HAYATI Journal of Biosciences, June 2008, p 61-66 Vol. 15, No. 2
ISSN: 1978-3019

Optimizing Angkak Pigments and Lovastatin Production By *Monascus purpureus*

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Received June 4, 2007/Accepted May 7, 2008

Angkak pigments and lovastatin had been reported very useful as natural coloring agents, as an agent to increase thrombocyte level in Dengue hemorrhagic fever, and also as a compound that was able to control blood cholesterol level. Three strains of fungus *Monascus purpureus* AKI, AKII, and 915 were selected to produce angkak pigments and lovastatin in potato dextrose agar (PDA) medium. The best fungus strain, which is AKII, was then applied in three kinds of rice media (white rice IR-42, red rice BP-1804-IF-9, and a combination of 1:1 (w/w) white IR-42 and red rice BP-1804-IF-9 for solid fermentation. The best medium and fermentation times were determined for the production of angkak pigments and lovastatin separately. Results showed that strains, media, and duration of fermentations gave significant effect on the amount of pigment produced. Strain AKII produced highest concentration of angkak pigments. The combination of rice (White IR-42 and red rice BP-1804-IF-9) produced the highest pigment than the individual white and red rie it self. The optimum duration of fermentation was 16 days for strains AKI and AKII, but only 15 days for strain 915. Therefore the strain AKII with media combination of rice and a fermentation time of 16 days were used to investigate the additional effect of various minerals. Addition of the mineral individually gave significant increased on angkak pigment production by AKII, where as the addition of minerals mixture in the forth tube did not.

Key words: angkak, pigments, lovastatin, *monascus purpureus*

INTRODUCTION

Nowadays, people are using more synthetic coloring in foods. However, previous reports have indicated that synthetic food colorings have a higher risk to human health as they may be carcinogenic (Blanc *et al*. 1994). Since this negative information about synthetic food colorings, people have attempted to develop natural food colorings.

Angkak pigment is an example of natural colorings and has been used in Asian countries e.g. Japan, China, Thailand, Philippines, and Indonesia. This pigment is not dangerous and does not cause allergic reactions in mice (Fardiaz *et al*. 1989). Besides being a natural coloring for food and beverages, angkak pigment has also been used as food preservative due to its antibacterial properties (Wong & Koehler 1981) and it is used for increasing thrombocyte level in Dengue Hemorrhagic Fever (DHF), a viral infection with drops of blood thrombocyte level (Nurhidayat 2004).

Angkak is a fermentation product from *Monascus purpureus* (Steinkraus 1983) although the species *M. rubropunctatus, M. rubiginosus, M. anka, M. major,* and *M. bakeri* can also produce the pigment (Carels & Shepherd 1977).

Monascus purpureus naturally produces the secondary metabolite monakalin K (Lovastatin) that can inhibit cholesterol biosynthesis in patients with hypercholesterolemia. Therefore, Lovastatin has been used as the first curative for patients with a high risk of heart attack due to hypercholesterolemia.

Lovastatin is as a competitive inhibitor for 3-hydroxy-3-methyl-glutaryl Coenzyme-A reductase (HMG-CoA reductase) that is an important enzyme for cholesterol biosynthesis. When this enzyme is inhibited, cholesterol synthesize will blocked (Szakacs *et al.* 1998). As natural secondary metabolite production of microbes is limited, the microbes and the media must be modified to obtain sufficient amounts of natural secondary metabolites for preparation of commercial products.

Pigments and lovastatin production is influenced by pH, temperature, moisture, the medium components (e.g. organic nutrients), and the fungal strain. Rice strains can be used as the medium to produce angkak and the researches have shown that rice gives better results than sticky rice. According to Santoso and Satiawiharja (1985), white rice contains high amylose and low amylopectin, giving a suitable medium to produce angkak, while the sticky rice has high amylopectin. Pigment production can also be increased by adding zinc (Zn) and magnesium (Mg; Lin & Demain 1991). Chiu and Poon (1993) also noted that Tween 80 [polyoxyethylene (20) sorbitan monoleate] can increase pigment production by *Monascus* sp.

Research objectives were to determine the optimum condition in producing pigments and lovastatin, parameters observed include the effects of *M. purpureus* strain, various combination of rice media, duration of fermentation, and also the effect of adding various minerals to fermentation mixtures.

MATERIALS AND METHODS

Potato Dextrose Agar Media (PDA). Ten mg of boiled D-glucose was added to 500 ml potato extract 50% (w/v). 20 g of agar was then added and dissolved by heating. The solution was made up to 1 using distilled water. When it had been cold, 5 ml PDA was poured into a reaction tube placed in a sloped position before being sterilized by autoclaving at 121 ^{o}C in 1 atmosphere pressure for 1 hour before being left to cool.

Rejuvenation and Inoculums (Starter) Preparation. Three strains of *M. purpureus* were used in this study; *M. purpureus* 915, AKI, and AKII are from the collection at the Microbiology laboratory, LIPI, Cibinong, Bogor, Indonesia. Rejuvenation of the *M. purpureus* isolates was carried out in laminar cabinet using aseptic conditions. Pure isolates of *M. purpureus* are inoculated into PDA, and then those were incubated at 25-30 ^{o}C for 16 days.

Starter medium was made from 40% (w/v) rice flour as a source of carbon (Sutrisno 1987), 0.15% (w/v) NH_4NO_3 as source of nitrogen, and minerals (KH_2PO_4 0.25% (w/v); $MgSO_4 \cdot 7H_2O$ 0,10 % (w/v)). The mixture was then dissolved in 100 ml distilled water (Jenie & Facda 1991). pH was adjusted to 6 before the medium was sterilized. Two scratches of spores were used for inoculation and the starter culture was incubated for a week at 25-30 ^{o}C. These *M. purpureus* cultures were then used as starter cultures for solid medium fermentation.

Growth of *M. purpureus* on Solid Media. The production of pigment and lovastatin were initiated by washing the rice in distilled water, draining it before placing 25 g rice and 12.5 ml distilled water into Erlenmeyer flask and adjusting the pH to 5 by adding of 0.1 M HCl or 0.25 M KOH. The rice media was then sterilized by autoclaving at 121 ^{o}C for one hour. After cooling, the medium was inoculated with 2 ml starter culture, and then incubated at room temperature (28-33 ^{o}C) for 9-16 days. Fermentation process was ended in 16 days; the substrate was dried in an oven at 56 ^{o}C for 3 days.

Growing at *M. purpureus* Solid Media with Minerals Variations. When medium supplemented with minerals was required, mineral salt mixtures were added before autoclaving the rice medium as follows. There were five different treatments: 1st treatment medium was added with 12.5 ml distilled water, 2nd treatment was added 12.5 ml mixture of minerals of KH_2PO_4 0.25% (w/v), $NaNO_3$ 0.75% (w/v), $MgSO_4 \cdot 7H_2O$ 0.5% (w/v), $CaCl_2 \cdot 2H_2O$ 0.005% (w/v), 3rd treatment was added 12.5 ml $ZnSO_4 \cdot 7H_2O$ 0.03 mM, 4th treatment was added 12.5 mg $MgSO_4 \cdot 7H_2O$ 12 mM, last treatment was added with 12.5 ml Tween 80 0.4%. All treatments were placed in Erlenmeyer glass. Fermentation process was ended in 16 days; the substrate was dried in an oven and refined in mortar.

Analysis of Angkak Pigment. Angkak powder (0.05 g) from the fermentation above was added to 10 ml methanol in glass tube in order to extract the pigment. Extraction was carried out for 24 hours in shaking incubator, and then it was vortexed and drained to obtain the filtrate. The pigment absorption was measured using a spectrophotometer. Yellow and red pigments were measured at 390 and 500 nm respectively with methanol as a blank.

Lovastatin Analysis. Standards of soluble lovastatin were prepared (120, 200, 500, and 1000 parts per million, ppm) then the absorption was measured at 237 nm using a UV spectrophotometer. Standard curves were prepared and used to determine the concentration of Lovastatin in samples.

Angkak powder (100 mg) was added into 900 ml of ethanol and then it was vortexed for a few minutes and centrifuged at 9,520 rpm for 15 minutes. The supernatant was removed and retained, while the pellet was extracted with another 900 ì l of 75% ethanol. The supernatant from these extractions was then mixed before the lovastatin content was analyzed as for the standards.

Statistical Analysis. The data was analyzed using analysis of variance (ANOVA) with confidence limits of 95% and a standard error of 0.005 using SPSS software.

RESULTS

The Growth of *Monascus purpureus* in PDA Medium. During the growth of *M. purpureus*, a process of granular liquid extrusion occurred through the point of hyphae growth. During culture growth in PDA medium, this granular liquid is initially white but gradually the color develops from yellowish to orange and finally to red, or (Table 1, Figure 1). This coloring consists of two pigments, a red pigment (*monascorubin*) and a yellow pigment (*monascoflavin*) (Wong & Kohler 1981). The mycelium growth of *M. purpureus* is also shown in Table 1.

Media and Strains Effect on the Red and Yellow Pigments Production by *M. purpureus*. To test the production angkak pigments by 3 strains of *M. purpureus* (AKII, 915, and AKI), we had inoculated cultures on white rice (IR-42) contained high amylase (25-33%), red rice (BP-1924-IE-52), and a 1:1 combination of red and white rice medium and then tested pigment levels between from 0 to 16 days (Figures 2, 3, and 4). Maximum production of red and yellow pigment by the AKII strain occurred on 16th day of fermentation (Figure 2). The maximum amount of red pigment was obtained from combination rice medium, while the maximum amount of yellow pigment was obtained from white rice. The maximum amount of both red and yellow pigment produced by strain 915 occurred

Table 1. The development of *M. purpueus* in PDA media

Duration of growth (days)	Change in colony color	Mycelium number		
		AKI	AKII	915
1	White	+	+	+
2	white	++	++	++
3	white	++	+++	++
4	White-yellowish	+++	++++	+++
5	White-yellowish	+++	++++	+++
6	White-yellowish	++++	++++	+++
7	White-yellowish	++++	+++++	++++
8	Orange	++++	+++++	+++++
9	Orange	+++++	++++++	+++++
10	Dark Orange	+++++	++++++	+++++
11	Dark Orange	++++++	++++++	+++++
12	Orange-red	++++	+++++	+++
13	orange-red	++++	+++++	+++
14	dark-red	+++	++++	++
15	dark-red	+++	++++	++
16	dark-red	++	+++	+

+: few, ++: medium, +++: many, ++++: numerous.

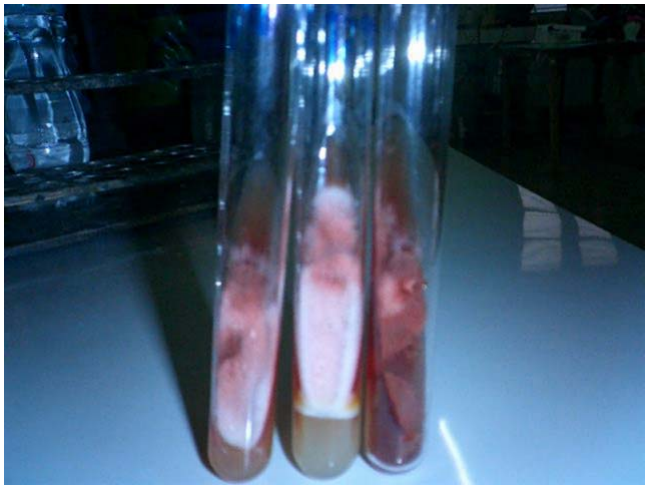


Figure 1. Production of red pigment by *M. purpureus*isolates AKI, AKII, and 915 in Potato Dextrose Agar (PDA) medium.

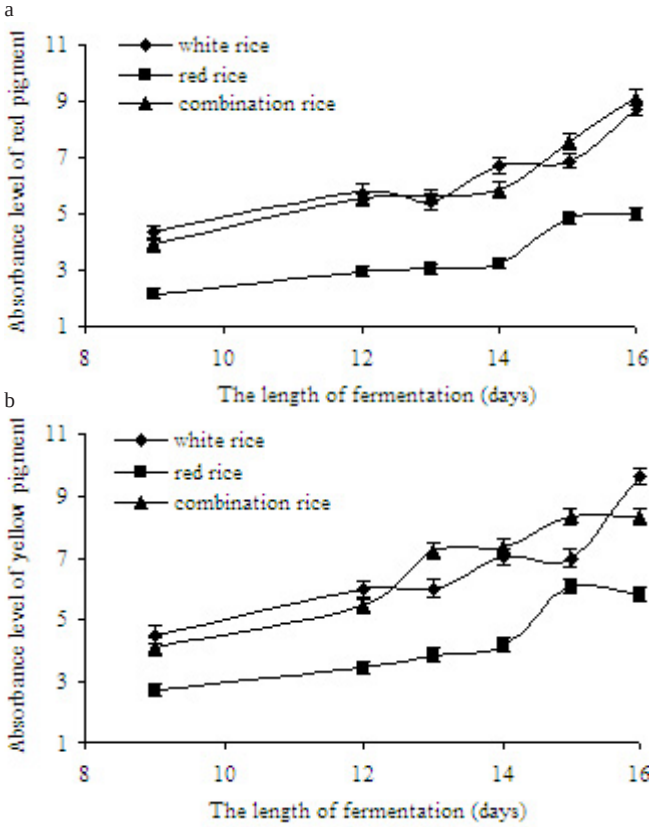


Figure 2. a: Red pigemnt production by *M. Purpureus* AKII in rice medium (measured in 500 nm); b: Yellow pigemnt production by *M. Purpureus* AKII in rice medium (measured in 390 nm).

on 15th day of fermentation on combination rice media (Figure 3). The AKI strain produced maximum pigments after 16 days fermentation with maximum red pigment produced using white rice medium and maximum yellow pigment using combination of rice medium. Analysis of variance (ANOVA) shows that the strains, rice media, and duration of fermentation significantly influence production of red and yellow pigments by *M. purpureus.*

The duration of fermentation influences pigment production. Table 1 shows that the potency of AKI and 915

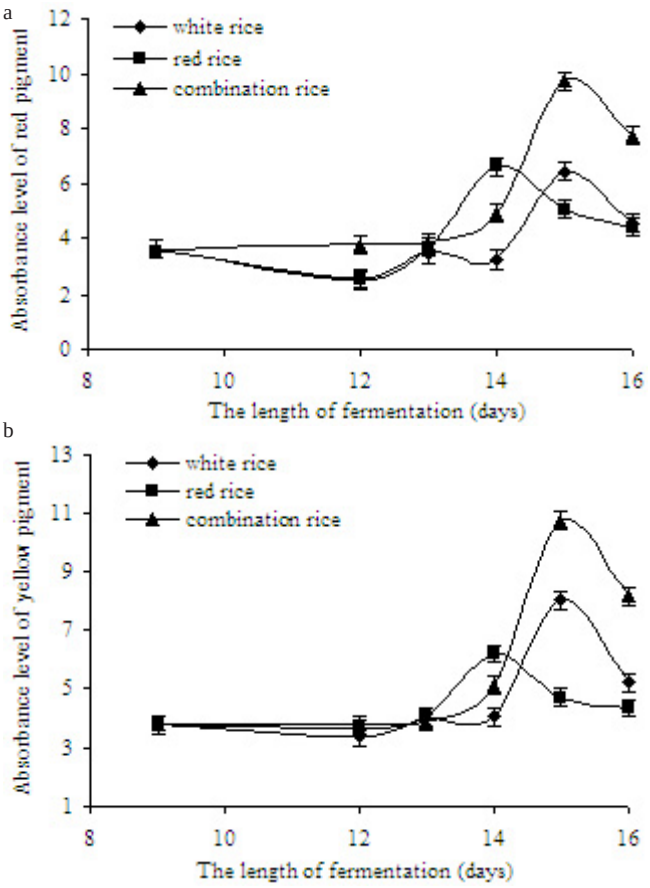


Figure 3. a: Red pigemnt production by *M. Purpureus* 915 in rice medium (measured in 500 nm); b: Yellow pigemnt production by *M. Purpureus* 915 in rice medium (measured in 390 nm).

produce pigment are similar, while AKII strains is differ to AKI and 915, in which pigment production of AKII is the highest. Concerning to the media in producing red and yellow pigment both white rice and combination rice is similar (Figure 2, 3, and 4).

Linear regression analysis indicates that red and yellow pigments have positive correlation ($r^2 = 0.933$), it means that when the red pigment increase, the yellow pigment produced will also increase (Table 2).

Effects of Minerals Addition on the Production of Red and Yellow Pigments by *M. purpureus* AKII Using White Rice and Combination Rice Media (White and Red 1:1). Table 1 indicates that *M. purpureus* AKII is the fungus strain which produce the highest pigment compared to the other strain. The variance analysis shows that red and yellow pigments produced by AKII with addition of minerals mixture, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$ and Tween 80 0.4% are significantly difference. The effects of different treatments on pigment production are shown in Figure 5 and 6. Using white rice medium, the red and yellow pigment production are decreased by 32.12 and 30.23% respectively, in comparison with the control treatment (distilled water) when mineral mixture (0.25% (w/v) KH_2PO_4, 0,75% (w/v) $NaNO_3$, 0.5% (w/v) $MgSO_4 \cdot 7H_2O$, 0.005% (w/v) $CaCl_2 \cdot 2H_2O$) was added to the medium. For combination rice medium, red and yellow pigment production are also decreased to 70.41 and 64.94%

respectively, when this mineral mixture is added. Statistical analysis showed these values are significantly difference to the control ($P < 0.05$).

Tween 80 is able to increase pigment production by *M. purpureus* (Figure 5, 6, and 7) Using white rice medium, tween 80 increases the production of red and yellow pigments by 15.05 and 9.39% respectively, while on combination rice the increase is 18.09 and 7.61% respectively. However, it is not significantly different to the control distilled water treatment ($P > 0.05$).

Minerals Addition Effects to Lovastatin Production by *M. purpureus* AKII Grown in White Rice Medium. Next, we investigated the lovastatin content that has been produced by angkak using white rice medium supplemented with mineral mixture and Tween 80 during fermentation by *M. purpureus* AKII. The results show that the content

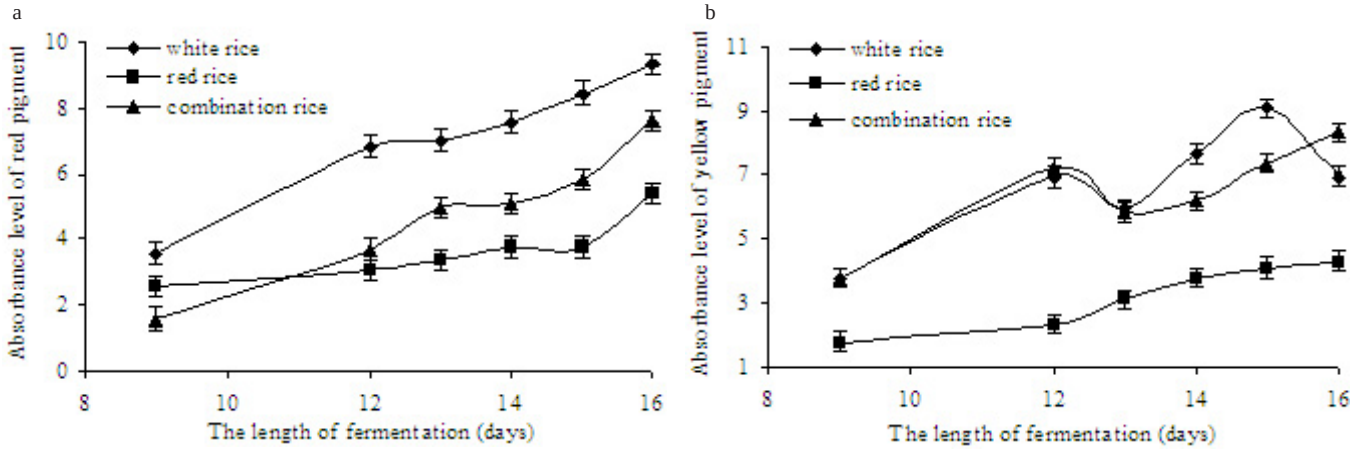


Figure 4. a: Red pigment production by *M. Purpureus* AKI in rice medium (measured in 500 nm); b: Yellow pigemnt production by *M. Purpureus* AKI in rice medium (measured in 390 nm).

Table 2. Correlation test result between red pigment, yellow pigment, lovastatin level, and fermentation length

Fixed variables	Correlation (r)				Sig probabilities (1-tailed)			
	Red pigment	Yellow pigment	Lovastatin	Fermentation length	Red pigment	Yellow pigment	Lovastatin	Fermentation length
Red pigment	1.000	0.964	0.520	0.633	-	0.000	0.403	0.000
Yellow pigment	0.964	1.000	0.088	0.665	0.000	-	0.338	0.000
Lovastatin	0.520	0.088	1.000	0.135	0.403	0.338	-	0.261
Fermentation Length	0.633	0.665	0.135	1.000	0.000	0.000	0.261	
R	0.971							
Determination Coefficient	0.944							
R^2	0.933							

Red pigment absorbance give positive correlation with yellow pigment absorbance (r = 0.964) and red pigment absorbance give no correlation with lovastatin production (r = 0.052), neither with the yellow pigment absorbance. Fermentation length also make a positive correlation with red and yellow pigment absorbance (r = 0.633), but lack of correlation with lovastatin production (r = 0.135).

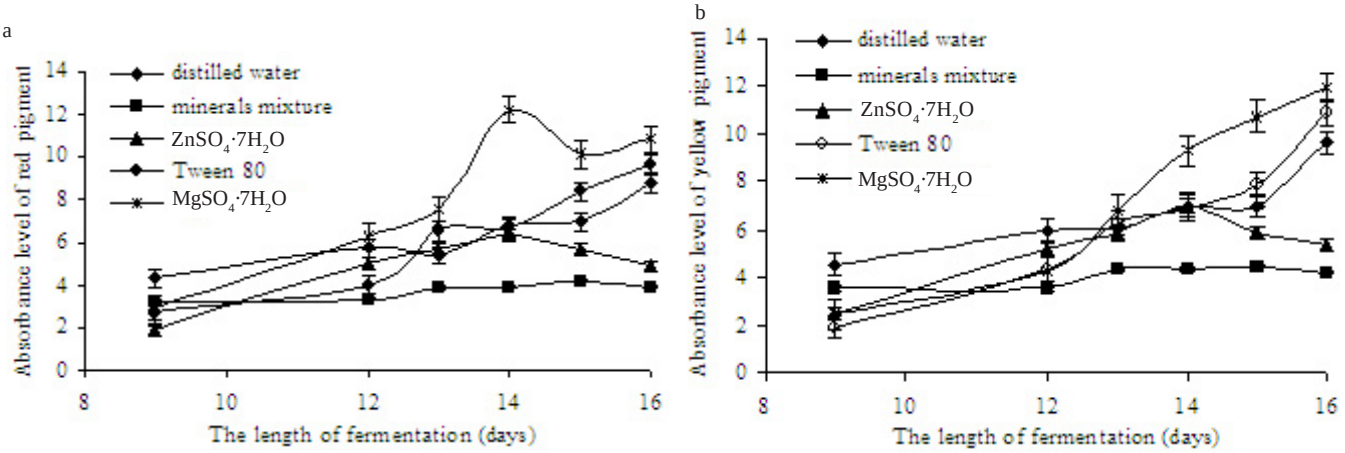
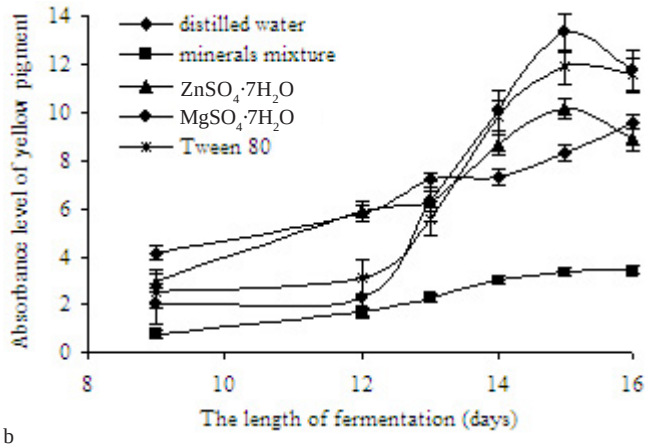


Figure 5. The effect of adding minerals to production of a: Red pigment in white rice medium by *M. Purpureus* AKII (measured in 500 nm); b: Yellow pigment in white rice medium by *M. Purpureus* AKII (measured in 390 nm).

of lovastatin is varying, depends on the treatment (Figure 7).

Variance analysis indicates that treatments gave significantly effect on lovastatin produced (Figure 7). The highest concentration of lovastatin is obtained from angkak when Tween 80 is added in its production. The average value for 5 replicates is 254.934 ± 9.656 ppm or an increase of 40.78% compared to the control. This result was supported by t-Test which shows a significant difference from the control. In contrast, the average lovastatin content from angkak produced with added mineral mixture was increased by 8.81% but this is not significantly different to control using the Duncan multiple range tests. The addition of $ZnSO_4 \cdot 7H_2O$ increased lovastatin content to 22.53% respectively (not significant compared to control group), whereas adding $MgSO_4 \cdot 7H_2O$ will decrease the pigment content by 4.11% (significant compared to control group). ANOVA test indicated that red pigment production correlated positively to the yellow pigment production (r = 0.96) and that there is no positive correlation between red pigment and lovastatin (r = 0.052) or yellow pigment and lovastatin (r = 0.088).

a



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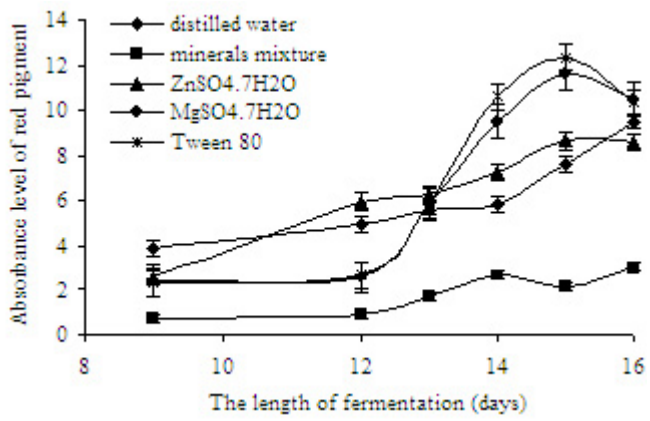

Figure 6. The effect of adding minerals on production of a: Red pigment in combination rice (red:white 1:1) by *M. Purpureus* AKII (measured in 500 nm); b: Yellow pigment in combination rice (red:white 1:1) by *M. Purpureus* AKII (measured in 390 nm).

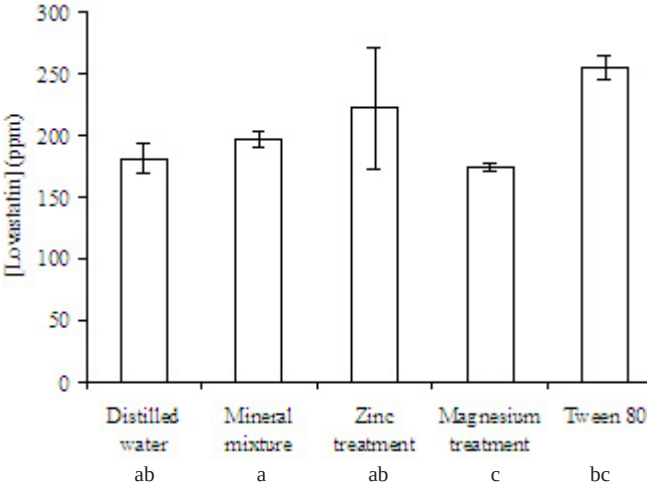

Figure 7. The effect of adding minerals on lovastatin levels (ppm) in angkak production by *M. Purpureus* AKII. Key: Same letter under each treatment indicates that the treatments give no significant results.

DISCUSSION

The results show that AKII and AKI strains produce maximum pigment on day 16 of fermentation, while 915 strain produced maximum pigment on the 15th day of fermentation. According to Jenie and Facda (1991), fermentation to produce pigment needs 16 days. The decrease of pigment during fermentation is caused by decomposition of pigment and change in pigment structure. The chromophor group of pigment is degraded, which results in decreased pigment absorption.

Wong and Koehler (1981) indicated that the growth of *M. purpureus* and pigment production are influenced by the amount of carbon and nitrogen in the media, and that the amount of carbon and nitrogen will determine the amount and the type of pigments.

Steinkraus (1983) said that pigment can be produced from several rice strains with high amylose (25-33%), more pigment production will be obtained using rice with low amylopectin (Santoso & Satiawiharja 1985). The use of sticky rice will inhibit the growth of *M. purpureus*.

M. purpureus produces protease and amylase enzymes which break down proteins and amylose respectively. Every strain of *M. purpureus* has the capability to produce enzyme amylase. The more amylase which the strain produces, the more amylose can be hydrolyzed into glucose that will produce more pigment. Glucose is needed for source of energy during the development of secondary metabolic. Amylose in white rice is higher than in red rice; therefore, white rice will produce more red and yellow pigment (Santoso & Satiawihardja 1985).

Rice consists of carbohydrate, protein, vitamin B1, phosphate, potassium, and zinc. Vitamin B1 in red rice is higher than in white rice (Santoso & Satiawihardja 1985). Vitamin B1 (Lin 1973), amino acids, and zinc (Lin & Demain 1991) can influence pigment production. Vitamin B1 (Thiamin Pyrophosphate) is as coenzyme or prosthetic group in complex of a pyruphate dehydrogenase enzyme that catalyses the conversion of pyruvate into acetyl-CoA in glucose metabolism. According to Chen and Johns (1994), *M. purpureus* pigment is produced through a polyketide pathway

and needs acetyl-CoA in which is produced from glucose by pyruvate acid. Therefore indirectly, vitamin B1 is involved in the production of pyruphate acid from glucose during the process of pigment biosynthesis. The study shows that pigment level produced using red rice medium is lower than that using white rice medium. This might be vitamin B1 in red rice is higher than in white rice.

The study shows that red and yellow pigments levels are lowered compared to the control (distilled water group) when a mineral mixture is added. Wong and Koehler (1981) stated that the growth of *M. purpureus* and pigment production are influenced by the proportion of carbon and nitrogen. Organic nutrients and minerals are important for the development of the angkak pigment, and mineral are supporting elements during metabolism of the microorganism during fermentation (Sutrisno 1987). When the amount of carbon is increased, more nitrogen and minerals are needed to reach the optimum growth. Decreasing pigment level in this study maybe caused by the proportion and composition of minerals added is optimal (Figures 5 & 6). Lind and Demain (1991) indicated that high phosphate level (> 70 mM) can hinder the growth of *M. purpureus* and pigment production. In contrast, high $MgSO_4$ (> 16 mM) will improve the growth of *M. purpureus* but hinder pigment production.

Figure 7 shows that Tween 80 can increase lovastatin level by *M. purpureus*. Tween 80 apparently an emulsifier that stimulates cell development and increases the permeability of the cell membrane, so that nutrient transport from both outside and inside the cell are increased and consequently cell metabolism will increase too.

Finally, pigment production will achieve best result using fermentation by *M. purpureus* AKII strains in combination of white and red rice for 16 day fermentation, addition of $MgSO_4 \cdot 7H_2O$ 12 mM will also increase pigment production. Whereas lovastatin production an be generated by adding Tween 80 in the fermentation medium. These results provide more information in producing pigment and lovastatin for commercial purpose described above.

ACKNOWLEDGEMENT

The author would like acknowledge and thank Dudi S. Sastraatmadja from LIPI for his kindness in allowing the use of *Monascus purpureus*; and Lisna Vitria Manjasari, Mega Safitri, and Dimas Andrianto for their help in this study.

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