The utilization of solid substrates on Monascus fermentation for anticholesterol agent production

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Abstract

Traditionally, Monascus fermented rice has developed as functional food for cholesterol lowering agent due to its secondary metabolite, monacolin K, was found to inhibit the biosynthesis of cholesterol. The formation of secondary metabolite in solid substrates fermentation is influenced by cultivation environment. Objective of the research was to study the influences of solid substrates in Monascus fermentation for production of anticholesterol agent, monacolin K with minimal citrinin content (M/C). The research aimed to select the best substrate on Monascus fermentation for better production of monacolin K. On this study we used waste tofu, gadung (Dioscorea hispida) and rice as the solid substrates. The fermentation was carried out by cultivation Monascus purpureus HD001 on dried substrates and incubated at 30°C for 14 days. Monacolin K was extracted with ethanol 95%, at 60°C, shaked at 110 rpm for two hours. The content of monacolin, citrinin and pigment in extracts was measured by spectrophotometric method and monacolin K was analyzed by HPLC method. The results showed that the highest M/C ratio was found on Monascus fermented waste tofu (M/C=4.4) with monacolin K content was 1,620 μg g⁻¹. However, the highest monacolin K content was found on Monascus fermented rice was 7,000 μg g⁻¹. Rice as the substrate of Monascus fermentation can produce monakolin K 4.6 times higher compared to gadung (Dioscorea hispida).

Keywords: Monascus sp, fermentation, monacolin K, citrinin

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Nomenclature

- **g**: weight (gram)
- **µg g⁻¹**: yield (microgram per gram)
- **°C**: Temperature in Celsius
- **rpm**: Speed (rotary per minute)
- **v/v**: ratio of concentration (volume/volume)
- **λ**: wavelength (nm)

1. Introduction

   *Monascus* species has been used for producing angkak or red fermented rice in East Asian countries for several centuries. This species can be cultivated in starch containing substrates. This fungi is still used for coloring and flavoring in food industry, mainly for fish, and meat products. *Monascus* fermented rice (MFR) has been used for treating hypolipidemia and developed as functional food for cholesterol lowering agent. The secondary metabolite, monacolin K inhibits biosynthesis of cholesterol by blocking the action of HMG-CoA reductase. In the hypercholesteremic activity, competitive inhibition of this enzyme due to structural homology between monacolin K (statins), β-hydroxy acid and HMG-CoA. However, the safety of MFR should be considered because the toxicity of citrinin. This compound is one of the mycotoxins produced by *Penicillium*, *Aspergillus* and *Monascus* species. Monacolin K and citrinin are polyketide derivative and always produced at the same time, but depends on the *Monascus* strain.

   The influence of different substrates on pigment production has been studied by Lin and Lizuka (1982). Beside rice, some other substrates such as bread, oat, corn and wheat grain can be used for *Monascus* fermentation in concern of pigment production. Several cereals have been used as substrates angkak production and the high quality fermentation product achieved by some suitable conditions of *Monascus* culture. The ratio of carbon-nitrogen sources influences to the production of *Monascus* metabolites such as pigment, mevinolin and citrinin. Chun-Lin Lee et al. (2006), has been studied the influences of some substrates such as potato, sweet potato, cassava, and dioscorea in monacolin and monascin production. They reported that dioscorea is the best substrate for *Monascus* fermentation in producing monacolin K as cholesterol lowering agent and also monascin as antiinflammation agent. The objective of this research is to study the best substrate on *Monascus* fermentation that could result in higher quantity of monacolin K production. On this study we use waste tofu, gadung (*dioscorea hispida*) and rice as the solid substrates.

2. Materials and Methods

2.1. Materials

   *Monascus purpureus* HD001 was obtained from microbiology ITB culture collections, rice was obtained from a traditional market in Bandung, gadung (*dioscorea hispida*) was obtained from Gombong, Central Java and waste tofu as solid substrates was obtained from a tofu manufacture in Bandung, mevinolin standard (Sigma-aldrich) and other chemicals for culture media and analysis.

2.2. Microorganisms and Cultivation

   The wild type *Monascus purpureus* HD001 was maintained in potato dextrose agar (PDA) slants. 10 mL suspension (A₆₆₀= 0.25) of *Monascus* strain was cultured in 100 mL YMP medium and incubated in shaker incubator (120 rpm), at room temperature for three days.

2.3. Solid State Fermentation of *Monascus purpureus*
300 g of dried solid substrates (waste tofu, dioscorea, and rice) was placed in a jar and autoclaved for 15 minutes. These substrates were cooled and inoculated with 10% of Monascus culture. Solid fermentation of Monascus strain was carried out at 30°C, for 14 days, these substrates was supplemented with 2 mL of YMP medium for five days and shaked for everyday. Monascus fermented products was dried at 50°C for overnight.

2.4. Extraction of Monacolin K in Solid Fermentation of Monascus purpureus

The dried of Monascus fermented products was ground into finely powdered material using a blender. Each gram of powdered materials were extracted with 10 mL of ethanol 95% by shaking on a rotary shaker at 65°C, 110 rpm for 2 hours. The content of monacolin, citrinin and pigment in extract was estimated by analysis of extract samples using a spectrophotometer UV-Vis at wavelength 238 nm, 330 nm and 500 nm. Furthermore, the extract samples were evaporated for HPLC analysis.

2.5. HPLC Analysis of Monacolin K

1.5 mg of mevinolin or monacolin K standard (Sigma-Aldrich) was dissolved in 0.5 mL of methanol. Ethanol extract was then extracted by 10 mL of ethyl acetate pH 3. The ethyl acetate fraction was separated and neutralized by sodium carbonate. The ethyl acetate fraction was then evaporated by using a rotary vacuum evaporator. The dried extract samples were weighed and dissolved in 5 mL of methanol. Monacolin K analysis was carried out by HPLC method based on Chun-Lin Lee et al. (2006) with modification. This method involved a Water system C18 column and the sample was eluted with acetonitrile/water (pH is adjusted to 2.5 with H3PO4) at the ratio 55:45, by volume. Analysis was carried out at 26°C for 20 minutes, flow rates were 2 ml/min and detection with a UV detector at 238 nm.

3. Results and Discussion

Solid substrates fermentation (SSF) is the best options for microbial metabolites production that produced from fungi. SSF of anticholesterol agent from Monascus sp using different substrates have been reported by some researcher. In this study, we utilize the cheap raw materials such as waste tofu production and gadung (Dioscorea hispida), and compared with rice as general substrate on Monascus fermentation. The Monascus fermented products are presented in Figure 1.

![Figure 1. Monascus fermented products by utilization of some substrates after fermentation at 30°C for 14 days](image)

(a: rice, b: waste tofu, c: gadung)

From the product fermentation shown that rice and gadung as the solid substrates produce high concentration of red pigment and waste tofu tends to produce orange pigment rather than red pigment. Based on the spectrophotometry data at λ500, the extract of waste tofu fermented product produced very low intensity (A= 0.04) compare to rice and dioscorea fermented product (A=0.381). Kasim et al. (2006) reported that the highest red
pigment was on the red rice Bah Butong cultivar with $A = 0.43$. Moreover, Mursalin (2010) reported that the formulation of dioscorea with byproduct of rice increase the red pigment intensity with $A = 0.889$. During fermentation process, the enzyme from *Monascus sp* degrade starch substrate into several metabolites. The structure of pigments is depend on substrate types and other specific factors during cultivation. *Monascus sp* can produce six pigment and categorized into three groups i.e. yellow, orange and red pigment.

The anticholesterol agent, monacolin K, was discovered in some species of *Monascus sp* i.e; *M. purpureus*, *M. ruber* and *M. koaliang*. The study on the secondary metabolites of *Monascus sp*, reported that the production and types of metabolites would be affected by the environment and methods of cultivation. In this study, the production of anticholesterol agent, monacolin K, was evaluated by estimation of monacolin K ($\lambda_{238}$) and citrinin ($\lambda_{330}$) production on *Monascus* fermented products. Based on the spectrophotometry data at $\lambda_{238}$ (figure 2), monacolin K which produced by fermentation on waste tofu was much lower than other substrates such as rice and dioscorea, that shown the similar intensity. However, the estimation of M/C ratio (monacolin/citrinin) produced on waste tofu fermented product (M/C=4.4) was higher than other fermented products. Citrinin content that produced on waste tofu fermented product is much lower than rice and dioscorea fermented products. *Monascus* fermented products may be contaminated by citrinin, a mycotoxin that could affect to kidney and liver damage. *Monascus* extracts and angkak have been used and commercialized as functional foods and food colorants. The citrinin level in all commercial *Monascus* samples was between 0.2 to 1.71 ug/g.

Some strategies to decrease the citrinin content in *Monascus* fermented products have been studied. F. Chen, X. Hu (2005), screened some strains of *Monascus* with low or non producing citrinin that isolated from red fermented rice rice samples. Moreover, Chun-Lin Lee et al. (2007) studied the extraction strategy for removing citrinin while monacolin K retained in the red fermented rice (RFR) product. They found that phosphate-ethanol extraction the effective strategy for minimize the citrinin level but monacolin K may retain. RFR product which extracted by 50% ethanol, 0.75% phosphate, for 60 minutes at 65°C give the M/C ratio 4.58. In our study, all *Monascus* fermented products were extracted by 95% ethanol by shaking on a rotary shaker at 60°C, 110 rpm for 2 hours. By this process, the extract was more dry when compared to the extract which extracted with 40% ethanol. The dry extract of *Monascus* fermented products would be easier in functional foods formulation rather than wet extract.

![Figure 2. Diagram of spectrophotometry data of *Monascus* fermented extracts for estimation of monacolin K content ($\lambda_{238}$), citrinin content ($\lambda_{330}$), and red pigment content ($\lambda_{500}$)](image)

Red yeast rice or Angkak has been known contain the active compound monacolin K, a HMG CoA reductase inhibitor, that responsible in inhibition of cholesterol synthesis in the liver. Chun-Lin Lee et al. (2006) reported that dioscorea (*Dioscorea batatas*) is the best substrate for *Monascus* species to produce monacolin K and monascin as the anti-inflammation agent. They reported that dioscorea can produce monacolin K at 2,584 μg g⁻¹, five times
higher compared to the rice as the substrate. In this study, we utilized gadung (Dioscorea hispida) as one of solid substrates that used in Monascus fermentation. Analysis of monacolin K of fermented products was carried out by HPLC method. Analysis data’s of monacolin K content in Monascus fermented products was shown on Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention time (minute)</th>
<th>Monacolin K content (µg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monacolin K (HPLC standard)</td>
<td>17.84</td>
<td>-</td>
</tr>
<tr>
<td>Monascus fermented of rice</td>
<td>18.06</td>
<td>7,000</td>
</tr>
<tr>
<td>Monascus fermented of waste tofu</td>
<td>18.07</td>
<td>1,620</td>
</tr>
<tr>
<td>Monascus fermented of gadung (Dioscorea hispida)</td>
<td>18.07</td>
<td>1,500</td>
</tr>
<tr>
<td>Monascus fermented of Dioscorea batatas¹</td>
<td>-</td>
<td>2,584</td>
</tr>
</tbody>
</table>

Data on Table 1 shown that rice is the best substrate to produce monacolin K. The results show that M. purpureus HD 001, with rice as the substrate can produce monacolin K at 7,000 µg g⁻¹. Even though Dioscorea hispida produced high concentration of red pigment, but monacolin K produced by this substrate was much lower than rice. This indicated that red pigment content on Monascus fermented products was not influenced to monacolin K production. During the fermentation, Monascus strain produce different amount of monacolin K and pigment. This fungi can produce yellow, orange and red pigment with their specific of structure compound. Chun-Lin Lee et al. (2006) reported that Monascus fermented product with Dioscorea (Dioscorea batatas) as the substrate, produced the yellow pigment with high concentration of monacolin K. Meanwhile, when rice is used, the red pigment was produced with low concentration of monacolin K, Dioscorea (Dioscorea batatas) can produced monacolin K five times to that resulted when rice is used as the substrate. When compared to our result, monacolin K produced by Monascus fermentation on gadung (Dioscorea hispida) was lower than on Dioscorea batatas as the substrate. However, rice as the substrate of Monascus fermentation can produce monakolin K 4.6 times compared to gadung (Dioscorea hispida). Dioscorea species is a member of the monocotyledonous family Dioscoreaceae. Amylose is one of starch type in dioscorea, and its content in the dioscorea is more than that in the rice grain. Amylose content in native gadung flour is 34.72%. This starch is quite fragile and tend to be fragmented by prolonged heating or agitation. The properties of starch as the solid substrate fermentation will influence to the growth of microorganisms. This properties can change by heat treatment and moisture content of substrates before fermentation is conducted. In this study, the substrates was dry and without any soaking treatment before autoclaved. Meanwhile, substrates which used by Chun-Lin Lee et al. (2006) was wet due to soaking treatment for 8 hours before autoclaved. According to this data, we assume beside the amylose content, the treatment of solid substrates before fermentation was also very important in growth of Monascus and metabolite production such as monacolin K.
4. Conclusion

Results from this study shown that the highest M/C ratio was found on *Monascus* fermented waste tofu (M/C=4.4) with monacolin K content of 1,620 μg g⁻¹. The highest monacolin K content was found on *Monascus* fermented rice of 7,000 μg g⁻¹. Rice as the substrate of *Monascus* fermentation can produce monakolin K 4.6 times compared to gadung (*Dioscorea hispida*).

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References