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HPLC analysis on citrinin and lovastatin and their genes expression of two selected *Monascus purpureus* strains

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Abstract. A physiological and genetic study was conducted to profile two selected of *Monascus purpureus* strains based on lovastatin produce which was analyzed by HPLC and its gene expression. After completed on producing angkak, a fermented rice product, by each *M. purpureus* strains at 25°C, HPLC analysis on the yield of citrinin and lovastatin was conducted. The expression of citrinin or lovastatin encoding gene of the two strains was analyzed by using reverse transverse (RT)-PCR of its RNA. Each RNA was extracted from mycelial masses by using Trizol (Invitrogen) kit. The primers used for RT-PCR were pksCT and *LovB* for citrinin and lovastatin encoding gene respectively. The results showed that the two strains were different physiologically and genetically. *M. purpureus* Skw2 was high potency as a good producer of lovastatin (38.5 mg/g). Unfortunately, this strain still produced citrinin although at low concentration (13.03 ppm). While *M. purpureus* Serasi was not good producer of lovastatin (7.4 mg/g). However, based on safety concern on health, *M. purpureus* Serasi was potential for future use for its benefits application because of its negative result based on the citrinin analysis result. Therefore, enhancement of lovastatin produce would be of one of the interests for further research of this *Monascus* strain.

1. Introduction

Monascus species are fungi belong to Monascaceae, Eurotiales, Ascomycota [1]. These fungi are well-known important fungi particularly related with traditional fermentation in China which has been ancient, thousands years ago. Distinctive value of food and also its usage as drug ingredients have been documented in ancient history [1, 2, 3]. *M. pilosus*, *M. purpureus* and *M. ruber* [4] are the major *Monascus* species known. *Monascus* strains are commonly found in traditional oriental food [5].

At recent, many researches are aimed at its potential in producing bioactive compounds such as secondary metabolites, pigments (yellow, orange and red pigments) [6, 7], anti hypercholesterolemic agents, (monacolin K and hypotensive agent, γ- amino butyric acid (GABA) [8, 9, 10, 11] and antibacterial substances including pigment and citrinin (as monascidin A) [12]. Much attention must be paid to citrinin since this toxic substance becomes as a limiting factor of acceptance commercially based on health safety concern. European countries have decided that 2 ppm are maximum concentration of citrinin as a standard maximum consumed based on health concern, while Japan administers at 0.2 ppm [13].

We reported our work on potential capacity based on citrinin and lovastatin analysed by HPLC and its gene expression of two selected *M. purpureus* strains. These two strains were local fungi originated from angkak or *Monascus* fermented rice (MFR) collected from Indonesian market.

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2. Materials and Methods

2.1. Monascus purpureus Strain

Two selected *Monascus purpureus* strains coded Skw2 and Serasi were used in this study. These two strains were originated from angkak sample purchased from market in Singkawang, Indonesia. These fungi were maintained in MEA 2% (Difco) agar slants.

2.2. Cultivation and purification

Fungal cultivation was prepared to refresh *Monascus* culture. Purification was carried out to ensure that the culture was free from contaminants. The purification process used MEA 2% with the addition of chloramphenicol 100 µg/ml. Cultivation on broth media (+ antibiotics chloramphenicol 100 µg/ml) was prepared for DNA extraction.

2.3. Making fermented rice product in the laboratory

A total of 25 grams of rice was soaked in water for one night and then sterilized by using autoclave for 15 minutes, at 121°C, one psi. *Monascus* inoculum 2 weeks old was inoculated as much as 5 ml (10% of rice medium) to rice which was previously placed in a Petri dish and homogenized. Incubation was carried out on an incubator at a temperature of 30°C for two weeks and then prepared for extracting citrinin and lovastatin.

2.4. HPLC analysis of citrinin

Extraction of the sample was prepared for analyzing citrinin content by using HPLC. The extraction was accomplished by dissolving 1.25 g of MFR with 50 ml ethanol 70% (pH 8.0) and homogenized by using the magnetic stirrer for three hours at 15-25˚C, filtered with 0,45 µm filter paper. The 20 µl of extract was injected in the column C18 and detector UV-Vis.

2.5. HPLC analysis of lovastatin

Before performing HPLC analysis of lovastatin, extraction process was carried. Add one gram of MFR was extracted by solution of 2 ml acetonitrile and 0.1 ml 0.1% phosphoric acid with 30 minute incubation time. The solute was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was concentrated by freeze dried and then diluted by mobile phase (acetonitrile+ 0.1% phosphoric acid (65:35). HPLC analysis was accomplished by injection 20 µl of lovastatin extract by using C18 column and a λ235nm UV detector with elusion rate at 1 ml/minute at 45°C.

2.6. RNA extraction

Fungal mass from three-day-old cultures that had previously been cultivated in media broth was used for DNA extraction. The RNA extraction procedure was performed using Trizol (Invitrogen) and all equipment is treated with DEPC to avoid RNAse activity.

2.7. RT-PCR

RT-PCR was performed to detect the transcript of *pksCT*. *SuperScript*™ One-Step RT-PCR System with *Platinum*™ Taq DNA Polymerase was applied to make cDNA from RNA produced which corresponded with citrinin or lovastatin encoding gene. Primer *pksCT* F (5'- TGCCATGCGCTTCCACCAGG -3'), and Primer *pksCT* R (5'- CACCATGCTCGCGTTGCTGC-3'). The conditions for RT-PCR amplication were as follows: denaturation stage: 95°C, 10 minutes; 30 cycles consisting of 94°C, 30 seconds, annealing 60°C 1 minute and extension 72°C 1 minute; then the last one was a single extension at 72°C for 7 minutes.

*2.8. Gel **e**lectrophoresis*

To find out the results of RT-PCR cDNA product, visualization was carried out under UV illumination on the electrophoresis gel. The data printed on photo paper and digital data that were made then analyzed to see the quality of the results obtained.

2.9. Measurement of quantity of cDNA

After accomplished RT-PCR, cDNA was run by gel electrophoresis. The gel was illuminated under UV and photographed by using PrintGraph. The cDNA quantity was analyzed by using CS analyzer v.2.08c.

3. Result and Discussion

The result was showing the results of HPLC analysis on the content of citrinin and angkak made in the laboratory using a starter of two strains, *M. purpureus* SKW2 and *M. purpureus* Serasi. The results showed that lovastatin and citrinin content in MFR using SKW2 starter *M. purpureus* were 38.5 mg/g (or 3.85 %) and 13.03 µg/g (or ppm) respectively, while using *M. purpureus* Serasi was 7.4 mg/g (or 0.74 %) and 0 µg/g respectively (Table 1, Table 2). Commercial product of MFR were reported containing citrinin. Table 1 shows *M. purpureus* Skw2 was higher citrinin content compared to commercial strains. But *M. purpureus* Serasi showed its superiority by zero citrinin content.

These results indicated that although MFR by *M. purpureus* SKW2 showed higher lovastatin content compare to the other collection strains, except to *A. terreus* UV 1718 (Table 2), but it was still produced more citrinin content above the health safety threshold of 0.2 ppm. On the contrary, the citrinin content of MFR by *M. purpureus* Serasi was null or undetectable but has a fairly low lovastatin content (7.4 mg/g). Accordingly, the MFR that meets the health safety standard is fermented by *M. purpureus* Serasi. Therefore, on regard of its low lovastatin content, further research is one of the interests in increasing lovastatin production by this fungus. On the contrary, the use of *M. purpureus* SKW2 was also interesting in reducing the level of citrinin content to below safety level allowed.

These results were in fact achieved on strain potency based. Other methods for enhancement bioactive production would be interested on physiological. Acidic condition or low pH of substrate for restricting citrinin production has been reported [15]. Addition of 0.3% ethanol has been succesfully reducing citrinin production, but production improvement occurred to monacolin K and c-aminobutyric acid (GABA) [16]. Genetic engineered *M. purpureus* by knock down of *pksCT* gene was reported in successful.

Table 1. Citrinin content of fermented rice product.

***Monascus* Strain used**	**Citrinin Content**	**Reference**
***Monascus* spp.**	0.47–11.82 µg/capsule	[18]
***Monascus* spp.**	0.2–140 mg/kg	[19]
***Monascus* spp.**	4.2–25.1 mg/kg	[20]
***M. purpureus* SKW2**	13.03 mg/kg	This paper
***M. purpureus* Serasi**	0	This paper

Table 2. List of lovastatin production in solid state fermentations.

Fungal Strain	**Lovastatin concentration**	**Incubation time (days)**	**Substrate**	**Reference**
***M. purpureus* Skw2**	38.5 mg/g	10	rice	This paper
***M. purpureus* Serasi**	7.4 mg/g	10	rice	This paper
A. terreus ATCC 74135	0.26 mg/g	8	Rice straw	[20]
A. terreus JPM 3	0.982.3 mg/g	15	Wheat bran	[21]
***A. terreus* ATCC 20542**	2.9 mg/g dry	11	Rice	[22]
***A. flavipes* BICC 5174**	16.65 mg/ g	6	Wheat bran	[23]
***M. purpureus* MTCC 369**	2.83 mg/g	14	Rice	[24]
***A. terreus* UV 1718**	3723.4 ± 49 µg/g	10	Wheat bran	[25]

Figure 1 shows cDNA products of the two *Monascus* strains were produced by RT-PCR of RNA transcript of *pksCT* gene which it is responsible for citrinin biosynthesis. Therefore, this results indicated that genetically, the two *Monascus* strain were the citrinin producer (Figure 1, Table 3). Probably during citrinin biosynthesis, there more factors including pH or medium which it made citrinin production was differentially expressed. This was similarly happened to *lovB* gene expression that the cDNA products was more expressed on *M. purpureus* Serasi (Figure 2, Table 3).

Figure 1. Photography on gel electrophoresis of cDNA product citrinin coding gene, *pksCT* by UV-illuminated RT-PCR method from two *Monascus purpureus* strains namely SKW2 and Serasi. The arrow shows the cDNA product of the pxCT gene.

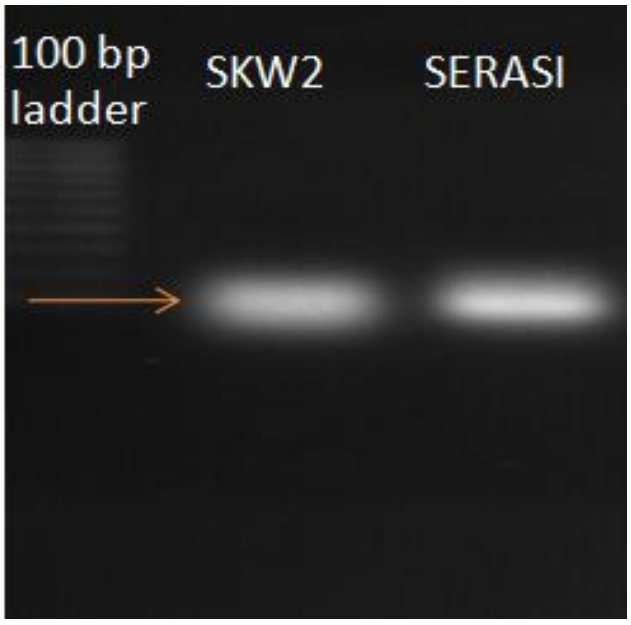

Figure 2. Photography of gel electrophoresis of cDNA produced by RT-PCR of *LovB* gene expression. Under UV illumination from two *Monascus purpureus* strains, SKW2 and Serasi. The arrow shows the *LovB* cDNA gene product.

Table 3. cDNA produced by RT-PCR of *LovB* and *pksCT* gene expression analyzed by CS analyzer.

Substance	*Monascus purpureus* strain	
	Skw2	**Serasi**
citrinin	10559	24468
lovastatin	202315	212716

M. purpureus-fermented rice (MFR) is an ordinarily used food colorant and dietary material in Asian countries such as China, Japan, Taiwan, Thailand, and Philippines. In these countries, MFR is usually used practically as a traditional preservative for storing fish and meat. Moreover, its flavor, aromatic fragrance, and vivid red color cause MFR regularly used as a seasoning agent for a variety of Chinese plates. Many examples of recipes using MFR are roast pork, roast duck, fermented bean curd, well-preserved dry fish, and vegetable pork stew. MFR is also common for its use as starter culture for brewing red rice wine. Other familiar names, including Hung-Chu, Hong Qu, Ang-kak, Ankak rice, Red Yeast rice, Red Mold rice, and Beni-Koji, are used as synonyms for this rice fermented rice product. MFR is designated as a mild folk medicine, which has the therapeutic effect to promote the health of cardiovascular system. Its pharmaceutical purpose has been definite in the ancient Chinese pharmacopeia, Ben Cao Gang Mu composed by Shi-Zhen Li (1518–1593 AD) [2, 3, 4, 5].

4. Conclusion

HPLC analysis results of citrinin and lovastatin showed that fermented rice products without citrinin could be achieved by using *M. purpureus* Serasi strain, but with a low lovastatin content. Whereas by using high *M. purpureus* SKW2 strain lovastatin content, but still produced high citrinin content. RT-PCR analysis showed that citrinin and lovastatin well expressed by its *pksCT* and *LovB* gene expression from the two strains tested.

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