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Separation and analysis of *Monascus* yellow pigment produced on durian seed substrate

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Abstract

This research was aimed to separate and analyse *Monascus* yellow pigment (MYP) with chromatography. MYP was in a mixture with red and orange pigments. MYP separation was performed in a silica gel column with eluent of ethyl acetate: ethanol: water = 90: 25: 4. The separated MYP were then subjected to analysis by using spectrophotometer, TLC, LC-PDA and LC-MS/MS. The results revealed that the separated MYP has a maximum absorption at 386 nm. Out of five MYP compounds detected by using LC-MS/MS, two of them were well known yellow pigment compounds, monascin and ankaflavin. Monascin and yellow II were the major pigment compounds in MYP.

1. Introduction

Monascus pigments are secondary metabolites of *Monascus* fungi biosynthesized through the polyketide pathway. It has been used as a natural food colourant, food supplement, and in traditional medicine. *Monascus* pigments consist of many pigment compounds which are categorized into three groups i.e. red, orange and yellow pigments. A total of six well-known pigment compounds are monascorubramine and rubropunctamine (red pigments), monascorubrin and rubropunctatin (orange pigments), monascin and ankaflavin (yellow pigments) (Feng *et al*., 2012; Srianta *et al*., 2014; Agboyibor *et al*., 2018). Until 2011, thirty-nine new pigment compounds have been identified (Feng *et al*., 2012).

Rice is commonly used as solid-state fermentation substrate in *Monascus* pigments production. Non-rice substrates have been developed in the *Monascus* pigment production e.g. corn, adlay, sorghum, dioscorea, jackfruit seed and durian seed (Pattanagul *et al*., 2007; Babitha *et al*., 2007; Hsu *et al*., 2011; Srianta *et al*., 2012; Srianta *et al*., 2016). Our previous studies revealed that *Monascus purpureus* produced red, orange and yellow pigments. Exploration of *Monascus* yellow pigments is still limited. MYP is not only a potential natural colourant for biscuit, mayonnaise, wheat noodle, etc. (Srianta *et al*., 2014) but also as functional food ingredient and in medicine, because MYP has various positive health effects i.e. antioxidant, antidiabetes and anticancer (Hsu *et al*., 2011; Chen and Wu, 2016). Monascin and ankaflavin have antitumor activity (Akihisa *et al*., 2005; Su *et al*., 2005), antiobesity activity, anti-atherosclerosis effect (Wang *et al*., 2013; Liu *et al*., 2018), improve memory and learning ability (Lee *et al*., 2015). Chen and Wu (2016) reported that MYPs have promising potential for application in food and pharmaceutical industries based on their bioactivities.

Silica gel has been applied in *Monascus* pigments mixture separation through thin layer chromatography and column chromatography systems with various eluents i.e. chloroform, methanol, benzene, acetone and petroleum ether (Kim *et al*., 2006; Vidyalakshmi *et al*., 2009; Hsu *et al*., 2011; Lee *et al*., 2011). In practice, ethyl acetate, ethanol and water are often more preferred for food and pharmaceutical processing compared to other solvents due to their safety and affordability. HPLC is utilized to analyze various *Monascus* pigments because of its high sensitivity and multiple detection systems including ultraviolet, fluorescent, photodiode array and mass spectrometer detectors (Feng *et al*., 2012).

This research was aimed to separate and analyse MYP produced on durian seed substrate. This is the first

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investigation in MYP separation using ethyl acetate, ethanol and water.

2. Materials and methods

2.1 Microorganism, substrate and chemicals

Monascus purpureus M9 culture was used in the solid-state fermentation on durian seed substrate. The culture was maintained periodically on Potato Dextrose Agar. Durian seed was obtained from a durian processing unit in Surabaya (Indonesia). The durian seed is a by-product of the food industry and was used as the SSF substrate. All chemicals were chromatographic and analytical grades.

2.2 Pigments production through solid-state fermentation with durian seed substrate

Pigments production was performed through solid-state fermentation on durian seed substrate according to our previous research (Srianta *et al*., 2012). After peeling, cutting, soaking, sterilization at 121°C for 10 mins and cooling, the durian seed substrate was inoculated with *Monascus purpureus* starter culture, and then incubated at 30°C for 14 days. The fermented matter was dried at 45°C for 24 hrs, ground and sieved.

2.3 Monascus pigment separation

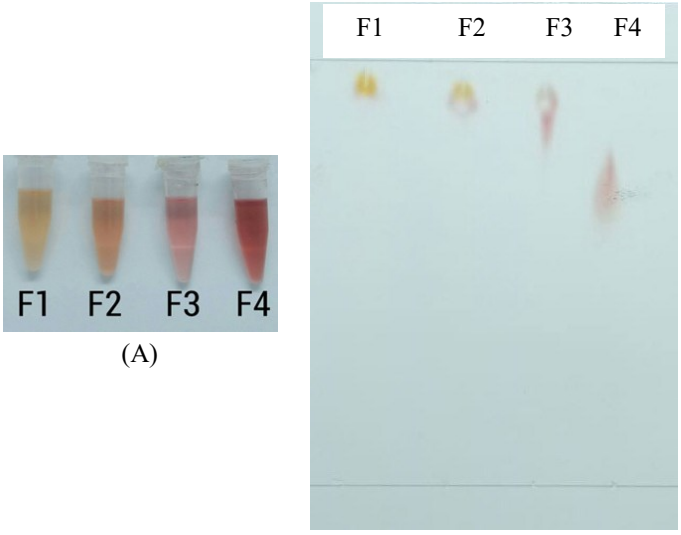
The *Monascus* pigment was extracted with soxhletation method in ethanolic solvent at a ratio of 1:50 for 2 hrs. The extract was then evaporated in a rotary evaporator. The evaporated extract was subjected to separation process. MYP separation was performed in a column chromatography (10 mm x 500 mm) with silica gel 60. The column was packed with silica gel by slurry method, then pre eluted with ethyl acetate: ethanol: water = 90:25:4. The extract was put into the column and eluted with ethyl acetate: ethanol: water = 90:25:4. The obtained fractions were subjected to analysis by using TLC and spectrophotometer. The MYP fraction was analyzed the pigment composition by using LC-MS/MS.

2.4 Pigment analysis

The separated pigments were subjected to the TLC analysis according to Nimnoi and Lumyong (2011) with modification, on silica gel F60 plate, with ethyl acetate: ethanol: water at 90:25:4. The separated MYP was scanned in a range of wavelength 380-600 nm with UV-Vis spectrophotometer (Shimadzu UV-1900, Japan). Pigment composition analysis was conducted by using LC-MS/MS (Shimadzu, Kyoto, Japan) with Q1 scan positive mode and SIM positive mode. The extract was filtered through a 0.2 µm PTFE filter membrane, put on to the autosampler. Pigments were separated on a Cosmosil 5 C18-MS-II (4.6 i.d. x 150 mm) column, with an isocratic program of mobile phase consists of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid (60:40 vol/vol), flow rate of 0.5 mL/min; oven temperature of 40°C, run time for 30 min. Pigment compounds were detected by MS/MS using electro-spray ionization in the positive ion mode, scan range: m/z 100 to 1000.

3. Results and discussion

In the separation of the pigments by using column chromatography, 4 fractions (F1 F2, F3 and F4) with different colours have been derived. The first fraction was MYP target, while other fractions were orange and red pigments. The MYP has the highest retention factor, followed by F2, F3 and F4. Those fractions and pigments profile on TLC shown in Figure 1. Those profiles describe that MYP was the pigments with the lowest polarity among the others. Although the chemical structure of *Monascus* pigments consists of β-ketoacid and chromophore, MYP compounds contain less polar groups than those of red pigments. The presence of $–NH_3$ group can increase the polarity of red pigments.



(A) (B)

Figure 1. Picture of 4 separated fractions of *Monascus* pigments (A) and their TLC chromatogram (B). F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

Figure 2 shows the fractions scanning by using spectrophotometer at 380-700 nm. F1, the MYP, has a λ max at 386 nm that was in yellow spectrum. F3 and F4 have 2 peaks in the orange and red spectrum. Those reflect that contain a mixture of orange and red pigments. This is the first investigation on the pigment compounds composition of MYP by using the LC-MS/MS. The LC-MS chromatograms and pigment composition of the extracts are presented in Figure 3 and Table 1, respectively. Five MYP compounds were detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and Yellow II.

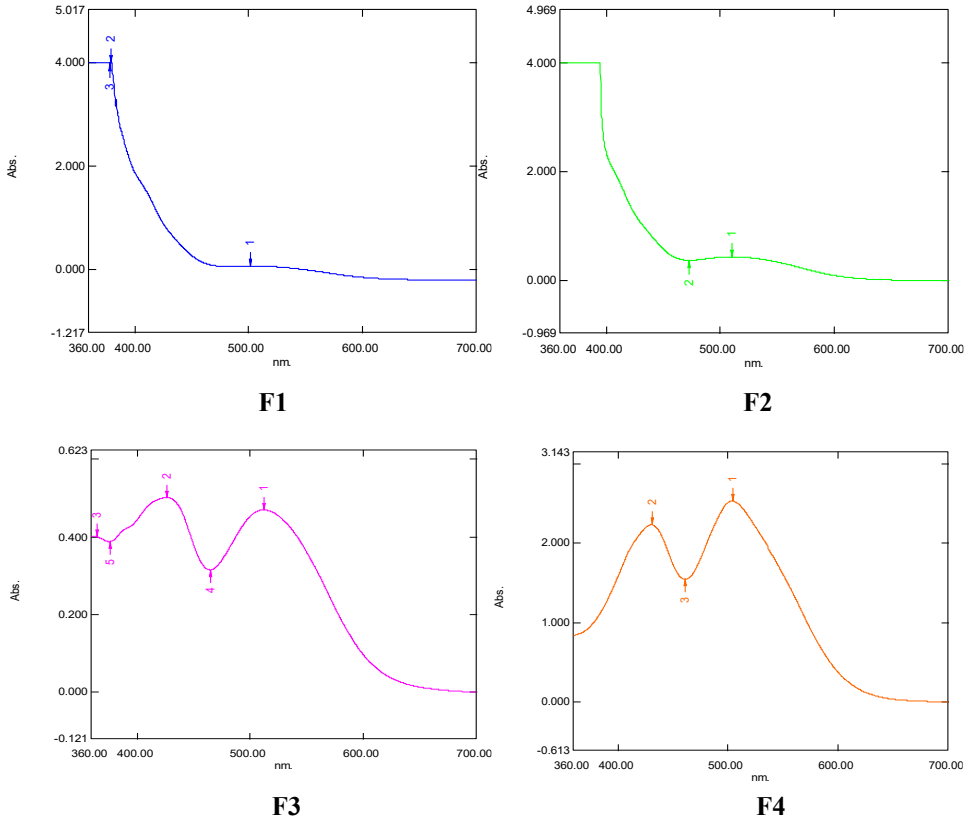


Figure 2. Scanning results of separated *Monascus* pigment fractions by using spectrophotometer. F1: Fraction 1; F2: Fraction 2; F3: Fraction 3; F4: Fraction 4.

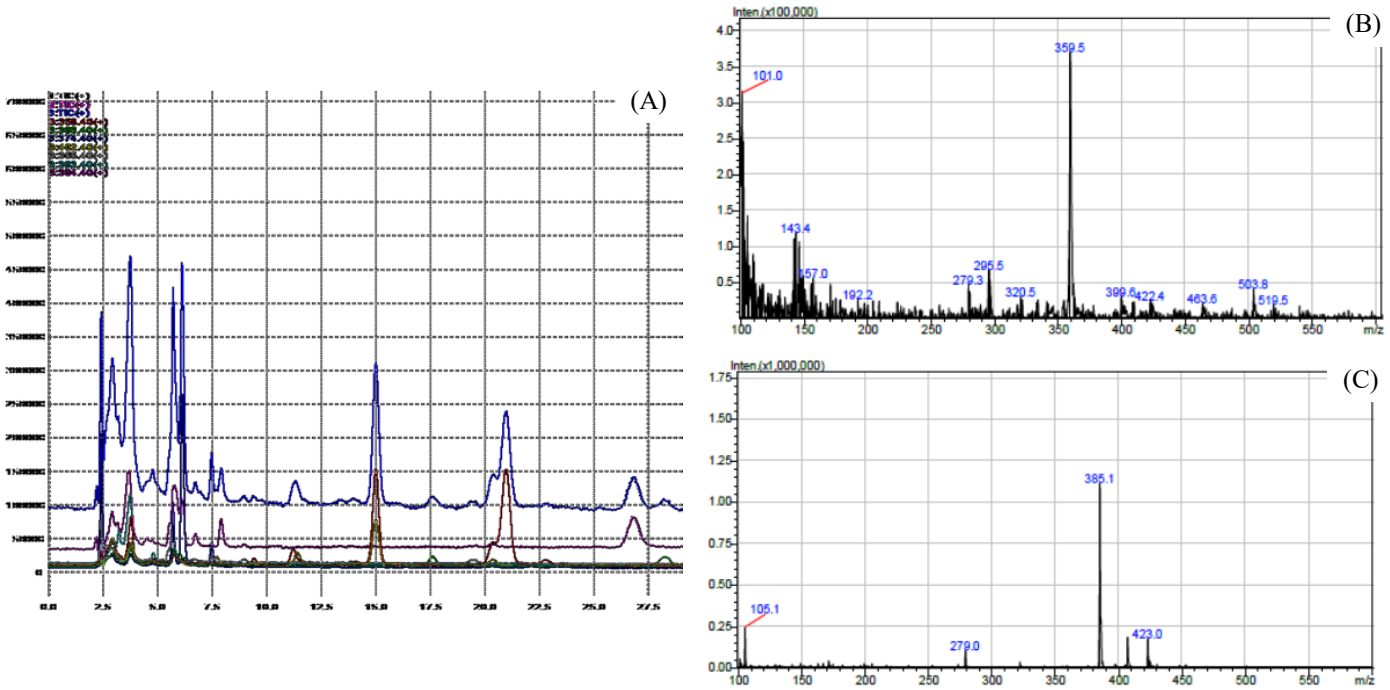


Figure 3. LC-MS analysis results: Total Ion Chromatogram of MYP (A) and representatives of the chromatogram of major MYP monascin (B) and yellow II (C).

Table 1. MYP composition

MYP compound	Content (%)
Monascin	59.81
Ankaflavin	6.32
XanthomonasinA	n.d.
XanthomonasinB	0.67
Monascopyridin A	n.d.
Monascopyridin B	5.39
Yellow II	27.80

n.d. = not detected

Monascin and Yellow II were the major pigment compounds in the MYP with the relative amount of 59.81% and 27.80%, respectively The MYP composition depends on the pigment composition in *Monascus* Fermented Durian Seed (MFDS), extraction and separation processes.

Pigments in MFDS produced through a complex polyketide biosynthesis pathway in the *Monascus* fungi, but it is still unclear and controversy. Some researchers proposed that only orange pigments (rubropunctatin and monascorubramine) were biosynthetic, and the others were transformed from them chemically. In orange pigment rubropunctatin biosynthesis, it consist of the β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue. Yellow pigments can be transformed from orange pigments via oxidation. Our previous research revealed that solvent type affected the *Monascus* pigment composition. The ethanolic extract contains higher MYP than those in the mixture solvent of ethanol and water. This research used ethanol as the extraction solvent. In the separation process, the chromatographic system used has successfully separated MYP from the red and orange pigments.

Monascin, the major MYP compound in this research, is attractive for its strong bioactivities such as anticancer, antitumor, antidiabetic, antioxidative stress, antiinflammatory, antiobesity and showed antiatherogenic activities, and also improves memory and learning ability. On the other hand, there is no report on the yellow II bioactivity.

4. Conclusion

The MYP has been successfully separated in a column chromatography packed with silica gel 60 and eluent of ethyl acetate:ethanol:water=90:25:4. Five MYP compounds have been detected by using LC-MS/MS i.e. monascin, ankaflavin, xanthomonasin B, monascopyridine B and yellow II. Monascin and yellow II were the major pigment compounds in MYP.

Conflict of Interest

The authors declare no conflict of interest.

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