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Production of Natural Colorant by *Monascus Purpureus* FNCC 6008 using Rice and Cassava as Carbon Substrates

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Abstract

Consumer recognition of the adverse effects of synthetic colorant has increased awareness in utilizing natural colorants as an alternative. One of them is a microbial colorant and it is already studied for its safety and functional properties in the human body, i.e. hypercholesterolemia, antimicrobial, antioxidant, and anticancer. The functional properties of microbial colorant have driven many kinds of research about natural colorant produced by the microorganism. Monascus pigment is one of the popular red pigment synthesized by mold *Monascus purpureus*. This research was conducted to investigate the effects of particle size and incubation temperature on color value and water solubility of the natural colorant produced by *M. purpureus* FNCC 6008 using rice and cassava as carbon substrates. Every experiment was conducted in an erlenmeyer flask filled by 15 g of substrates, sterilized and incubated in an incubator chamber. Three particle sizes of 8, 10, and 16 mesh were employed on the incubation temperatures of 30, 32, and 34 °C for 14 days. Two parameters were measured from the sample to evaluate the results of the fermentation process, i.e. color intensity and water solubility of product. The highest color intensity of 59.6 CVU/gds was obtained from the rice substrate at the particle size of 10 mesh and incubation temperature of 32 °C. That particular fermentation condition resulted in a product with 71.4% product solubility.

INTRODUCTION

The growth of the food and beverage industries is increasing the demand of colorants for improving product appeal to consumers. For this purpose, the use of synthetic colorants has been widely applied. However, in the last few decades, it grows a new public awareness on usage of natural colorant as a substitute for synthetic colorants. Apart from being useful for food coloring, these natural colorants have several functional benefits, they are antimicrobial, antioxidant, and anticancer (Parmar & Singh, 2018; Tuli et al., 2015). Thus, this colorants are natural, safe, and multifunctional. This condition has encouraged research about the synthesis of natural pigments by microorganisms. The research included pigments synthesized by actinomycetes and fungi *Monascus* sp. (Agboyibor et al., 2018; Huang et al., 2017; Parmar & Singh, 2018; Suraiya et al., 2018; Hilares et al., 2018).

Monascus pigment, or better known as angkak, is a popular colorant for a food coloring because it has long been used by the community. Angkak is a red pigment synthesized by *Monascus* sp. and it has a consistent color and safe. Angkak contains lovastatin compounds having characteristics of inhibiting cholesterol synthesis in the body and also several other functions, namely antioxidants and antimicrobials (Agboyibor et al., 2018; Parmar & Singh, 2018; Tuli et al., 2015; Zubaidah & Sriherfyna, 2015). The secondary metabolite of *monacolin K.* is produced by the mold *Monascus* sp. and it can inhibit cholesterol synthesis

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so that it can be used as a functional food to lower cholesterol (Priatni et al., 2014).

Various grains have been studied as substrates for the production of Angkak, several of them are rice, corn, barley, and wheat. The studies show that *Monascus purpureus* produces the best angkak using the millet substrate (Huang et al., 2017; Heer, 2017). Hydrolyzaate of sugarcane bagasse can also be used as a substrate for angkak production using *Monascus ruber* with variable irradiation using a light-emitting diode (LED). The research shows that the best results are when the fermentation takes place in the dark, namely 18.71 UA490 nm for 288 hours of fermentation (Hilares et al., 2018).

There are two fermentation methods for the production of monascus pigments, i.e. solid-state fermentation (SSF) and submerged fermentation (SmF). Research shows that the SSF method has more benefits than SmF. Monascus cells grow faster in SSF than SmF. SSF method is more suitable for the production of food colorants due to its simple finishing process, i.e. it can be treated by direct drying without separation and purification processes (Zhang et al., 2015). Pigment production in an industrial scale has been carried out using submerged fermentation (SmF) method (Babitha et al., 2006). However, the SSF method is more prospective because it provides a more adequate habitat for molds resulting in a high pigment product.

Some factors affecting the growth and pigment production of microorganism are temperature, pH, carbon source, nitrogen source, and method of fermentation (Joshi et al., 2003). Velmurugan et al. (2011) obtained an observation that *Monascus* sp. was in maximum growth and pigment production at the temperature of 30 ^oC, while at the substrate pH of 5 and 6 resulted in maximum red and yellow pigment respectively. Other factor of the particle size, for SSF method, is also important for the growth and pigment production of *Monascus* sp. (Babitha et al., 2006).

This research was intended to study the fermentation process of angkak using the SSF method. The variables studied included the type of substrate, particle size, and incubation temperature. Cassava and rice substrates were investigated and compared. Cassava is an inexpensive substrate that has not been studied yet, while rice is a substrate that is commonly used for Angkak production. Another novelty in this research is the solubility test of the product in water. This solubility test is to see the opportunities of angkak product as a natural food colorant in terms of color homogeneity during the applications.

MATERIALS AND METHODS

Mircroorganism and Medium

Monascus purpureus FNCC 6008 was obtained from Food and Nutrition Culture Collection, Universitas Gadjah Mada, Yogyakarta. Carbon substrates consisting of rice and cassava was obtained from Pasar Gede and Pasar Legi, Surakarta. Nutrient salt solution was prepared from pro analysis quality materials from Merck (Damstadt, Germany) with the composition: KH_2PO_4 2 g / L, NH_4NO_3 5 g / L, NaCl 1 g / L, and $MgSO_4$.7H2O 1 g / L. Potato dextrose agar (PDA) was obtained from Sigma-Aldrich (St. Louis, MO, United States).

Stock Culture

M. purpureus FNCC 6008 was grown on agar slant using PDA medium for stock culture utilization. It was incubated at 30 ^oC for 7 days and then stored in a refrigerator (at 4 ^oC). This stock culture was regenerated regularly before the agar slant run out.

Inoculum Development

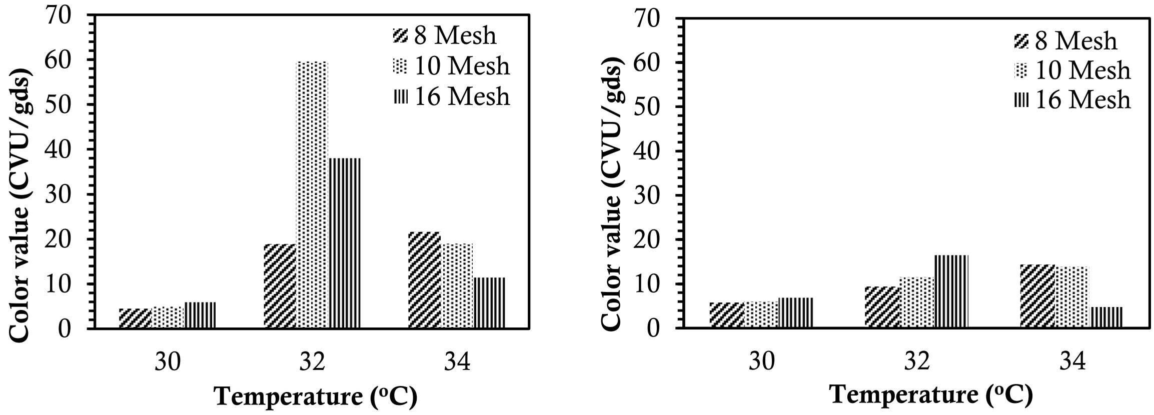
Inoculum development is carried out with reference to previous work (Babitha et al., 2006). Spore suspension was obtained from 5 mL of sterile distilled water poured into the agar slant stock culture. One milliliter of the spore suspension was diluted to 10 mL with sterile distilled water and ready for fermentation inoculum.

Preparation of Rice and Cassava Substrates

The rice was washed, steamed to cook and dried in an oven at 70 ^oC for 24 hours. Dry rice was crushed and sieved into 3 sizes, namely 8 mesh (equivalent to 0.24 cm), 10 mesh (equivalent to 0.2 cm), 16 mesh (equivalent to 0.14 cm). The same way is done with cassava, but the cassava was sliced thinly before cooking.

Solid State Fermentation Methods

Fermentation was carried out using 15 g of carbon substrate placed in an Erlenmeyer flask of 250 mL. Five milliliters of nutrient salt solution and



(a) (b)

Figure 1. The effects of particle size and incubation temperature on color value for 14th days of incubation ((a) rice substrate , (b) cassava substrate).

10 mL of sterile distilled water were added to the substrate and homogenizely blended . The substrate was inoculated with 3 mL of inoculum and incubated in an incubator at 30, 32, or 34 °C for 14 days.

Analysis of Color Intensity and Solubility of Product

The Color intensity is expressed in color values, namely the color value unit (CVU) per gram of dry substrate (gds). It was determined by extracting 1 g of the sample with 5 mL of 96% methanol. The sample was homogenized using a rotary shaker at 124 rpm for 1 hour and let stand for 15 minutes. The color solution was separated from the residue by filtering it using filter paper and diluted. The adsorbance (ads) of the filtrate was measured using a spectrophotometer at 500 nm with a blank solution of methanol 96%. The color value (CVU / gds) is calculated using the Eq. (1).

$$Color\ value = \frac{ads\ x\ degree\ of\ dilution\ x\ volume\ of\ exstract}{grams\ of\ sample} \quad (1)$$

The solubility test is carried out by dissolving every 0.5 g of the product sample in 100 mL of water. The sample was stirred until homogeneous then let stand for 10 minutes. The sample is filtered and the solid obtained is dried. The dissolved solids were calculated by substracting 0.5 g sample by weight of dry residue.

The influential of experimental variables on the color value and product solubility was statistically analized using analysis of variance (ANOVA) method with a significance level (α) of 0.05, while the pattern of variable relationships was shown visually in the form of a wireframe graphic.

RESULTS AND DISCUSSION

The fungus of *M. purpureus* FNCC 6008 grows well on carbohydrate (starch) substrates, especially the well known substrate of rice. The alternative substrates are other agricultural products, which are carbon source nutrients in the form of carbohydrates, including cassava. Furthermore, environmental conditions which greatly affect its growth in solid state are particle size and incubation temperature. Their effects are investigated on the color value (color intensity) of monascus pigment and product solubility. The number of color values for 14 days incubation are shown in Figure 1.

The best incubation temperature for *Monascus* sp. is between 30-35 °C (Asben & Permata, 2018). The recent study, it observed more detail temperatures of 30, 32, and 34 °C. The highest color value was 59.6 CVU/gds and it was obtained using a 10 mesh particle size of rice substrate and incubated at 32 °C. The achievement of this color value was higher than the result of 1.1 CVU/gds obtained using a corncob substrate with size of 18 mesh and incubated at 30 °C (Ardini et al., 2014)). The color value of monascus pigment on cassava substrate is lower than that of rice substrate. The highest color value on the cassava substrate is 16.5 CVU / gds and this was achieved at an incubation temperature of 32 °C and particle size of 16 mesh. The results show that although the cassava substrate can be used for monascus pigment production, the red color intensity in cassava cannot replace the red color intensity in rice yet.

Table 1. The effects of carbon source, particle size, and incubation temperature on color intensity of monascus pigment for 14th days incubation (n=4).

Treatment		Particle Size (mesh)					
		8		10		16	
		Rice	Cassava	Rice	Cassava	Rice	Cassava
Incubation Temperature (°C)	30	4.5	5.5	4.5	7	6.5	7.5
		5	6	5	7	6	6.5
		4	5	4.5	4.5	5.5	7
		4.5	6.5	5.5	5.5	5.5	6.5
	32	18.5	8.5	56.5	11	38	14.5
		15.5	9.5	60.5	12.5	33.5	17
		20	9	55	10.5	39.5	19
		21.5	10.5	66.5	12	41	15.5
	34	21.5	15	15.5	12	14	4
		25.5	12.5	21	14.5	10.5	5.5
		20.5	15.5	19	15	11.5	5
		19	14.5	20.5	14	9.5	4.5

Table 2. Table of ANOVA for the effects of carbon source, particle size, and incubation temperature on color value of monascus pigment. (n= 4)

Variation	df	SS	MS	$F_{calculation}$	F_{table} (α=0.05)
Mean	1	16531.7	16531.7		
Treatment					
A	2	600.26	300.13	73.878	3.174
B	1	1995.01	1995.01	491.080	4.024
C	2	4835.01	2417.50	595.078	3.174
AB	2	467.01	233.50	57.478	3.174
AC	4	1732.56	433.14	106.619	2.554
BC	2	2427.09	1213.55	298.719	3.174
ABC	4	1101.01	275.25	67.755	2.554
Error	54	219.38	4.06		
Total	72	29909	-	-	-

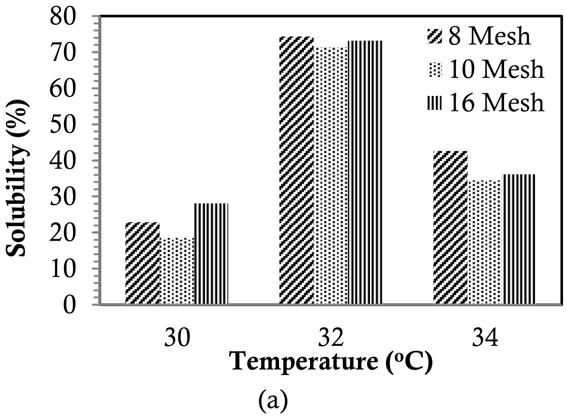
Note: A: particle size ; B: carbon source ; C: incubation temperature

However, it is in line with those achieved by previous researches using several cereal substrates (rice, maize, wheat germ, and sorghum bran) and rice is the most suitable substrate for the growth of *M. purpureus* and pigment production (Srianta et al., 2016).

Theoretically, the smaller particle sizes the better fermentation process or the higher color intensity, because it provides a greater surface area for mold penetration. On the other hand, the larger particles provide better aeration because the space between particles is increased even though the surface of the mold penetration is less (Babitha etal., 2006). However, the data show that particle size of 16 mesh produces lower color intensity than 10 mesh. This is presumably because the soft particles resulted aeration blocking in smaller particle size so that it inhibits the cell growth and pigment production.

The significancy of variable effects (substrate type, particle size, and incubation temperature) on the color intensity of monascus pigments was analysed using ANOVA method with a significance level (α) of 0.05 in experiments conducted with four replications. The data resulted in the experiments are presented in Table 1, while the results of ANOVA calculations are presented in Table 2.

The ANOVA analysis in Table 2 show the variables of carbon source, particle size and incubation temperature each of them affects the color value of monascus pigment. This influence is indicated by each value of $F_{calculation}$ which is higher than F_{table}. The results of the interaction test also show that there is an interaction between the variables studied. The interaction between these variables is shown by $F_{calculation}$ of each interaction higher than that of F_{table}. The pattern of influence of



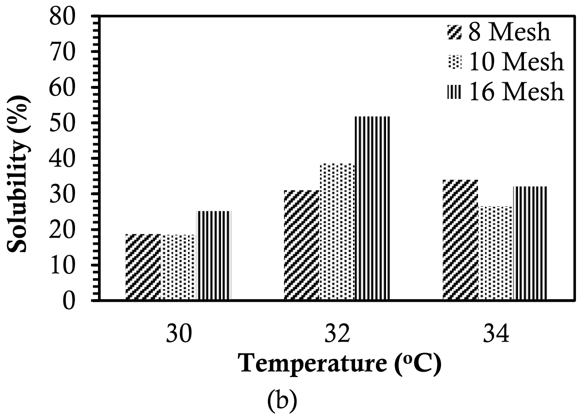


Figure 2. The effects of particle size and incubation temperature on the product solubility at the 14th days of fermentation ((a) rice , (b) cassava).

Table 3. The effects of carbon source, particle size and incubation temperature on the solubility of fermentation product at the 14th days of fermentation (n=4).

Treatment		Particle Size (mesh)					
		8		10		16	
		Rice	Cassava	Rice	Cassava	Rice	Cassava
Incubation Temperature (°C)	30	26.4	19	16.6	16.2	30.6	26.4
		19.6	20.4	20.4	20.6	26.8	29
		20.8	18	15.6	17.8	29.6	21.8
		24.6	17.4	21.4	19.4	25.4	23.4
	32	73.6	33.2	68.2	40.6	74.2	52.2
		74	30.2	82.4	31.4	76.2	54.2
		74.2	32.4	63.4	47.2	72.2	51.4
		75.4	28.2	71.4	35	70.2	49.4
	34	46.2	36.8	32.6	24.2	36.4	33.8
		43.8	31.6	35.6	27.6	41.2	29
		41.2	35	35	27.4	35.6	31.2
		39.4	32.6	34.6	26.8	31.4	34.4

particle size (mesh) and incubation temperature (°C) on the color value follows a non-linear relationship pattern as shown in Figure 3 (a).

This study also investigated the product solubility in water system. The effect of the variables on product solubility shows a similar pattern as their effect on color values. The effect of particle size and incubation temperature on product solubility is shown in Figure 2. In general, the solubility of products using cassava substrate is lower than that of rice, which is 51.8% for cassava compared to 74.3% for rice substrate. The best product solubility is produced by fermentation which is incubated at 32 °C, using either rice or cassava substrates.

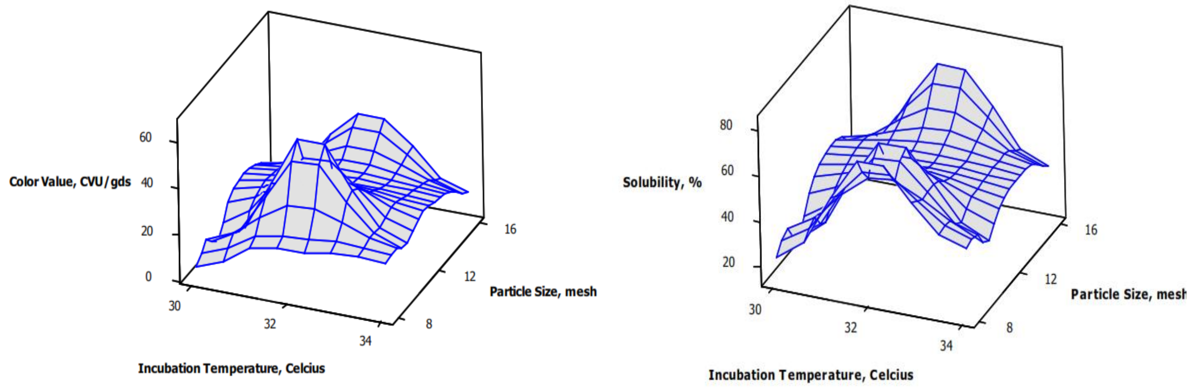
The Complete data of product solubility throughout the fermentation process are shown in Table 3. The ANOVA analysis with a significance level (α) of 0.05 was carried out on the data in Table 3 to determine whether the variables had an effect / had no effect on the product solubility. The ANOVA list calculated from the data in Table 3 is presented in Table 4.

Table 4 show the variables of carbon source, particle size and incubation temperature have a significant effect on product solubility. This influence is shown by each value of F_{count} higher than F_{table}. The ANOVA analysis also show interactions effect between variables, namely the interactions between carbon source-particle size, carbon source-incubation temperature, particle size-incubation temperature, and the interaction of the three variables. Each interaction analysis shows that F_{count} is greater than F_{table}. The pattern of

Table 4. Table of ANOVA for the effects of carbon source, particle size and incubation temperature on the product solubility (n=4)

Variation	df	SS	MS	$F_{calculation}$	F_{table} (α=0.05)
Mean	1	102107	102107		
Treatment					
A	2	503.94	251.97	21.655	3.174
B	1	3486.13	3486.13	299.605	4.024
C	2	14880.90	7440.43	639.446	3.174
AB	2	256.76	128.38	11.033	3.174
AC	4	448.89	112.22	9.645	2.554
BC	2	3169.26	1584.63	136.187	3.174
ABC	4	265.93	66.48	5.714	2.554
Error	54	628.33	11.64		
Total	72	125747	-	-	-

Note: A: particle size ; B: carbon source ; C: incubation temperature



(a) (b)

Figure 3. The profile of the effects of particle size and incubation temperature on color value and product solubility ((a) color value , (b) product solubility).

variable influences on product solubility are non-linear pattern as shown in Figure 3 (b).

Based on the described parameters of the color value and product solubility, it can be concluded that monascus pigment is potential to be developed into natural dye. It has a benefit of functional properties making it suitable for applying on medicinal and food products. The high solubility of the product, reaching an average of 71.4%, also illustrates that the production of natural dye from monascus pigment by the SSF method has a high possibility to be used for further research. On the other hand, the cassava substrate does not have characteristics as good as the rice substrate for the production of monascus pigment.

CONCLUSION

In solid state fermentation method, the particle size and incubation temperature have significant effects on color value and solubility of product. The highest color value of monascus pigment was 59.6 CVU/gds and it can be achieved by 10 mesh particle size of rice substrate incubated at 32 °C. While the product solubility was 71.4% when it produced using rice substrate at 16 mesh of particle size incubated at 32 °C. However, the cassava substrate does not become alternative substrate for rice in production of monascus pigment due to the lower of color value and solubility of product.

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