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Isolation and Identification of New Pigment from *Monascus purpureus*

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ABSTRACT

This research was aimed to isolate and identify the new pigment from the main pigments of Monascus purpureus, which derive from five different isolates and through with solid fermentation used IR-64 rice as substrate. The measurement of pigment was done by extracting t samples using methanol in the 14th day of fermentation process. The absorbance of main pigment was measured by a UV-Visible spectrophotometer at the wavelengths of 364, 365, 375, 400, 480 and 500 nm. The results showed that the absorbance of each pigments isolate varied within ranges from 0.219 to 0.559. Result followed of Thin Layer Chromatography (TLC) method with chloroform: acetone (9: 1). TLC results were followed by isolating IPB-B samples using Thin Layer Chromatography- Preparative (TLC-P), whereas the band obtained was identified by using Liquid Chromatography-Mass Spectrum (LC-MS) instrument. The isolated and identified IPB-B samples showed that there was found a new compound of Molecular Weight (MW), which was Monaphilol A with Molecular Weight (MW) of 384.47.

Keywords*: Monascus purpureus, pigment,* LC-MS.

INTRODUCTION

The pigment has been widely used in various kinds of product such as textiles, cosmetics and especially food. The pigment was needed to make a product more variative and esthetically valuable. In more recently, the synthetic pigment is seemingly more used than the natural pigment. This happens because the synthetic pigment is more practical, cheaper and easier to be purchasedin the market (Parmar & Singh, 2018).

In addition to plants, one of the alternative sources of other natural and potential pigment substances is that naturally produced by microorganisms. Various types of microorganisms, especially from the class of *Ascomycetes*, are known as potentially producing various kinds of alternative pigment-substance materials, one of them is *Monascus purpureus* (Gmoser et al., 2017).

Research on *Monascus purpureus* shades is developing quickly, including the revelation of new colors, which are gotten from the principle shades: yellow, orange and red. The exploration information acquired is as yet a different information from any examination diaries, so that total information

are required, especially data in regards to organic action. Monascus purpureus microorganisms are basically known to deliver six principle color parts of which can be gathered into three gatherings, they are monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$) yellow pigments, monascorubrin ($C_{23}H_{26}O_5$) and rubropunctatin ($C_{21}H_{22}O_5$) orange pigments and monascoubramine ($C_{23}H_{27}NO_4$) and rubropuntamine ($C_{21}H_{23}NO_4$) red pigments (Yuliana et al., 2020).

MATERIAL AND METHODS

Chemicals and Apparatus

The equipments used in this study were a UV-visible light Spectrophotometer (GENESYS), HPLC (*High Performance Liquid Chromatography)* (HITACHI L 6200) Mass Spectrum detector (LC-MS) (MARINER BIOSPECTROMETRY), autoclave (Hirayama), stir bar, otter (*misselium* crusher), test tubes (PYREX), *rotary shaker*, tweezers, flame heater, analytical balance, sterilizer, centrifuge, and TLC plate of GF 254 silica gel.

They were *Monascus purpureus* microorganisms obtained from several places, collection of ITB-Bandung, LIPI-Bogor or INACC (*Indonesian Culture Collections*), and IPB- Bogor or IPBCC (*IPB Culture collections*). Substrate medium used was IR-64 rice obtained from rice traders in Tasikmalaya.

The materials were PDA (Potato Dextrose Agar), distilled water, methanol, formic acid, chloroform, and acetone.

Preparation of Breeding Medium:

The synthetic PDA medium in a 250ml Erlenmeyer, was heated while stirred until it was formed aclear solution, and then it was autoclaved by sterilized at 121 ° C for 15 minutes. The medium was then inserted into the tube, tilted then solidify.

***Monascus purpureus* Inoculation**:

The isolation began with sampling the mold, then inoculated into PDA medium, then had it incubated at 25°-32° C for 7-14 days (Yuliana, 2020).

***Monascus purpureus* suspension**:

Monascus purpureus that had been aseptically incubated for 7 days was removed using a spatula into a sterilized potter, then added some distilled water, so that the suspension was made in homogeneous stirring.

Fermentation of *Monascus purpureus*:

The red yeast rice was made by inserting approximately 100g of IR-64 rice into Erlenmeyer, then was sterilized by autoclave at 121°C for 15 minutes. As soon as it was cooled, the rice was inoculated with *Monascus purpureus* suspension, then homogenized and incubated at 25-28° C for 7-14 days.

The Extraction of Pigments:

The fermented rice was dried in an oven at 105° C, and then mashed up until fine powder was formed, extracted with 5 mL of

methanol per gram and was centrifuged at 2500 rpm for 10 minutes and then was decanted.

Analysis of Fermentation Metabolites

Analysis of UV-Vis spectrophotometry

The pigments extract solution was determined by the absorbance and its maximum wavelength. The measurements of pigments absorbance were performed at maximum wavelength identified by spectrophotometer which was at wavelength of 364, 365, 375, 400, 480, and 500 nm (Yuliana, 2020).

Analysis of Thin Layer Chromatography

The TLC was tested usingplate of GF 254 silica gel, eluent chloroform and acetone (9: 1) (Feng, 2012), then it was eluted, more over the spotting that occurred was observed visually and under UV light at a wavelength of 254 nm and 365 nm (Srianta et al., 2017).

Analysis of preparative Thin Layer Chromatography

The Preparative Thin Layer Chromatography was tested using plates of GF 254 silica gel, the spottingwas madeusing the band and then the band was eluted by motion phase using chloroform and acetone (9: 1) (Feng et al., 2012), and then observed visually and under UV light at a wavelength of 254 nm and 365 nm.

LC-MS Analysis

The results of TLC-P isolate were identified using LC-MS using C-18 column (Higa et al., 2020).

RESULT AND DISCUSSION

Monascus purpureus was used as the research sample obtained from multiple isolates, which was from the collection of ITB-Bandung, LIPI-Bogor or INACC (*Indonesian Culture collections*), and IPB-Bogor or IPBCC (*IPB Culture collections*). IR -64 rice was the medium of solid substrate, as used for breeding medium of *Monascus purpureus* purch asedatone of rice traders in TasikmalayaTotal phytochemical content

Table 1. The Characteristics of *Monascus purpureus* Isolate

Isolate	Color of Isolate
ITB–Bandung	Red
IPB–Bogor	dark red
LIPI-Bogor	Orange

LIPI-Bogor F01; F147 | ITB-Bandung | IPB-Bogor IPB-A; IPB-B

Figure 1. *Monascus purpureus* Isolates

Inoculation of *Monascus purpureus* was carried out on PDA. The morphology of five *Monascus purpureus* isolates on PDA for 14 days, visually experienced the same change, which begun with the widening of a whitespot, the widening of dispigmentation,

then followed by the forming of shriveled and pigmented layer on each isolate.

The fermentation process had been carried out for 14 days in whichIR-64 rice medium was used as the solid fermentation substrates. The mold formation in the solid medium was characterized by the formation of pigment in each *Monascus purpureus* isolates. The pigment was formed in a accordance with the growth of *Monascus purpureus* . The rapid growth rate of the pigment could also indicate that the fermentation medium was also suitable for growing the mold (Yuliana et al., 2019). IPB Bogor and ITB-Bandung isolates had resulted an evenly red pigment formation on the rice, and was considered the best. While LIPI Bogor F01 and LIPI Bogor F147 isolates had formed an unevenlyred pigment. This happened due to the agitation was less perfect and unequal. Fermentation results using IR-64 rice of each isolate was shown in Figure 2. Fermented rice.

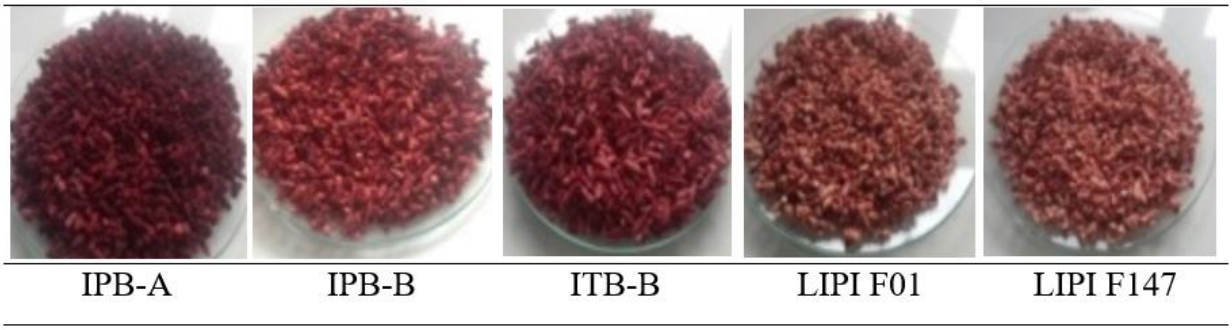

Figure 2. Fermented rice by *Monascus purpureus*

The pigment extract solution was determined by the absorbance and its maximum wavelength. The result showed that each sample had pigment intensity in the wavelength of 300-800 nm range. The absorbance measurement of *Monascus purpureus* pigment was performed at maximum wavelength, which was identified by spectrophotometer, at the wavelength of 364, 365, 375, 400, 480, and 500 nm (Singgih et al., 2019). The following is the description of analysis results using UV-Vis spectrophotometry:

The TLC was tested using plate of GF 254 silica gel, eluent chloroform and acetone (9: 1) (Feng et al., 2012), then it was eluted, moreoverthe spotting that occuredwas observed visually and under UV light at a wavelength of 254 nm and 365 nm. Angkak (*Monascus* rice) used as standard.

Table 2. The measurement of *Monascus purpureus* pigment formation on each isolate

(λ)	Absorbance (Abs)				
	IPB-A	IPB-B	ITB	LIPI F01	LIPI F147
364	0,475	0,493	0,514	0,558	0,346
365	0,481	0,499	0,519	0,559	0,343
375	0,519	0,540	0,543	0,558	0,321
400	0,519	0,550	0,511	0,495	0,261
480	0,363	0,440	0,369	0,284	0,219
500	0,430	0,533	0,432	0,338	0,231

Based on the measurement results in table 2, it can be seen that the LIPI F01 isolate has the highest absorbance at 3 wavelengths, at a wavelength of 364 nm, 365 nm and 375 nm with the absorption obtained is 0.558; 0.559 and 0.558. Meanwhile, IPB B isolates also had the highest absorbance at 3 wavelengths, at wavelengths of 400 nm, 480 nm and 500 nm with the obtained absorptions of 0.550; 0.440 and 0.533.

The 364 and 365 nm absorbance was characterized as the yellow pigment absorbance which were monascin and ankaflavin.The 375 and 400 nm absorbance was characterized as the orange pigment Preparative Thin Layer Chromatography (PTLC) is an isolation process built upon the difference of absorbance and partitions, as well as the solubility of chemical components that stirs following eluent polarity level. The compound separation or purification occurs due to the unbalance of absorbance against chemical components, so that the components stir in different speeds. PTLC isolation test was conducted to obtain a single absorbace which were monascorubrin and rubropuncstatin, and the 480 and 500 nm absorbance was characterized as the red pigment absorbance which were monascorubramin and rubropuntamine (Singgih et al., 2018).

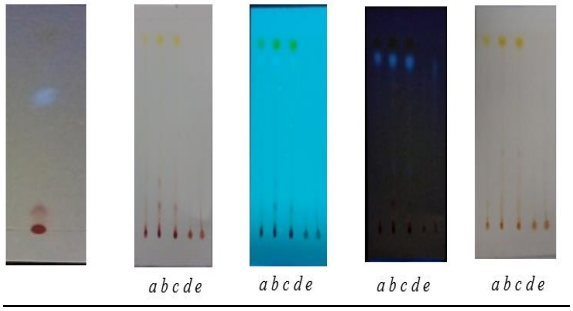

Figure 3. TLC Results of each *Monascus purupreus* isolates

(a) IPB-A (b) IPB-B (c) ITB B (d) LIPI F01 (e) LIPI F147

Preparative Thin Layer Chromatography (PTLC) is an isolation process built upon the difference of absorbance and partitions, as well as the solubility of chemical components that stirs following eluent polarity level. The compound separation or purification occurs due to the unbalance of absorbance against chemical components, so that the components stir in different speeds. PTLC isolation test was conducted to obtain a single pigment, whereas the eluent used was chloroform: acetone (9: 1). The band elution result could be visually observed and under UV light at 254 nm and 365 nm wavelength. The band that lasted to thethird subfraction was the blue band that reached 365 nm UV floressence. This band sample had much further identification.

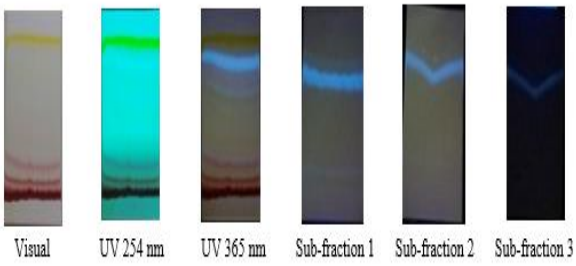

Figure 4. PTLC test result of IPB-B isolates.

Analysis of new pigment substance was done by using High Performance Liquid Chromatography-Mass Spectrum (LC-MS) instrument. This instrument wasthe combination of two instruments, which function was to separate some compound or compound mixture based on the polarity (the chromatography principle). As the compound mixture was secluded, the pure compound could identify the molecular weight (Isbrandt et al., 2020). The LC-MS analysis was performed by using HPLC, whichwas connected to mass spectrometer,was fitted with ESI source, full-scan mode from m / z 100-1200 and was carried out at a temperature of 140°C. The analysing HPLC column was Phenomenex 5µ C18 (150 × 1 mm) wherein the injected sample volume was 2µl as much (Mondal et al., 2019).

The commonly solvent used as an LC reversed-phase solvent was methanol. The motion phase was 0.3% formic acid. The solvent was flowed at a total 0.1 mL/min flow rate. The solvent proceded with isocratic elution, when the sample was injected into the column whichduring the analysis the motion phase composition did not change, it was performed until samples were eluted from the column. Based on the analysis performed on IPB-B isolate samples, and identifyed by the Mass Spectrum (MS) detector, the molecular weight of *Monascus purpureus* main pigments were obtained, which those were monascin, ankaflavin, monascorubrin, rubropunctatin, and monascorubramin pigments. The molecular weight predicted to be derivative compound of new pigment substancewas 384.47 as much. Based on the literature study, The derived and predicted MW compound was monaphilol A, with literature MW was 384.19 (Kraboun et al., 2019).

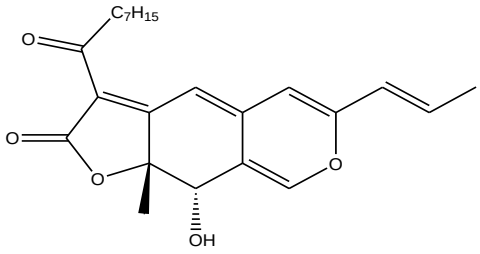

Figure 5. Molecular Structure of Monaphilol A Compound

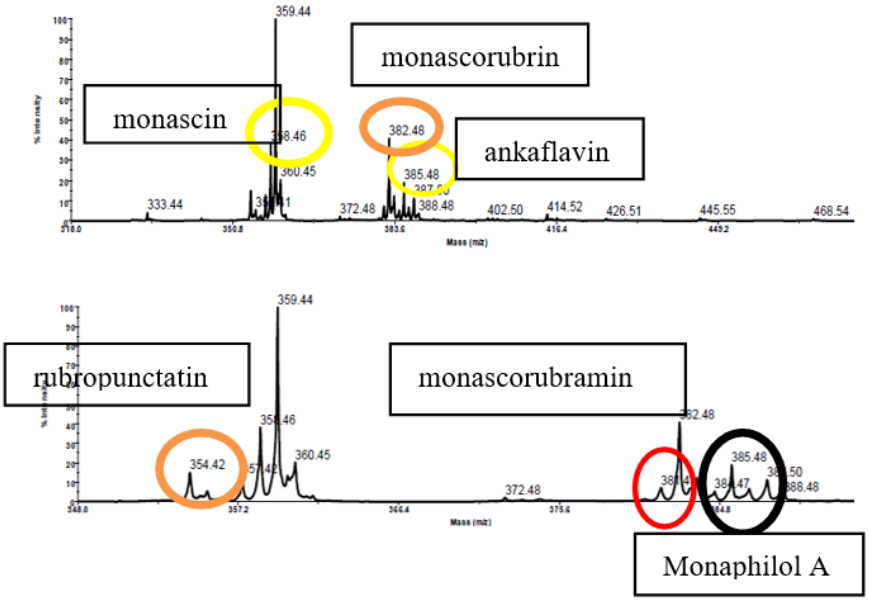

Figure 6. The mass spectrum of IPB-B Isolates

CONCLUSION

The PTLC isolation result, the band obtained was analyzed by LC-MS.The MW gained was 384.47, which was predicted as monaphilol A compound and gained 384.19 MW literature. The further research is suggested to specifically identify monaphilol A compounds. Nevertheless, the other isolates have the same potential to generate new color substance, so it is necessary to identify other isolates as well.

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