

ENHANCED PRODUCTION OF RED PIGMENT AND LOVASTATIN BY CO-CULTURE WITH *SACCHAROMYCES CEREVICIAE* IN ANGKAK RICE-MUNG BEAN

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Abstract - Angkak known as fermentation product by *Monascus purpureus* that can be used as food colorant and has a valuable component known as lovastatin. Lovastatin, a secondary metabolite of *Monascus* species is one of the competitive inhibitors of hydroxymethylglutaryl-coenzyme A reductase, that has been widely used as a potent drug for lowering blood cholesterol. The low productivity of red pigment and lovastatin is the main problem in Angkak. Addition of 5% mung bean and *Saccharomyces cerevisiae* as co-culture was used to enhance lovastatin and red pigment production. The result show that addition of *Saccharomyces cerevisiae* could increase lovastatin production. Whereas 10^5 cell/ml *Saccharomyces cerevisiae* was added on the 12th day of the fermentation, the final lovastatin production reached by 26,035 mg/100mg which was improved 39% compare to the control. Red pigments production is 4,067, which was improved 69% than that of control.

Keywords: Lovastatin, *Monascus purpureus*, Red pigment, *Saccharomyces cerevisiae*

I. INTRODUCTION

The uses of synthetic coloring are increasing every year. However, there are major concerns about using synthetic coloring in the food and beverage because of its safety problems [1]. Therefore we need to explore natural coloring such as Angkak. Angkak is a fermentation product of filamentous fungus *Monascus purpureus* made of rice although another species like *M. ruber*, *M. anka*, and *M. pilosus* can also produce the pigment [2]. Red pigment from Angkak commonly used as food coloring. In Japan, Philippines and China, Angkak has been applied in meat, fish and wine [3]. Angkak known to produce high value secondary metabolites like lovastatin, pigments and γ -aminobutyric acid (GABA) [4]. Lovastatin has been widely used as a potent drug for lowering blood cholesterol [5]. Lovastatin is a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which is a regulatory of limiting enzyme in cholesterol biosynthesis [6][7]. Many factors influence the production of pigment and lovastatin during fermentation like adding another nutrition sources and co-culture [8][9][10].

In this study, Mung bean and *Saccharomyces cerevisiae* was used to enhance red pigment and lovastatin production. Danuri [8] reported that when nutrition like B1 vitamin, zinc and amino acid was added, red pigment and lovastatin had significantly increased. Vitamin B1 from mung bean become cofactor in acetyl-CoA formation which is used in pigment and lovastatin formation. Zinc will inhibit NADPH formation but stimulate production of secondary metabolites [8]. Based on previous results, red pigment and lovastatin can be increased by adding 5% (w/w) of mung bean [11]. Addition of

S. cerevisiae as a co-culture can also optimize the number of metabolite that is produced by *Monascus purpureus* during the process of fermentation. In addition, Lim and Shin [10][12] showed that co-culture with *S. cerevisiae* can stimulate red pigment and lovastatin production because *S. cerevisiae* secreted hydrolytic enzymes outside of cells. Hydrolytic enzymes like chitinase and glucoamylase could degrade *M. purpureus* cell walls. This action causes *M. purpureus* produce hydrophobic substances such as pigment, lovastatin and citrinin as a defense mechanism for blocking attacks from *S. cerevisiae*. The objective of this study was to find out the best co-culture condition for red pigment and lovastatin production.

II. MATERIAL AND METHODS

A. Microorganisms

Cultures of *Monascus purpureus* and *Saccharomyces cerevisiae* were obtained from Microbiology Laboratory, Faculty of Agricultural Product, Brawijaya University (East Java, Indonesia). *M. purpureus* was maintained routinely on potato dextrose agar (PDA) medium and sub cultured in every 2 weeks. Yeast *S. cerevisiae* was maintained routinely on peptone glucose yeast extract agar (PGYA) medium and sub cultured in every 1 week. Both of cultures are incubated at 30°C. *M. purpureus* was incubated for 7 days and *S. cerevisiae* was incubated for 3 days.

B. Preparation of seed cultures

Two loops of *Monascus purpureus* was inoculated to 100 ml medium that contains 4 g of rice flour, 0,15 g of NH₄Cl, 0,25 g of KH₂PO₄ and 0,1 g of MgSO₄.7H₂O. Medium was adjusted to pH 6,0 using KOH or HCl and sterilized by autoclaving at 121°C for 15 minutes. The seed cultures was incubated for 7 days at 30°C [8].

C. Preparation of *S. cerevisiae* culture

One loop of *S. cerevisiae* was inoculated into 5 ml PGYB (Pepton, Glucose and Yeast extract broth) and incubated at 30°C for 2-3 days. The culture was incubated until the concentration of culture was 10^4 , 10^5 and 10^6 cell/ml. The concentration of *S. cerevisiae* was counted by Haemocytometer.

D. Preparation of Angkak Fermentation

The substrate that was used in Angkak fermentation is IR36 Rice because it has high amylose (27%) [13]. Mung bean was added in fermentation medium because of B1 vitamin (0,64 mg/100 g) and zinc (0,8 mg/100 g) that can

stimulate synthesis of secondary metabolite [14]. 5% (w/w) of mung bean was used based on best treatment in previous research [11].

40 g IR36 Rice was soaked into distilled water (1:1) for 8 hours. Presoaked rice was transferred into petri disk and 5% (w/w) of mung bean was added. The mixture was then dissolved in 24 ml starter medium. Starter medium was made from 4% (w/v) of rice flour, 0,15% (w/v) of NH_4Cl , 0,25% (w/v) of KH_2PO_4 and 0,1% (w/v) of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and adjusted to pH 6,0 using KOH or HCl. The media was sterilized by autoclaving at 121°C for 15 minutes. After that, the media was inoculated with 8 ml seed cultures and then incubated at room temperature for 14 days. Co-culture by *S. cerevisiae* was added at 8th and 12th fermentation days. The media was added 2,5 ml of yeast's suspension with different concentration (10^4 , 10^5 and 10^6 cell/ml). Fermentation process was ended in 14 days and angkak was dried in an oven for 8-12 hours at 70°C [15].

E. Analysis of Red Pigment

0,05 g of dried angkak was suspended in 10 ml 96% of methanol. This mixture was incubated at shaker 120 rpm for 24 hours and then it was drained to get the supernatant. The red pigment absorbance was done using spectrophotometer with absorbance at 500 nm [17].

F. Analysis of Lovastatin

Standard curve was prepared using 5 tablets of cholvastin. The tablets was suspended in 100 ml acetonitrile and incubated in shaker for 30 minutes at 120 rpm. Then the mixture was drained to get the supernatant. The supernatant was concentrated with nitrogen gas. The concentrate was added 10 ml 70% of ethanol and distilled water until 100 ml and diluted until the concentration was 10 mg/100ml, 20 mg/100 ml, 40 mg/100ml, 60 mg/100ml, 80 mg/100 ml and 100 mg/100ml. The mixture was absorbance at 237 nm and the data was used for standard curve [8].

1,0 mg of dried angkak was suspended in 9 ml 70% of ethanol and centrifuged at 9500 rpm for 15 minutes. The pellet was suspended again with 9 ml 70% of ethanol and centrifuged at 9500 rpm for 15 minutes. The supernatant from first extraction and second extraction was mixed with vortex. Lovastatin concentration was determined using spectrophotometer with absorbance at 237 nm [8].

III. RESULTS AND DISCUSSION

The result from Angkak fermentation using *S. cerevisiae* as co-culture are show at table 1. An experimental design was made according to Analysis of Variances (ANOVA) method. Table 1 show an average value from 18 runs for 2 parameters (time of inoculation and concentration of co-culture).

A. Red Pigment Production on Co-culture Treated

Red pigment concentration as shown in Fig.1 seemed enhanced in co-culture with *S. cerevisiae* compared to the control (without co-culture). The addition of *S. cerevisiae* at the concentration 10^5 cell/ml and fermentation day 12 has the highest red pigment compared to the other. Pigment production during Angkak fermentation can decrease if the concentration of *S. cerevisiae* is too large and the inoculation time is too early [9]. Red pigment production at the concentration 10^6 cell/ml was lower than concentration 10^4 and 10^5 cell/ml because the inoculum size is too large. *S.*

cerevisiae cells can outgrow and decreasing growth of *M. purpureus* below the original levels to stimulated production of red pigment. It can make result in low production of red pigment in Angkak fermentation.

Adding of Co-culture		Red Pigment Concentration	Lovastatin Concentration (mg/100g)
Control		2,801	18,685
Fermentation day 8	10^4 cell/ml	3,743	23,242
	10^5 cell/ml	3,388	20,121
	10^6 cell/ml	2,383	21,531
Fermentation day 12	10^4 cell/ml	3,206	20,981
	10^5 cell/ml	4,067	26,035
	10^6 cell/ml	2,668	21,655

Table 1. The Effect of Co-culture in Production of Red Pigment and Lovastatin

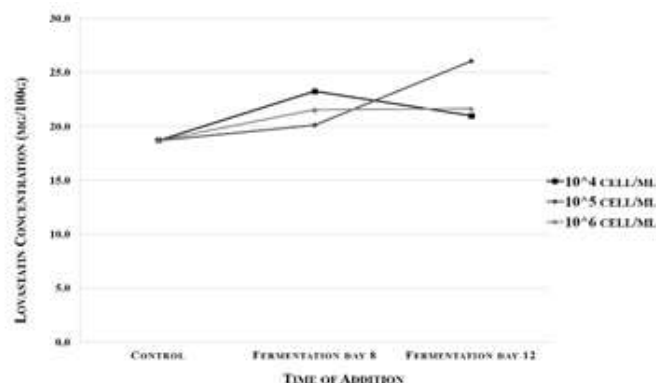


Fig. 1. The effect of inoculation time and concentration *Sachharomyces cerevisiae* in concentration of red pigment

Another factor that can influence productivity of red pigment is ethanol. Ethanol from *S. cerevisiae* could change pH to base. This condition will stimulate the change of pyranoid oxygen atom from orange pigment with functional group of $-\text{NH}$ and it was made change of orange pigment to red pigment [17]. But if concentration of ethanol is too large it'll decrease the growth of *M. purpureus* [18]. So that, the concentration of *S. cerevisiae* must be equal with the growth of *M. purpureus*.

Secondary metabolite like pigment, lovastatin and citrinin is production in stationery growth phase. *M. purpureus* will use product from log growth phase like pyruvate to produce secondary metabolite [20]. The addition of co-culture is better when *M. purpureus* in stationery growth phase. If co-culture add before stationery growth phase, *S. cerevisiae* will outgrow and growth of *M. purpureus* will hampered. The addition of co-culture at 12th fermentation day has higher result than 8th fermentation day, it assumption that *M. purpureus* was in stationery growth phase so that production of secondary metabolite will be higher than other.

B. Lovastatin Production on Co-culture Treated

The fermentation medium that was used in the experiment according to the procedure described above. Result at Fig. 2 showed that co-culture and addition of mung bean stimulated lovastatin production during fermentation. Maximum yield was observed with 10^5 cell/ml concentration of *S. cerevisiae* with inoculation time on the 12th day of fermentation.

Production of lovastatin was effected by the concentration of *S. cerevisiae*. If concentration of *S. cerevisiae* that was added is high, the amount of metabolism that was secreted is high too. Ethanol will converted to acetyl-CoA that can be used as substrate for lovastatin formation [21]. Ethanol and hydrolytic enzymes that was secreted by *S. cerevisiae* can stimulated production both of pigment and lovastatin. But *M. purpureus* cell walls can lysis if concentration of ethanol and hydrolytic enzymes are too large.

The main problem when using co-culture is concentration of co-culture can't too large, because it will decrease growth of *M. purpureus* and production of secondary metabolites like pigment and lovastatin will be hampered [9]. The higher lovastatin was reached with 10^5 cell/ml concentration at 12th fermentation day of *S. cerevisiae*. It can be assumed that this concentration was the best treatment and the amount of *S. cerevisiae* was balance with the growth of *M. purpureus*. So that, metabolite from *S. cerevisiae* such as ethanol can used as energy and carbon source for metabolism of *M. purpureus*. The concentration of lovastatin was lower when co-culture added in 8th fermentation day, it can be assumed that *M. purpureus* still in log growth phase so that metabolism of *S. cerevisiae* not fully usage and nutrition for *M. purpureus* will decrease. Stationery growth phase of *M. purpureus* was signed with changes of media fermentation to orange and it would take after 8th days of fermentation [22].

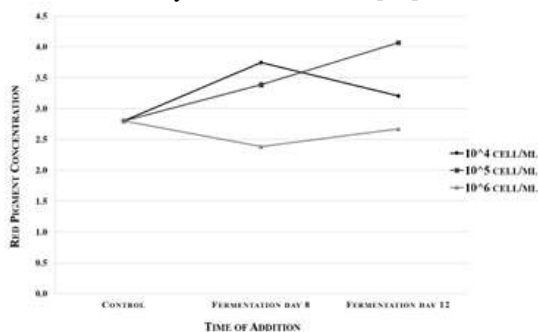


Fig. 2. The effect of inoculation time and concentration *Sachharomyces cerevisiae* in lovastatin concentration



Fig. 3. Angkak Rice-Mung Bean from different co-culture a: 8th day fermentation with 104 cell/ml of *S. cerevisiae*, b: 8th day fermentation with 105 cell/ml, c: 8th day fermentation with 106 cell/ml, d: 12th day fermentation with 104 cell/ml, e: 12th day fermentation with 105 cell/ml, f: 12th day fermentation with 106 cell/ml

Total pigment production can indicate the production of lovastatin because they used same precursor that is tetraketide (one molecule of acetyl-CoA and three molecules of malonyl-CoA) [23]. *S. cerevisiae* as co-culture can provide acetyl-CoA to produce lovastatin and red pigment. *S. cerevisiae* can produced acetyl-CoA from glucose via pyruvate [4].

IV. CONCLUSIONS

Red pigment and lovastatin from angkak are useful. Development of new nutrition sources and fermentation method to increase production of pigment and lovastatin is essential. Here, we added mung bean as B1 vitamin and zinc source and *S. cerevisiae* as co-culture to improve the production levels of secondary metabolites from *M. purpureus* including red pigment and lovastatin. Red pigment and lovastatin production in Angkak has high productivity using 5% (w/w) mung bean and 10^5 cell/ml of co-culture in inoculation time at fermentation day 12. This suggest that co-culture and addition of other nutrition sources might result in high quantity of red pigment and lovastatin in Angkak.

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