Pigments extraction from monascus-fermented durian seed

by Ignatius Srianta

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Abstract. Durian seed has been studied as a new substrate for *Monascus* solid state fermentation. *Monascuspurpureus* produce yellow, orange and red pigments during the fermentation. The purposes of this research were to study the pigment extraction from the *Monascus*-fermented durian seed (MFDS) by using ethanol and water at various ratios and to analyze the pigment composition of the extracted pigment. The extraction was conducted in a 250 mL Erlenmeyer with 1 g of powdered MFDS and 50 mL of solvent at various ethanol:water ratios (10:0; 9:1; 8:2; 7:3; 6:4 and 5:5), in a shaking waterbath at different temperature (30°C and 60°C), 100 rpm agitation for 2 hours. The extracts were subjected to pigment content analysis by using a spectrophotometer and thin layer chromatography (TLC). Extract with the highest pigment content was then subjected to pigment compounds detection by using liquid chromatography-mass spectrometry (LC-MS). The results showed that extraction at 30°C was more effective than that at 60°C. The lower the ethanol:water ratio until 7:3, the higher the pigment content extracted. However, the lower the ratio tends to lower pigment content. Interestingly, the lower ethanol:water ratio, more viscous extract resulted. The TLC analysis showed that the extracts contained various pigments. Consistent with those results of TLC, various pigment compounds detected by LC-MS.

1. Introduction

Monascuspurpureus fermentation on rice substrate has been produced traditionally for centuries in Asia. The products are commonly consumed as a natural food colorant, food flavoring, brewing agent, supplement, and in traditional medicine [1]. Monascuspurpureus produces pigments during the fermentation, which is categorized into 3 color group i.e. yellow, orange and red pigments. Six well-known pigment compounds are monascin and ankaflavin (yellow), rubropunctatin and monascorubrin (orange), rubropunctamine and monascorubramine (red). Many other pigment compounds e.g. xanthomonascin, monascopyridine, yellow II and monapilol have been reported [2-4]. Red pigment, as well as yellow pigment, has been used as a natural food colorant [5]. Various bioactivities of the pigment compounds have been also reported such as antioxidants, antiinflammation, anticancer, antimicrobes, antidiabetes, antiobesity, anticancer immunosuppressive and antimicrobes [2,3,6-13].

Our previous studies revealed that durian seed can be utilized as a new substrate of Monascuspurpureus fermentation. Monascuspurpureus grew well and produced pigments during the fermentation [14,15]. Miyake *et al* (2008) reported that the Monascus strain and composition of fermentation medium affected the pigment composition [16]. Srianta *et al* (2016) also reported that

different substrates affected pigment production and composition [3]. However, pigment extraction from MFDS and its composition has not been studied yet.

Many factors can affect the pigment extraction from a fermented material. There is no universal ideal technique in the extraction due to the diversity of compounds. The factors in employing extraction techniques are solvent type, solvent ratios, extraction temperature, extraction times and solid to liquid ratios. In practice, water and ethanol are often more preferred in food applications compared to other solvents due to their safety [17]. Acetonitrile, dimethyl sulphoxide and isopropanol may be used, but the only common solvent which extracts pigments more efficiently than ethanol is methanol, however, it is not recommended for food use because it is toxic [18].

The major Monascus pigment compounds are slightly polar molecules which soluble in ethanol, not soluble in water [19]. However, some pigment compounds of red pigment can react with the amine to form pigment compounds soluble in water [2]. Carvalho *et al* (2007) reported that ethanol:water ratio affects the pigment content extracted from Monascusfermented-rice. Other researchers also reported ethanol application in the monascus pigment extraction at various concentrations [20,21,22, 23]. Singgih *et al* (2014) reported that temperature and ethanol concentration affect the secondary metabolites extraction from Monascus fermented-rice. There is no report on the extracted pigment profile and composition with different ethanol:water ratios. Various methods have been developed for monascus pigment detection and identification [24].

The aims of this research were to study the effect of temperature and ethanol:water ratio on the pigment extraction from the *Monascus*-fermented durian seed; and to analyze the pigment composition of the extracted pigment.

2. Materials and method

2.1. Microorganism

Monascuspurpureus M9 culture was used in the fermentation. It was routinely cultured on PDA slant. Starter culture was prepared with inoculating 8 loops of the culture scrabbed from the PDA slant, then incubated at 30°C for 10 days. The starter culture was then used in the durian seed fermentation.

2.2. Durian seed fermentation.

The durian seed fermentation was performed according to [14]. The process steps were: washing, boiling in 5% Ca(OH)2 solution, peeling, cutting, sterilization, inoculation with 5% of starter culture, incubation at 30°C for 14 days, drying at 45°C for 24 hours, grinding and sieving through 40 mesh siever. The MFDS was used for the next step, pigment extraction.

2.3. Pigment extraction

An accurately weighed MFDS of about 1 g was extracted with different ethanol:water ratios of 10:0; 9:1; 8:2; 7:3; 6:4 and 5:5 at 30°C or 60°C at 100 rpm for 2 hours, then filtered through Whatman no. 1 filter paper. The extracts were subjected to pigment content, profile and composition analysis.

2.4. Pigment content analysis

Pigment content analysis was conducted by using spectrophotometry. The extract was measured the absorbance at 400 nm (yellow pigment); 470 nm (orange pigment) and 500 nm (red pigment). The pigment content was calculated sum of absorbance at those 3 different wavelengths, expressed as Absorbance Unit (AU)/g [26].

2.5. Pigment profile analysis

The pigment profile analysis was performed by using Thin Layer Chromatography according to Nimnoi and Lumyong (2011) [27]. The extract was applied on the Silica Gel 60 F254 plate (Merck, Germany), then the pigments were separated with a mobile phase consist of chloroform:methanol:water which is 90:25:4.

2.6. Pigment composition analysis

Pigment composition analysis was conducted according to Miyake *et al* (2008) [16] 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 um PTFE filter membrane, then 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 consisting 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 electrospray ionization in the positive ion mode, scan range: m/z 100 to 1000.

2.7. Data analysis

The obtained data of pigment content were subjected to a one-way analysis of variants with randomized nested block design at α=5% and then further analysis using DMRT at α=5%. The analysis was performed with SPSS version 19.

3. Results and discussion

3.1. Effect of temperature and ethanol:water ratio on the pigment extraction

Figure 1 shows that temperature and ethanol:water ratio affected the pigment content. Temperature affected pigment extraction significantly (p<0.05). Extraction at 30°C was more effective than that at 60°C. About 43 AU/g of pigment can be extracted from the MFDS at 30°C, which was higher than that of about 25 AU/g at 60°C. Rasoamandrary *et al* (2013) also reported that 30°C was the optimum temperature in vanillin extraction with ethanol and water mixture solvent [28]. Below 30°C, the vanillin released was lower. Beyond that temperature was found to be detrimental for the extraction process. In the ethanol and water mixture solvent for extraction, the temperature can affect its diffusion and extraction ability [29].

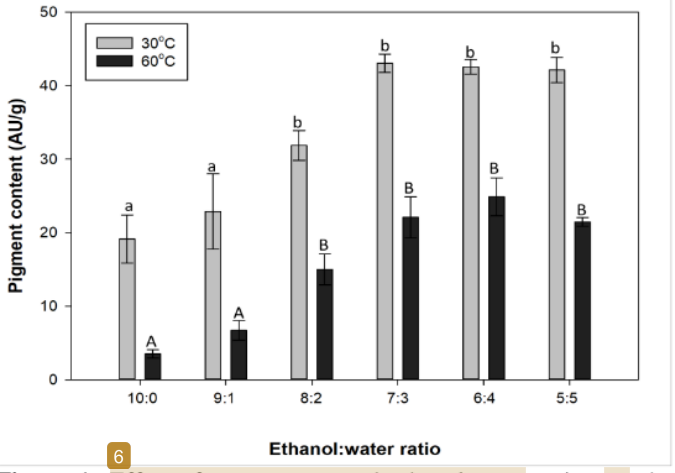

Figure 1. Effect of temperature and ethanol:water ratios on the monascus pigment extraction.

The solvent extraction method is based on the "like dissolve like" principle. In the employing of ethanol/water solvent, different ratio generates different polarity which can affect the extraction ability. Since the monascus pigment is a mixture of various pigment compounds with various polarities, the extracted pigment amount and composition may be affected by the ethanol/water ratio. Figure 1 shows that ethanol:water ratio affected the pigment extraction significantly (p<0.05) both at

30ºC and 60ºC. The lower the ethanol:water ratio until 7:3, the higher the pigment content. Ethanol:water ratio in the range of 7:3 and 5:5 were not significantly different. However, the lower ratio tends to lower pigment content. Interestingly, the lower ethanol:water ratio, more viscous extract observed, which may be because the extract contains higher gum, that naturally exist in the durian seed.

The pigment profile of the extracts at different extraction conditions is presented in figure 2. In general, the intensity of the separated pigment spots of extraction at 30ºC was higher than those at 60ºC. These results supported that pigment extraction at 30ºC was more effective than that at 60ºC. It can be seen that pigment profiles are different at various ethanol:water concentration. This reflected that the pigment composition is different. At the ratio of 10:0 and 9:1, the yellow pigment was clearly separated, but at 8:2 and higher water proportion, the yellow pigment tends to disappear.

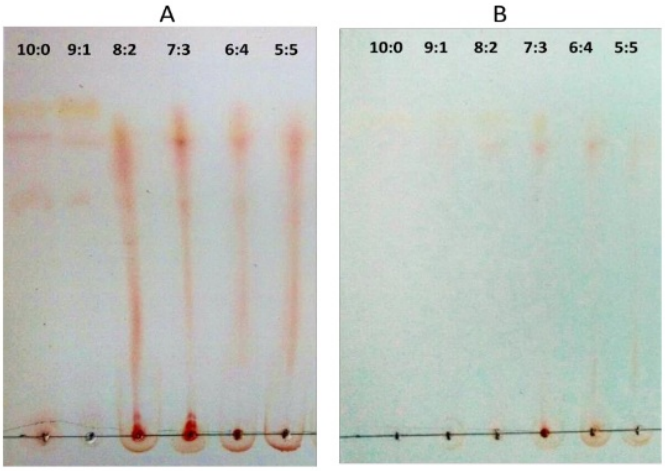

Figure 2. TLC analysis of MFDS extract derived from different temperature and ethanol:water ratios extraction: (A) at 30ºC and (B) at 60ºC.

3.2. Pigment composition of MFDS extracts

This is the first investigation on the pigment compounds composition of MFDS. Pigment extracts derived from MFDS extraction at ethanol:water ratio of 10:0 and 7:3 at 30ºC were analyzed for their pigment composition by using LC-MS. Twelve pigment compounds were detected at different relative amounts. The LC-MS chromatograms and pigment composition of the extracts are presented in figure 3 and table 1, respectively. Xanthomonascin A, which was detected at 6.5 minutes retention time, become a major pigment compound in both extracts. Yellow pigments amount in the extract of 10:0 was higher than that of 7:3. Rubropunctamine, a red pigment compound, level in the extract of 7:3 was higher than that of 10:0. These results agree to Carvalho *et al* (2003) who reported that red pigment solubility is highest in an aqueous solution containing 60-70% ethanol [18].

The monascus pigment composition depends on the complex biosynthesis pathway in the Monascus fungi. Orange pigments are the first biosynthesis product, which can be transformed into red and yellow pigments. In orange pigment rubropunctatin biosynthesis, it is consists of β-ketide pathway to form acetate and malonate and another pathway to form n-hexanoyl acetyl residue [1]. Rubropunctatin then can be transformed into red pigment Rubropunctamine through Schiff base with the nitrogen of the primary amino group of various compounds such as amino acids, peptides, and protein contribution. The MFDS contain lower Rubropunctamine than the Monascus-fermented cereals (rice, corn, and sorghum). This may be due to protein content in durian seed is lower than of those cereals [3].

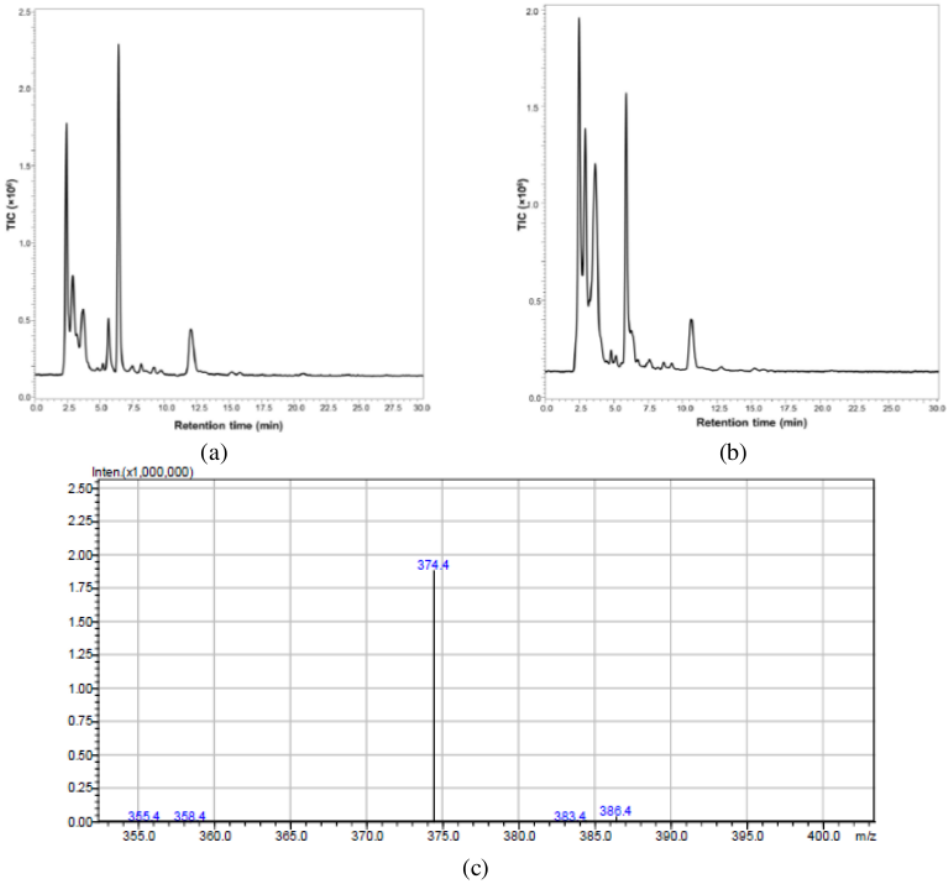

Figure 3. (a) TIC of MFDS extract with ethanol:water ratio of 10:0; (b) TIC of MFDS extract with ethanol:water ratio of 7:3; (c) A representative of SIM chromatogram of MFDS extracts at the retention time of 6.5 minutes.

Table 1. Pigment composition of MFDS extract.

Pigment compound	Extract with ethanol:water ratio of 10:0	Extract with ethanol:water ratio of 7:3
Monascin	8.39%	4.69%
Ankaflavin	0.26%	0.79%
Monascorubrin	3.27%	8.51%
Rubropunctatin	17.69%	9.61%
Monascorubramine	0.94%	0.77%
Rubropunctamine	5.24%	13.89%
Xanthomonascin A	50.26%	47.40%
Xanthomonascin B	6.23%	3.75%
Monascopyridine A	n.d.	n.d.
Monascopyridine B	0.30%	0.06%
Yellow II	7.42%	10.53%

4. Conclusions

It can be concluded that the temperature and ethanol/water ratio affected significantly the pigment extraction from MFDS. Extraction at 30°C was more effective than that at 60°C. Ethanol/water ratio of 7:3 extracted the highest pigment amount from MFDS. Pigment profile and composition of the extracts were different with different temperature and ethanol/water ratio. Interestingly, xanthomonascin A was the major pigment compound in the MFDS.

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