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FULL PAPER

Simultaneous detection of monacolins and citrinin of angkak produced by *Monascus purpureus* strains using Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

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Abstract

Angkak or red mold rice is produced from the fermentation of white rice using *Monascus purpureus,* which results in the red color of the fermented rice. Angkak has been used as a natural coloring agent for food, spices, and medicine. The active compound in angkak that contributes to lower blood cholesterol is known as monacolin (monacolin K). However, the presence of citrinin, the byproduct of angkak fermentation, needs to be considered as it can cause hepato-nephrotoxic mycotoxin. The contents of pigments, monacolins, and citrinin as secondary metabolites depends on the *Monascus* strain and fermentation conditions. This study aims to analyze simultaneously monacolins and citrinin in angkak produced by *M. purpureus* strains using Liquid Chromatography-Mass Spectrometry (LC-MS/MS). The angkak was prepared by fermentation using *M. purpureus* FNCC 6008 and *M. purpureus* JK2. A total of 10^7 *M. purpureus* spores/mL was inoculated into the rice. The fermentation was carried out at room temperature (25-30^{o}C) for 14 days. The detection using LC-MS/MS showed that the monacolin K in angkak from both strains was below LOQ (< limit of quantification). The analysis of citrinin content in angkak showed that JK2 strain produced lower citrinin (1.10±0.021µg/g) compared to FNCC 6008 (3.01±0.072 µg/g). The other monacolins found in angkak from both strains were including dehydromonacolin K, monacolin J (qualitative), and mevastatin. Based on t-test, the amount of both mevastatin and citrinin in angkak produced by two different strains were significantly different. In contrast, the amount of dehydromonacolin K in both angkak was comparable. The simultaneous detection result of LC-MS/MS could determine the choice of *Monascus* strains quickly. JK2 strain was considered as safe, thus it could be chosen to be applied to food products.

1. Introduction

Angkak or red mold rice (RMR) is a product of *M. purpureus* fermentation via solid-state fermentation using rice as the primary media. There are many names to refer to angkak, in China and Taiwan angkak is called "Hong Qu", "Hon-Chi", "Anka", or "Ang-kak"; whereas the Japanese people use the name of "Beni Koji" or "red Koji". In America and Europe angkak is called as "red rice", "red mold rice" or "red Chinese rice". However, the name red yeast rice (RYR) is often used in many publications and commercial products even though it is not an appropriate name for filamentous fungi (Wang and Lin, 2007; Song *et al*., 2019).

Monascus can produce beneficial secondary metabolites, such as pigments (food-grade colorants), monacolins (cholesterol-lowering agents), γ-butyric acid (an antihypertensive substance), dimerumic acid (an antioxidant) (Chung *et al*., 2009, Chen *et al*., 2015; Song *et al*., 2019). The red pigment of angkak is mainly used as a natural coloring for food, but it can also be used as a preservative in meat and fish products (Wang and Lin, 2007; Chen *et al*., 2015), cheese (Devi and Meera, 2015) as well as an agent to increase platelets in dengue hemorrhagic fever (Danuri, 2008; Ristiarini *et al*.,

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2017a). Monacolins can be used as a cholesterol-lowering agent (Campbell and Vederas, 2010; Heinz *et al*., 2016) by inhibiting the enzyme of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase in cholesterol biosynthesis (Song *et al*., 2012; Heinz *et al*., 2016). Approximately 14 structural forms of monacolin have been identified from the genus of *Monascus*, namely: monacolin K (monacolin K lactone form; monacolin K acid form), monacolin J, monacolin J acid form, monacolin L, monacolin L acid form, monacolin M, monacolin M acid form, monacolin X, monacolin X acid form, and dehydromonacolin K, dihydromonacolin L, compactin, 3α hydroxy 3,5 dihydromonacolin L (Li *et al*., 2004; Avula *et al*., 2014).

Citrinin was reportedly detected in various *M. purpureus* fermented products (Vuković *et al*., 2017). Citrinin, a mycotoxin which has harmful effects on the function and structure of the kidney and is able to modify liver metabolism (Mornar *et al*., 2013; Ji *et al*., 2015). The maximum level of citrinin in red yeast rice-based food supplements is 2000 µg/kg (2 ppm) (Commission Regulation (EU) No 212/2014 of 6 March 2014). The presence of citrinin in food products needs to be considered to ensure product safety.

The content of secondary metabolites of angkak depends on the *Monascus* strain and fermentation conditions. Detection of the amount of monacolin K and citrinin has been carried out on various *Monascus* fermented products in the form of angkak and food supplements. Detection of monacolin K (Li *et al*., 2004; Song, *et al*., 2012; Di Donna *et al*., 2018) and citrinin (Ji *et al*., 2015; Vuković *et al*., 2017; Vuković *et al*., 2019) can be carried out individually or simultaneously (Nigović *et al*., 2013; Mornar *et al*., 2013; Avula *et al*., 2014). Chromatographic techniques are often used in the detection of monacolin K and/or citrinin (Avula *et al*., 2014). Although the chromatographic technique is sensitive and selective (Nigovic*et al*., 2013), it is time-consuming and requires expensive equipment and solvents. Chromatographic techniques that have been developed to simultaneously detect monacolin K and citrinin that are fast and accurate include: chromatography electromagnetic capillary chromatography (Nigovic*et al*., 2013), liquid chromatography with array diode detectors (DAD) and/ or with mass spectrometry (MS) (Mornar *et al*., 2013) and liquid chromatography-DAD-QtoF-MS (Avula *et al*., 2014).

Fast detection of monacolin K and/or citrinin in *Monascus* fermentation products have been carried out for screening products from adulteration, including labels that do not match the contents of product (Li *et al*., 2004; Song *et al*., 2012, Monar *et al*., 2013; Avula *et al*., 2014; Di Donna *et al*., 2018; Vuković *et al*., 2019). Simultaneous detection of monacolin K and citrinin of angkak produced by *M. purpureus* strains has not been widely reported. This study aimed to analyze simultaneously monacolins and citrinin in angkak produced by *M. purpureus* strains using Liquid Chromatography-Mass Spectrometry (LC-MS/MS).

2. Materials and methods

2.1 Microorganism

There were three strains of *M. purpureus* used in this study. The strain of *M. purpureus* FNCC 6008 was purchased from the collection of Food and Nutrition Collection Center (FNCC), Universitas Gadjah Mada Indonesia; *M. purpureus* JK2 strain was obtained from Widya Mandala University, Surabaya, Indonesia that was isolated from angkak at a pharmacy shop in Jakarta; and *M. purpureus* HD-CC 001 was obtained from the collection of Microbiology Laboratory, School of Pharmacy, Bandung Institute of Technology. The culture strains were inoculated into potato dextrose agar (PDA; Merck) slants, and then incubated at room temperature (25-30^{o}C) for 14 days. Spore suspensions were prepared by the addition of sterilized water into the grown culture on PDA agar slant. The spore concentration was 10^{7} spores per mL as counted by using haemocytometer.

2.2 Solid-state fermentation

Solid-state fermentation was conducted by inoculating *M. purpureus* culture into the rice. The IR 64 rice was purchased from Giant supermarket in Yogyakarta. The rice was soaked in water (1:1) for 24 hours. After soaking the rice was rinsed and drained. A total of 100g of rice was put into a 500 mL erlenmeyer and sterilized at 121^{o}C for 15 mins and then cooled to room temperature. The sterilized rice was inoculated with 10mL spore suspension of *M. purpureus* (10^{7} spores/ mL). After cultivation at room temperature (25-30^{o}C) for 14 days, the angkak (fermented rice) was dried with cabinet dryer at 40°C for 5 hours (until the final moisture content of less than 10% was achieved). The dried angkak was blended to a fine powder before extraction.

2.3 Chemicals

Monacolin K (lovastatin) and citrinin standard were ordered from Sigma-Aldric RTC, WY, USA. Acetonitrile gradient grade and formic acid were purchased from Germany. Water used from the purification process (Evoqua).

2.4 Sample extraction

The sample extraction and analysis using LC-MS/MS followed Avula *et al*. (2014) and Di Dona *et al*. (2018) with modification. Approximately 0.04 g of angkak powder was extracted using 10 mL of acetonitrile in a sonicator (Branson 2200, USA) at 30°C for 30 mins. The obtained mixture was heated with a waterbath at 60° C for 1 hr and then centrifuged (Zenith Lab, LC-04S, China) at 3500 rpm for 10 min. The amount of 0.01g adsorbent (MgSO4: Na-Acetate, modification) was added into 1 mL supernatant and then centrifuged (Hitachi, Japan) at 10000 rpm for 5 mins. It was followed by filtration through nylon 0.2 µm (Agilent, USA). The filtrate (2µL) was injected into the LC-MS/MS.

2.5 Mass spectrometry condition

The sample analysis was carried out using LC-MS/MS, Thermo Scientific UHPLC instrument, Accela LC type 1250 (Thermo Fisher Scientific, USA). The chromatographic separation used Hypersil Gold (50 mm x 2.1 mm x 1.9 µm) column. Solvent A consisted of 0.1% formic acid in aquabidest and solvent B consisted of 0.1% formic acid in acetonitrile. The flow rate was set at 300 µl/min and the sample injection volume was 2µl. The linear gradient with the adjustment of the mobile phase was as follows: 0-0.6 min, 75% A; 0.6-3.0 min, 90% B; 3.0-4.0 min, 90% B; 4.0-4.5 min, 25% B and 4.5 -6.0 min, 75% A. The column was setat 30°C, and the autosampler compartment was set to 16°C.

The use of MS/MS Triple Q (quadrupole) TSQ Quantum Access Max mass spectrometers from Thermo Finnigan with ESI (electrospray ionization) as ion sources was controlled by TSQ Tune software which was operated in a positive ion mode. ESI ionization conditions were adjusted as following: 3kV spray voltage; evaporation temperature, 300°C; capillary temperature, 300°C; nitrogen as a sheath gas pressure, 40 psi; and Aux gas pressure, 10 psi with argon gas. Determination of quantity used the SRM (selected reaction monitoring) method. Monacolin K molecules were adjusted with precursor ions (m/z) 405, and product ion (m/z) 199 while the citrinin was adjusted with precursor ion (m/z) 251, and the product ion (m/z) 233.

2.6 Color measurement

Color measurement was conducted using chromameter CR 400 (Konica Minolta Co. Ltd., Osaka, Japan). White calibration using the white calibration plate was done before measuring. The ground samples (angkak) were poured in a cuvette. The measurement button was pressed after making sure the ready lamp ON. Measurement was done, and the data was displayed. The measurement was expressed in three parameters of L*, a* value, and b* value. The L* value is a lightness variable which has a value from 0 (black) to 100 (white). The a* indicates redness (+ value) or greenness (- value), whereas b* indicates yellowness (+ value) or blueness (- value).

2.7 Statistical analysis

The results were statistically analyzed using t-test.The statistical product and service solution (SPPS) statistical software version 22 was used for statistical analysis.

3. Results and discussion

3.1 Growth of Monascus purpureus in PDA medium

M. purpureus spore was grown on PDA medium and it germinated to form branched hyphae. A mass of hyphae is termed mycelium (Manan *et al*., 2017). In this study, the color development of 3 strains *M.*

Table 1. The color change of *M. purpureus* in PDA medium during incubation at room temperature (25-30°C) for 14 days

Incubation (days)	Colony color	Strain		
		FNCC 6008	JK2	HD-CC 001
1	White	+	+	+
2	White	++	++	+
3	White	++	++	+
4	White-yellowish	+++	+++	+
5	White-yellowish	+++	+++	+
6	White-yellowish	++++	++++	+
7	Orange	++++	+++++	+
8	Orange	+++++	+++++	+
9	Rich orange	+++++	+++++	+
10	Rich orange	+++++	++++++	+
11	Orange-red	++++++	++++++	+
12	Orang-red	++++++	++++++	++
13	Rich red	++++	+++++++	-
14	Rich red	++++	+++++++	-

+: few; ++: medium; +++: many; ++++: numerous

purpureus mycelium that incubated in PDA medium at room temperature (25-30°C) for 14 days was visually observed (Table 1). In the early stages of growth, mycelium was white, then it changed to vibrant orange and finally became red. The color development of *M. purpureus* mycelium followed Danuri (2008).

At the early stage, the colony was observed having white color until the third day of incubation. On the fourth day of incubation, the color production started and continued to increase along with incubation period (Dikshit and Tallapragada, 2011). However, this rapidly changedinto a vibrant orange (8-9 days) and later to a distinctly rich red color (14-16 days) (Manan *et al*., 2017; Tallapragada *et al*., 2017).

The strain of *M. purpureus* FNCC 6008 and JK2 produced yellow, orange, and red pigments with the red pigment being predominant. The red pigment of JK2 was stronger than that of FNCC 6008 at the final stage (14 days). The HD-CC 001 strain produced mycelium which was still white until incubation for 14 days, even though the closest part to the PDA medium appeared orange-red. The final color of 3 strains *M. purpureus* mycelium on

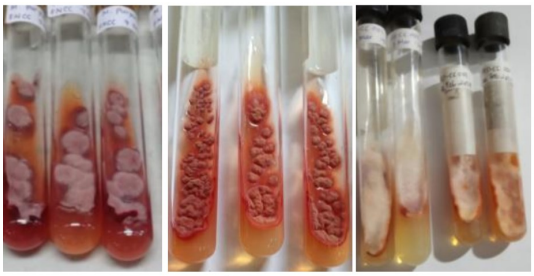

Figure 1. The mycelium pigment of *M. purpureus* strains FNCC 6008, JK2 and HD-CC 001 in PDA medium at room temperature (25-30°C) for 14 days incubation

14 days of incubation as shown in Figure 1.

The above result indicated that pigment production of HD-CC 001 strain was lower than strains FNCC 6008 and JK2. It was shown that pigment production depended on the strain. In the following part of the study, the strains FNCC 6008 and JK2 were used to produce angkak with rice as the substrate.

3.2 Solid-state fermentation

M. purpureus strains FNCC 6008 and JK2 was cultivated on sterilized rice at room temperature for 14 days. Based on the produced angkak, it could be visually observed that the JK2 strain gave more red pigment than FNCC 6008 strain (Figure 2). The starch content in the rice was the major carbon source during the growth of *M. purpureus.* Yang *et al*. (2015) reported that in rice medium, the biomass of *M. purpureus* increased rapidly during the first stage of the logarithmic growth phase (approximately on the 4thday), and the synthesis of pigment occurred rapidly in the near end of the

Figure 2. Angkak fermented by M. purpureus strains: a. FNCC 6008; b. JK2 at room temperature (25-30°C) for 14 days incubation

logarithmic growth phase (approximately on the 8thday).

The differences in the color of angkak indicated the differences in pigment synthesis during fermentation (Table 2). Significant differences were found in lightness (L*), yellowness (b*), and redness (a*) of angkak. Angkak fermented by FNCC 6008 possessed higher lightness (L*) and yellowness (b*), whereas angkak fermented by JK2 had higher redness (a*). The high value of lightness and yellowness, as well as the low redness, resulted in a pale red of angkak. In contrast, the low value of lightness and yellowness, as well as the high redness, showed an intense red of angkak (Ristiarini *et al*., 2017b). Angkak fermented by JK2 was significantly red (24.05±0.028 vs. 15.14±0.104, P = 0.000).

Table 2. Color value L*, a*, b* of angkak fermented by *M. purpureus* strain FNCC 6008 and JK2 at room temperature (25 -30°C) for 14 days incubation

Variable	FNCC 6008	JK2	P-value
L*	71.54±0.534	56.74±0.284	0
a*	15.14±0.104	24.05±0.028	0
b*	19.25±0.182	17.91±0.123	0

The difference in the color of angkak produced by the strains of FNCC 6008 and JK2 was associated with the differences in the ability to utilize glucose from glycolysis. Therefore, it affected the availability of acetyl -CoA, which could have an impact on the formation of secondary metabolites, including pigment production. On the eight days of fermentation, it was clear that the expression of acetyl-CoA carboxylase converts acetyl-CoA into malonyl-CoA, as a precursor of pigment synthesis. Increased formation of malonyl-CoA and less use of malonyl-CoA for fatty acid biosynthesis can provide more malonyl-CoA precursors for pigment biosynthesis (Yang *et al*., 2015).

Based on the previous reports, *M. purpureus* pigment biosynthesis involves two synthesis pathways, namely the polyketide pathway and the fatty acid pathway.

Pigment formation starts from the formation of chromophore hexaketide derived from 1 mole of acetate with 5 moles of malonate by the enzyme polyketide synthase through the polyketide pathway. Simultaneously, through the biosynthetic pathway of fatty acids, the medium-chain fatty acid, such as octanoic acid forms ß-keto acids. Beta-keto acids bind to the chromophore structure through trans-esterification reactions to form monascorubrin orange pigments (or rubropunctatin by binding with hexanoic acid). The reduction of monascorubrin orange pigment forms ankaflavin yellow pigment (or monascin from rubropunctatin). The amination reaction of the orange pigment (monascorubrin) with NH_3 derived from glutamic acid forms a red color (monascorubramine and rubropunctamine) (Hajjaj *et al*., 2000; Chen *et al*., 2015).

3.3 Monacolins and Citrinin Detection by LC-MS/MS

Detection of monacolin K and citrinin content in angkak powder fermented by FNCC 6008 and JK2 was carried out using LC-MS/MS. The monacolin K (m/z 405) and citrinin (m/z 251) in angkak were identified by comparing the chromatograms obtained from both samples with the chromatograms from the standard monacolin K and citrinin (Figure 3). Using the LC-MS/MS, various compounds such as dehydromonacolin K (m/z 387), mevastatin (compactin m/z 391), monacolin J (m/z 321, qualitative) (Avula *et al*., 2014) were also detected in both angkak powder samples. The number of various compounds detected by LC-MS/MS was shown in Table 3. Detection of several compounds with a single run at the same time requires a smaller number of samples and solvents, shorter time, and more sensitive high resolution (Monar *et al*., 2013). LC-MS/MS method obtains a high concentration of analytes (Song *et al*., 2012), informative detection (Ajdari *et al*., 2011) and provides very high sensitivity and accuracy (Vuković *et al*., 2019). The detection of specificity was determined by mass spectrometry (Avula *et al*., 2014; Di Dona *et al*., 2018).

As shown in Table 3, the angkak powder produced with FNCC 6008 and JK2 had a low content of monacolin K, as the analysis using LC-MS/MS showed that the monacolin K from both samples were under the limit of quantification (<LOQ). This indicated that the ability of both strains in synthesizing monacolin K was low. Monacolin K level of various *M. purpureus s*train in rice medium that analyzed by LC-MS/MS was vary in amount, NTU 601 produces 530±32 (µg/g), BCRC 31499 produces 119±21 (µg/g) (Lee *et al*., 2006), while angkak using FTC5391 did not contain monacolin K (Adjari *et al*., 2011).

The monacolin K was converted into dehydromonacolin K via dehydration, during storage. It eliminates the therapeutic effects of monacolin K (Jirasatid *et al*., 2013). The amount of dehydromonacolin K in both angkak was not significantly different. JK2 strain was able to produce higher mevastatin than FNCC 6008. Considering its structure and biosynthesis, the mevastatin or ML-236A or known as compactin, is similar to lovastatin (monacolin K). It also acts as an inhibitor of HMG-CoA and is highly effective in lowering plasma cholesterol levels in animals and man

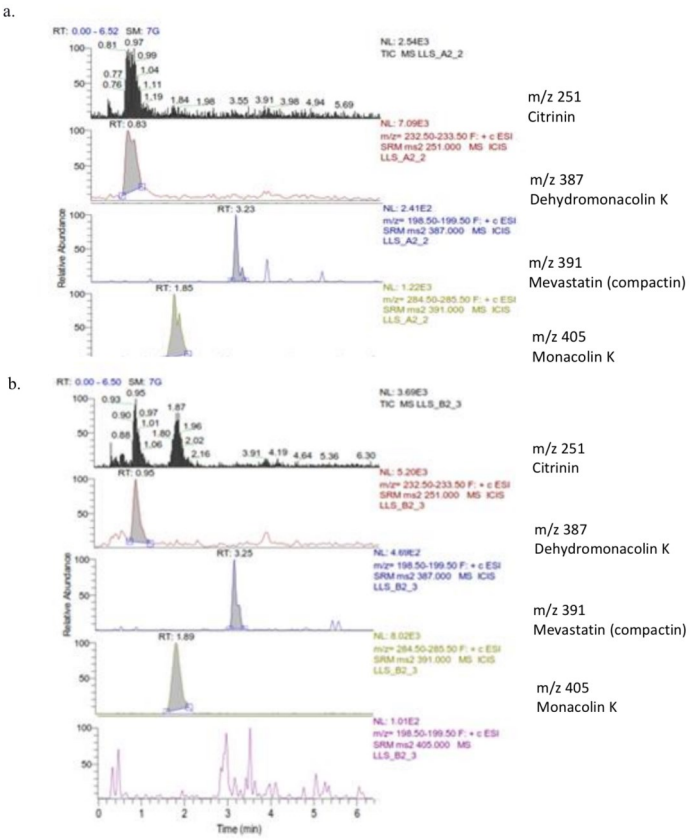

Figure 3. Chromatograms of angkak fermented by *M. purpureus* strains: a. FNCC 6008; b. JK2

Table 3. Quantitative analysis of angkak fermented by *M. purpureus* strain FNCC 6008 and JK2 at room temperature (25 -30°C) for 14 days incubation using LC-MS/MS

Compounds (µg/g)	FNCC 6008	JK2	P-value
Monacolin K	< LOQ	< LOQ	-
Dehydromonacolin K	74.62±2.161	71.82±1.245	0.14
Mevastatin	86.60±2.753	183.26±1.108	0
Citrinin	3.01±0.072	1.10±0.021	0

(Endo *et al*., 1985; Campbell and Vederas, 2010).

Analysis of the citrinin concentration in angkak powder showed that the strain JK2 produced lower concentration of citrinin (1.10±0.021 µg/g) compared to the strain FNCC 6008 (3.01±0.072 µg/g). The citrinin concentration in both samples was significantly different. Lee *et al*. (2007) reported that *M. purpureus* NTU 568 produce citrinin in angkak 1.89 ppm, NTU 601 0.46 ppm and NTU 301 0.37 ppm, while strain TISTR3541

produce citrinin 0.26 ppm (Adjari *et al*., 2011), it was detected by LC-MS/MS. Ristiarini *et al*. (2017b) reported the citrinin content of the commercial angkak from several region in Indonesia ranged from 17.94 ppm to 142.74 ppm.

The citrinin concentration in angkak fermented by *M. purpureus* JK2 strain was under the maximum level of citrinin allowed in foods based on the fermented rice with *M. purpureus* according to EU 212/2014 (2000 µg/kg or 2µg/g). Although Lee *et al*. (2010) reported that the concentration of 200 ppm citrinin in a *Monascus* fermentation product did not affect liver and kidney function as well as cause nephrotoxicity and hepatotoxicity in the animal test using rat, citrinin concentration of 2 ppm (2µg/g) was considered as a safe concentration. Therefore, the JK2 strain was considered as safe to be chosen and applied to food production.

4. Conclusion

The simultaneous detection of monacolin K and citrinin on angkak using LC-MS/MS was able to be carried out quickly. In addition, LC-MS/MS could be used to detect other monacolins. The *M. purpureus* strain JK2 produced citrinin lower than FNCC 6008, so it was preferable considering the safety aspect. However, the ability of both strains to produce monacolin K was still very low.

Conflict of interest

The authors declare no conflict of interest.

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