sulfate, then filtered to remove anhydrous sodium sulfate. The filtrate obtained was evaporated in a weighed round bottom flask (W₁) at a temperature around 102-105 °C until the weigh remained constant (W₂). Fatty acid content was calculated by equation (2).

$$\text{Fatty acids content} = \frac{(W_2 - W_1)}{\text{Sample weigh}} \times 100\% \quad (2)$$

A number of free fatty acids were measured by titration method [6]. 30 mL of ethanol with 0.5 mL phenolphthalein indicator was boiled at a temperature of 70 °C, then neutralize with 0.1 N alcoholic KOH solution. After that, around 5 grams of soap sample (W) was added, then the mixture was refluxed for 30 minutes. After the mixture was no longer alkaline, the solution was titrated with 0.1 N alcoholic KOH solution (N) until the appearance of red color remained for at least 15 seconds (V in mL). Free fatty acid content was determined by equation (3). Multiplication by 0,205 is equivalent with the weight of lauric acid.

$$\text{Free fatty acids content (\%)} = \frac{V \times N \times 0,205}{W} \times 100\% \quad (3)$$

The nonsaponifiable fat was measured by calculating the remaining solution from free fatty acids determination above. The remaining solution from the above procedure was added 5 mL of 0.5 N alcoholic KOH, then refluxed for 1 hour. After refluxing process, the solution was titrated with 0.5 N alcoholic HCl (N) until red color appeared (V₂). Titration was also carried out for blank with 0.5 N alcoholic KOH (V₁). The saponified fats can be calculated using equation (4). The value of 0.0561 is equivalent to the weight of KOH; 0.1981 is the saponification value of average oil.

$$\text{Nonsaponifiable fat content} = \frac{(V_2 - V_1) \times N \times 0,0561}{0,1981 \times W} \times 100\% \quad (4)$$

The pH value was measured by dissolving 1 gram of soap into 10 mL of distilled water, then measured the pH of the soap solution [7].

The stability of foam was measured by dissolving 1 g of soap into 10 mL of distilled water, shook vigorously for 1 minute, then measured the foam height. The soap solution was allowed to stand for 60 minutes, and then the foam height was measured again [8]. The foam stability was calculated following the equation (5).

$$\text{Foam stability (\%)} = \frac{\text{foam height after 60 min}}{\text{initial foam height}} \times 100\% \quad (5)$$

2.5. Organoleptic test of glycerine soap

An organoleptic test was conducted using 15 panelists who were randomly selected based on the difference between the sexes (male and female). The soap samples were blindly given to all panelists to know their preference based on color, aroma, texture and the impression of the glycerine soap before and after used application.

2.6. Data analysis

Resulted data from soap characterization were analyzed using two-ways ANOVA (Analysis of Variance) with a 95% confidence level ($\alpha = 0.05$). The resulted analysis which showed significant diversity was further analyzed using DMRT (Duncan's Multiple Range Test) with a 95% confidence level ($\alpha = 0.05$). The organoleptic tests were analyzed using data scoring from 1 to 5.

3. Results and Discussion

3.1. Pigment from *Streptomyces* K-4B

Actinomycetes are microorganisms that originally have the antibiotic potential. Excitingly, some of the actinobacteria express both extracellular and intracellular pigments with various colors. It was observed that *Streptomyces* K-4B has antibacterial activity and also produced red mycelium. Thus, in this study, the ability of *Streptomyces* K-4B (figure 1) to produce natural red pigment was thoroughly explored.

Streptomyces K-4B was cultivated in starch-casein nitrate agar supplemented with nystatin. The fermentation process for pigment production and ethanol extraction of pigment were done following the similar procedure reported by Asnani *et.al.* [4]. For mass extraction of mycelium, the soxhlet extraction method using ethanol 96% was applied. The ethanol extract was then evaporated to give concentrated red pigment (52.37%).

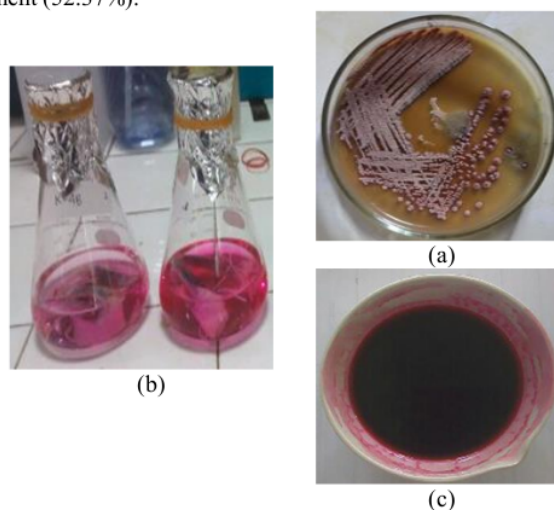


Figure 1. *Streptomyces* K-4B: (a) the colony, (b) ethanol extract, and (c) concentrated red pigment

3.2. Pigment from Dayak onions

Bawang Dayak (*Eleutherine palmifolia* (L.) Merr) is one of the indigenous plants from Kalimantan [9]. In Indonesia, this plant is also known as the Mecca onions, garlic ghost, and Sabrang Arab garlic.

Local people in the area have used Dayak onions as traditional medicine. The part of Dayak onions that can be utilized is its bulb (figure 2a). Specific features of this plant are red bulb with a very smooth surface, leaves paired with double-finned leaf composition, and small white flowers. An extraction of red pigment from Dayak onions is easily done by maceration method using ethanol 96% as extraction solvent. The ethanol extract is then evaporated to obtained concentrated extract of red pigment which was readily used as the red pigment for colouring glycerine soap.

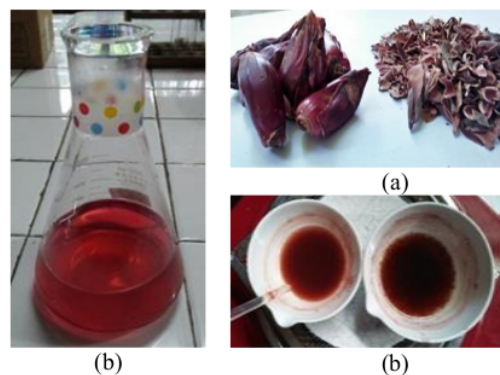


Figure 2. Dayak onions (*Eleutherine palmifolia* (L.) Merr), (a) the dried bulb, (b) the ethanol extract, and (c) the concentrated ethanol extracts.

3.3. Glycerine soap products

According to the Indonesian National Standard [6], bath soap is a compound of sodium or potassium fatty acids from vegetable oil or animal lipid solid, soft or liquid form, foaming used as a cleanser, and with the addition of fragrances or other non-harmful ingredients. The standard quality requirement of soap is in accordance with SNI 06-3532-1994. The term glycerine soap is based on the translucent apparent of the soap due to the addition of glycerine to the formula. The process of making glycerine soap is begun with heating the oil at a temperature of 70 °C and then added NaOH solution slowly. The mixture is stirred thoroughly until saponification process completed. The glycerine and alcohol are then added until the soap becomes transparent.

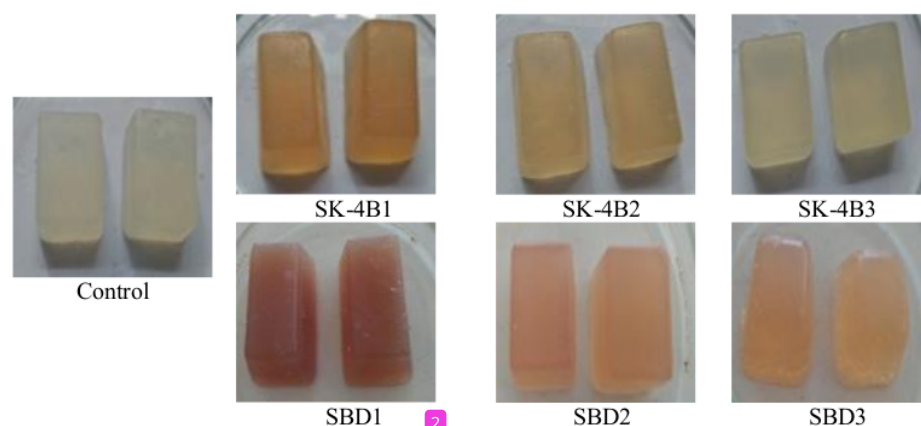


Figure 3. Glycerine soap products with red pigments from *Streptomyces* K-4B and Dayak onions

Color of glycerine soaps will depend on the choice of starting oil material. If the quality of oil used is poor, then it is likely to produce yellow soap [10]. In this research, the glycerine soaps were colored with various concentrations of natural red pigments from *Streptomyces* K-4B and Dayak onions. Control soap has a solid white to transparent, whereas soaps with the addition of pigments from *Streptomyces* K-4B and Dayak onions has increasingly reddish color with the increasing concentrations of red pigments (figure 3).

The coding used for glycerine soap products were as follow:

SK-4B1 = soap with 400 μ L of pigment from *Streptomyces* K-4B

SK-4B2 = soap with 300 μ L of pigment from *Streptomyces* K-4B

SK-4B3 = soap with 200 μ L of pigment from *Streptomyces* K-4B

SBD1 = soap with 400 μ L of pigment from Dayak onions

SBD2 = soap with 300 μ L of pigment from Dayak onions

SBD3 = soap with 200 μ L of pigment from Dayak onions

The characteristics evaluation of glycerine soaps is presented in Table 1. The water content indicates the amount of water contained in the product which will greatly affect the quality of the shelf life of the soap. Soap with high levels of water will have a lower shelf life because it gives an opportunity for the growth of microorganisms. In this research, the water content of glycerine soap products ranged from 0.36% to 12.56%. Those results indicated that all glycerine soaps products had met the requirement for water content when compared to SNI 06-3532-1994 (<15%). The lowest water content was SBD3 (0.36%) which contained 200 μ L of pigment from Dayak onions. The highest water content was SK-4B2 (12.56%) which contained 200 μ L of pigment from *Streptomyces* K-4B. The results from analysis of variance showed that the addition of pigment gave significant affected on water content at 95% confidence level ($\alpha = 0.05$). Further Duncan test showed that glycerine soaps SK-4B1, SK-4B2, SBD1, SBD2, and SBD3 were significantly different from that of SK-4B3.

A number of fatty acid is the total fatty acids contained in the soap. Both fatty acids occur as saponifiable or nonsaponifiable fats. Naturally, fatty acids are obtained by hydrolysis of the triglycerides [10]. The principle of measurement of a number of fatty acids is hydrolysing the bond between the fatty acids with Natrium (Na) on the soap using strong acids.

In this study, the average amount of fatty acids ranged from 14% to 38.5%. The lowest amount of fatty acid was SBD3 (14%), whereas the highest amount of fatty acids was SK-4B2 (38.5%) which was glycerine soap with 300 μ L pigment from *Streptomyces* K-4B. However, those results have not met the standard requirement of SNI 06-3532-1994 (>70%), yet. The decreasing amount of fatty acid was probably due to the increasing amount of water used. The amount of fatty acid also has a tendency to decrease with the increasing stirring time and the ratio of water [11]. In addition, the amount of fatty acid in the soap product highly depended on the type of oils or fats used for soap production.

Table 1. The average characteristics value of glycerine soaps

Glycerine Soap	Water content (%)	Fatty Acids (%)	Free Fatty Acids (%)	Nonsaponifiable lipid (%)	pH	Foam Stability
Control	0.36	29.40	0.00	0.001	11.06	79.16
SK-4B1	0.59	35.00	0.37	0.015	10.02	85.87
SK-4B2	12.56	38.50	0.30	0.010	10.32	89.09
SK-4B3	0.87	32.25	0.16	0.010	10.35	82.16
SBD1	0.45	36.75	0.00	0.002	10.72	0.61
SBD2	0.36	17.50	0.14	0.002	10.74	0.70
SBD3	0.38	14.00	0.00	0.018	10.33	0.78
SNI	<15%	>64%	<2.5%	<2.5%	-	-

In glycerine soaps, there are possibilities that these soaps contain free fatty acids or free-alkaline. Free fatty acids are fatty acid contained in the soap but are not bound as soap or triglycerides. High contain of free fatty acids in soap will reduce the cleaning power of the soap against oil or grease. Alkaline-free is the amount of alkaline in the soap which is not bound to a compound. Excess alkaline in the soap can cause irritation to the skin. Analysis of free fatty acid aims to determine the amount of free fatty acid or free-alkaline contained in soaps.

The analysis of free fatty acid showed that glycerine soap with the addition of pigments from *Streptomyces* K-4B and Dayak onions contained free fatty acids ranged from 0% to 0.37%. These findings indicated that all soap products has free fatty acid less than 2.5%, which met the standard requirement based on SNI 06-3532-1994. The results from analysis of variance showed that the addition of pigment significantly affected the value of free fatty acids at 95% confidence level ($\alpha = 0.05$). Further Duncan test showed that the type and concentration of pigment from *Streptomyces* K-4B (SK-4B1 and SK-4B2) were significantly different from SK-4B3 and SBD2.

Nonsaponifiable lipids are triglycerides or other fats in the soap that cannot be saponified by alkaline lye. The nonsaponifiable lipid will reduce the ability of soap to clean the dirt because the soap will tend to bind these nonsaponifiable lipids of the soap itself. The analysis gave the value for nonsaponifiable lipid ranged from 0.010% to 0.018%. The highest content of nonsaponifiable lipid was obtained from SBD3 (0.018%), whereas the lowest was 0.010% from both SK-4B2 and SK-4B3. All soap products has met the standard requirement for nonsaponifiable lipid (<2.5%) according to SNI 06-3532-1994. Based on the analysis of variance, the addition of the pigment concentration significantly affected the value of nonsaponifiable lipid at 95% confidence level ($\alpha = 0.05$). Further Duncan test showed that SK-4B1, SK-4B2, SK-4B3, SBD2, and SBD3 were significantly different towards each other.

The pH value of the soap can be influenced by the content of free alkaline and other raw materials. The pH value of soap, whether it is too low or too high, can cause skin irritation. Normal skin has a pH of about 5 and it will increase if the skin is washed with soap solution. However, the increment in the pH value on the skin will not exceed to pH 7 [12]. In this research, the soap obtained has pH ranged from 9.02 to 10.74. The highest pH value of 10.74 was SBD2, and the lowest value were SK-4B1. Based on the results of analysis of variance, the addition of pigments significantly affected the pH on the 95% confidence level ($\alpha = 0.05$). Further Duncan test showed that SBD3 was significantly different with SK-4B1 and SBD3, as well as with SBD1 and SBD2.

Foam is the dispersed gas in the fluid with the relatively stable structure consisting of air pockets encased in a thin layer [12]. Foam stability test is conducted by measuring the decrease in volume of foam after the soap solution is shaken and allowed to stand for 1 hour. Based on the measurement, the foam stability ranged from 0.61% to 89.09%. The highest value of the foam stability was SK-4B2 (89.09%), and the lowest value was SBD1 (0.61%). Based on the analysis of variance, the type of pigments significantly affect the foam stability on the 95% confidence level ($\alpha = 0.05$). Further Duncan test showed that the increasing concentration of pigment has no effect, but the type of pigments (*Streptomyces* or Dayak onions) significantly affected the foam stability.

The tendency of the value of foam stability decreased with the increasing concentration of added pigments. The foaming capability is correlated with surface tension. The lower of the surface tension of soap solution, the greater the resulting foam. Low surface tension might occur because of the attraction of surface molecules are weak, and it will easily disturb the surface of the coating solution, hence increasing the ability for foam formation [7]. The addition of pigment eventually decreases the foam stability. This happens because of the presence of dissolved substances in the liquid will increase viscosity which in turn will increase surface tension and result in reduced foaming ability.

3.4. Consumers preference

Consumer preference towards a certain product begins with the assessment of the appearance, flavor, and texture of the product itself. In the end, the target is consumer acceptance to particular product. Consumer preference can be evaluated using an organoleptic test that uses human senses to measure

the color, aroma, texture, appearance, and impression of soap products. To do so, the organoleptic test employs panelists who are asked to evaluate the characteristics of the product by the level of assessments that are very disliked (1), disliked (2), average (3), like (4), and very like (5).

In this research, the organoleptic test was a test for favorite or hedonic. The glycerine soap products to be evaluated were SK-4B1, SK-4B2, SK-4B3, SBD1, SBD2, SBD3, and control soap (without addition any pigment). The hedonic test was conducted to determine the level of consumer acceptance of products based on the characteristics of the standard soap (SNI 06-3532-1994). The test involved 15 panelists (eight women and seven men), who evaluated the soap characteristics which were color, aroma, texture, foam, and usage impression. The research results were presented in Table 2.

Color assessment was done visually. Increasing concentration of pigment gave significant effect on colour soap produced. Based on the color, panelists preferred SK-4B3 soap with an average value of 4.3 (like to very like). SK-4B3 soap was glycerine soap with the addition of 200 μ L pigment from *Streptomyces* K-4B.

Aroma assessment was done by smelling the scent of soap products. Since the soap was originally made from coconut oil, the distinctive scent from coconut oil also added to the soap aroma. Apparently, an addition of pigment from Dayak onions could overcome the odor from coconut oil, whereas the pigment from *Streptomyces*-4B contributed no aroma at all. Hence, the panelists favored SK-4B3 and SBD1 products with the average value of 3.87 (average to like). SK-4B3 was glycerine soap with addition of 200 μ L pigment from *Streptomyces*-4B, whereas SBD1 was added 400 μ L of pigment from Dayak onions.

Texture assessment was done by touching and feeling the texture of the products. Evaluation results showed that panelists preferred SBD1 soap, which was glycerine soap with the addition of 400 μ L of pigment from Dayak onions. The texture average value was 4.67 (like to very like).

Foam assessment was done by washing hands with the soap products. The amount of foam produced during the washing was observed by the panelists. The average ratings from panelists for resulted foam designated the SK-4B3 as the most favored soap with the value of 3.67 (average to like). SK-4B3 was glycerine soap with the addition of 200 μ L pigment from *Streptomyces* K-4B.

Assessment of the utilization of glycerine soap was done by washing hands with soap products. The impression on the skin was observed during and after applying the products. The results indicated that panelists preferred SBD1 soap for impression during washing, but preferred SBD3 soap for impression after washing. SBD was the soap with the addition of pigment from Dayak onions. The application of glycerine soap SBD1 gave the average value of 3.97, whereas the application of SBD3 gave that of 4.37.

Table 2. The results from hedonic tests

glycerine soap	color average score	aroma average score	texture average score	foam average score	on usage average score	after usage average score
Kontrol	3.80	3.0	3.73	3.60	3.60	3.67
SK-4B1	3.53	3.53	4.06	3.60	3.80	3.87
SK-4B2	3.87	3.40	3.93	3.40	3.87	4.13
SK-4B3	4.30	3.87	4.06	3.67	3.73	4.13
SBD1	3.67	3.87	4.67	3.40	3.97	4.00
SBD2	3.86	3.64	3.86	3.36	3.50	4.00
SBD3	3.73	3.40	3.97	3.40	3.93	4.37

2 Conclusions

Red pigments from *Streptomyces* K-4B and Dayak onions can be used to color glycine soap. The soap products mostly met the standard requirement based on SNI 06-3532-1994. The water content ranged from 0.36% to 12.56% (SNI > 15%), the number of acid fatty acids ranged from 14.00% to 36.75% (SNI > 70%), the number of free fatty acid ranged from 0% to 0.37% (SNI < 2.5%), the amount of

nonsaponifiable fat ranged from 0.001% to 0.019% (SNI <2.5%), pH value ranged from 10.02 to 11.06, and the foam stability ranged from 0.61% to 89.09%. The organoleptic tests indicated that the most preferred color was SK-4B3, aromas were both SK-4B3 and SBD1, the texture was SK-4B3, the foam was SK-4B3, impression on usage was SBD1, and impression after usage was SBD3. Above all, the most preferred glycerine soap was SK-4B3 (addition of 400 μ L pigment from *Streptomyces* K-4B) and SBD3 (addition of 100 μ L pigment from Dayak onions).

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