Isolation and Determination Antibacterial Citrinin From Various Fungal Monascus Purpureus using Rice as a Fermentation Substrate

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Abstract. The red yeast rice was the product of fermented rice by Monascus purpureus. Metabolic processes during fermentation produce contents of secondary metabolites, such as pigment, monacolin K and citrinin. Citrinin has antimicrobial activities but its have nephrotoxic and hepatotoxic mycotoxin effects. The isolation and determination of citrinin by solid fermentation Monascus purpureus from various isolates have been done using rice as a substrate. The red yeast rice product was extracted using methanol. Citrinin levels of several red yeast rice products were measured using High-Performance Liquid Chromatography at 330nm and 500nm with acetonitrile: phosphoric acid 0.2% (1:1), using a C18 column with a flow rate of 1ml/min. The curve of Citrinin standard linearity was created with some concentrations and produced the linear equation which was y = 71048x + 47811 with a value of r = 0.9998. The citrinin levels from several different red yeast rice products were red yeast rice of A 369.97 ng/ml, B 0.85 ng/ml, C 70.48 ng/ml, D 3.59 ng/ml and of E 790.17 ng/ml. The research showed that the different isolates of Monascus purpureus could influence the production of citrinin levels whereas the isolates of Monascus purpureus E has the highest citrinin levels. This citrinin level was considered safe to use because have not effected for nephrotoxic and hepatotoxic.

1. Introduction
Red yeast rice is the product of rice fermented by Monascus purpureus. Red yeast rice can be referred to by other names as Chinese red rice because the product is red, made of rice and the history mentioned that red yeast rice came from China. Angkak is known as the different name in many countries, such as beni-koji, hong qu, hang-chu, Monascus, red koji, red leaven, red yeast rice, xue zhi kang, ang-ka, angkhak and zhi tai [5].

The mold of the genus Monascus purpureus have been used for many centuries by the Asian people. Some species of mold that have been used for producing red yeast rice are Monascus purpureus, Monascus floridanus, Monascus ruber and Monascus pilosus [6].

Monascus purpureus produces secondary metabolites, ie Monakolin K, pigment and Citrinin. Citrinin is a mycotoxin compound (toxic compounds) produced by Monascus purpureus. Citrinin is
both nephrotoxic and hepatotoxic because it causes kidney malfunction and dis-structure as well as a liver malfunction and metabolism changes. Citrinin also has antibacterial activity [1].

2. Methods
2.1 Material
Materials used in this research were the HPLC (Alliance), Colom C18 (Sunfire), oven (Memmert), autoclave (All Americana), a petri dish, Erlenmeyer glass, test tube beaker (Pyrex), os potter (misselia crushers), stir bar, cotton, tweezers, Bunsen, sterilizer, centrifugation (Health HC-12), a magnetic stirrer (IKC C-MAG HS 7), refrigerator (Sharp) and other laboratory equipment commonly used.

The materials required were PDA (Potato Dextrose Agar), distilled water, physiological saline, ethanol (BRATACO), methanol (BRATACO), phosphoric acid, acetonitrile, Citrinin, IR 64 rice, and Monascus purpureus.

2.2. Microorganism
The research sample was microorganisms that was Monascus purpureus obtained from several institutions namely as the collection of the Laboratory of Microbiology-ITB Bandung, Indonesia Culture Collection-Lipi Bogor and Culture Collection-IPB Bogor.

2.3. Sterilization
The glassware tools and materials used in the experiment were sterilized in an autoclave at 121 ° C for 15 minutes. Ose needle and tweezers were sterilized by burning them down in flames. All other works of microorganism involvement were done aseptically by the help of bunsen blue flame.

2.4. Monascus purpureus Breeding in slanted Agar
Monascus purpureus breeding was started with making of PDA medium (Potato Dextrose Agar) which 19.5gram PDA mixed with 500ml of distilled water then heated until it formed a clear colorless liquid medium, slanted the sterilized 5 mL medium then incorporated into a sterile test tube, then the side of tilt tube media was slanted to 30 ° and let it freeze. Monascus purpureus was streaked onto the medium, then incubated at 28 ° C for 7-14 days.

2.5. Monascus purpureus suspension.
Monascus purpureus in slanted agar that had been aged for 14 days was aseptically removed using a spatula into PDB liquid medium (Potato Dextrose Broth) 50ml in 250ml Erlenmeyer, then was put in the shaker for 3 days at a temperature of 30-35 ° C at a speed of 150-160 rpm, after entering the stationery phase then the mold suspension was fermented on rice as a substrate.

2.6. Fermentation of Monascus purpureus.
A total of 100-200 grams of washed rice was put into 250ml Erlenmeyer then was sterilized by autoclaving, the rice was inoculated with 3 mL Monascus purpureus suspension and was incubated for 14 days at 28 ° C and agitation were done every 3 days.

2.7. Citrinin Extraction
After rice being fermented with Monascus purpureus was formed, then it was deposited over aluminum foil and then dried in an oven at 60 ° C and subsequently comminuted to form a powder. A total 0.3g red yeast rice powder was mixed with 10ml of methanol in a beaker, stirred for 3 hours using a magnetic stirrer and centrifuged. Then each sample of the red yeast rice was filtered by Whatman filter paper.

2.8. Determination of Citrinin Levels
The red yeast rice solution was analyzed using HPLC using C-18 column with a mixture movement phase of acetonitrile: 0.2% phosphoric acid (1: 1), with a flow rate of 1 mL/min and Fluorescence detector at a 330nm and 500nm wavelength. The pure Citrinin is a standard solution made with several concentrations of 1.5625ng / mL, 3.125ng / mL, 5.25ng / mL, 12.5ng / ml, 25ng / ml, 50ng / mL,
100ng / mL, 200 ng / mL in methanol. The level calculation was determined using a linear regression equation of Citrinin levels towards the chromatogram area.

3. Result and discussion

The research sample used was \textit{Monascus purpureus} obtained from several institutions. Table 1 shows the characteristics of the isolates.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{Isolates} & \textbf{Characteristics} \\
\hline
Lipi F01 & Mycelia shaped like flowers, strands, and orange isolates \\
Lipi F147 & Mycelia shaped like flowers, saffron and white strands and orange isolates \\
IPB A & Dispersed mycelia have intense red colored isolates \\
ITB & Dispersed mycelia have orange colored isolates \\
IPB-B & Wrinkled mycelia have intense red colored isolates \\
\hline
\end{tabular}
\caption{Characteristics of Isolates}
\end{table}

Temperature is one of the main factors that can affect the growth of molds [2] whereas the temperature of the growth of mold was at 25 °C-30 °C. The temperature for mold fermentation used was 28 °C so that the mold grew well. If the temperature is less or more than the optimum temperature of the mold, then the mold was difficult to grow and susceptible to bacterial contamination or other microorganisms.

\textit{Monascus purpureus} can grow well in substrates contain carbohydrates, fats and proteins, such as potato, corn, cassava, wheat, and rice. The substrate used in this study was rice because rice is a good substrate for \textit{Monascus purpureus}, furthermore the red yeast rice product generally found in the market is rice.

The rice used is IR 64 rice or known as Setra Ramos. In addition, to easily be found in the market, IR 64 rice also contains about 25%-30% amylose. IR 64 rice is also known having the characteristics of rice pera, has high levels of amylase and low levels of amylpectin which is not sticky when cooked. This situation makes it spacious enough for the oxygen to flow among the rice grains as to maximize the growth of mold because the mold has aerobic characterism that requires oxygen to grow. The space among the rice grains can make mycelium completely closed. Rice is the best substrate for pigment production because of its microscopic structure that is good for hyphae penetration [7]. Rice fermented should be exposed to the morning sunshine and shuffled so that the growth of mold and color formed equally spread on all substrates. The morning sunshine is also good for the fermented product to remain dry and for the growth of molds. During the fermentation process, the fermented products disburse water or fluid, either which the substrate contains water, the mold will not grow and susceptibility contaminated. Here is where the morning sunshine is useful to keep the fermented products dry and not humid. The higher the water content in the substrate, the more difficult for the mold to grow and it is highly possibility for the substrate to decay.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{Curve of Citrinin Linearity}
\end{figure}
The curve of citrinin standards linearity was made in advance to determine the citrinin levels found in the red yeast rice product and used to compare the retention time of citrinin standard with a retention time of the red yeast rice product, so the citrinin compound on the red yeast rice product can be discovered as well as the linearity which was \( y = 71048x + 47811 \) with \( r = 0.9998 \) value. Table 2 shows the concentration and AUC standards and citrinin standards linearity.

The citrinin qualitative determination of fermentation was made by comparing the retention time of citrinin standard with a retention time of the red yeast product. While quantitative was viewed of AUC using the HPLC method with fluorescence detector and movement phase acetonitrile: phosphoric acid 0.2% (1:1), flow rate 1mL / min with Sunfire column system C-18 and a maximum wavelength of 330nm and 500nm [3].

Table 2. Concentration of Citrinin Standard

| Concentration (ng/ml) | AUC     |
|----------------------|---------|
| 1.5625               | 89447   |
| 3.125                | 217120  |
| 5.25                 | 478277  |
| 12.5                 | 953011  |
| 25                   | 1898210 |
| 50                   | 3716925 |
| 100                  | 7083240 |
| 200                  | 14254265|

As the results of the fermentation of red yeast rice product with several different isolates of the analyses, secondary citrinin metabolites was revealed with various levels. This case was exactly in accordance with the purpose of the research which to prove the citrinin levels of red yeast rice products with different isolates produced different levels of citrinin as well. The highest citrinin produced was of the red yeast rice products by IPB-B ie 790.17 ng / ml and the lowest citrinin levels of the red yeast rice products was generated by Lipi F01 ie 0.85 ng/ml. The results of citrinin content measurement of all red yeast rice products can be seen in Table 3.

Table 3. Citrinin levels of red yeast rice product with some isolates

| Red Yeast Rice Product | Citrinin Levels |
|------------------------|-----------------|
| Lipi F147              | 3.59 ng/ml      |
| IPB-A                  | 369.97 ng/ml    |
| ITB                    | 70.48 ng/ml     |
| Lipi F01               | 0.85 ng/ml      |
| IPB-B                  | 790.17 ng/ml    |

The differences of citrinin levels were generated due to several factors, which among others was different isolates Monascus which contributed in having different genes as well as capable of producing secondary metabolites during different fermentation, the source of carbon and nitrogen, as well as the environment (pH, temperature and oxygen intake), so that it was understood why the citrinin content of the five red yeast rice products was indifference. The citrinin content allowed to consume is as a maximum of 200 mg / mL [4]. While the citrinin levels of the five red yeast rice products are far below the consumed maximum levels so that those five red yeast rice products are relatively safe to use and be publicly consumed.
Figure 2. Citrinin Concentration of various *Monascus purpureus*

4. Conclusion
The analyses of citrinin levels using the HPLC fermentation method of red yeast rice products with some Monascus purpureus isolates has a retention time approached citrinin standards so that it can be concluded that the fermented product contains citrinin secondary metabolites and the outcome of citrinin levels of some fermentation products of red yeast rice has different levels which were Lipi F147 3.59 ng / ml, ITB 70.48 ng / ml, IPB-B 790.17 ng / ml, Lipi F01 0.85 ng / ml and IPB-A 369.97 ng / ml.

So some isolates of different sources and breeders used can influence the citrinin levels produced. The highest citrinin levels produced by fermentation of red yeast rice products were from IPB-B isolates. The citrinin levels produced was far below the consumed maximum of citrinin levels so that the citrinin levels produced by all isolates were considered not to cause nephrotoxic and hepatotoxic effects which means those are publicly safe to be consumed.

5. References
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