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Formation analysis of primary and secondary metabolites during angkak fermentation based on GC-TOF-MS, GC-FID, and HPLC and metabolomics analysis

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ABSTRACT

The significant primary and secondary metabolites occurred during angkak fermentation are composed of organic acids, amino acids, monacolin K, pigments, and citrinin (a mycotoxin), which their variance is able to inform how the *Monascus*-fermented produce characteristics. Here, gas chromatography time of flight mass spectrometry (GC-TOF-MS), gas chromatography-flame ionization detector (GC-FID), and high-performance liquid chromatography (HPLC) were utilized for analysis of the metabolites in angkak *(Monascus purpureus)* for 30 days. Principal component analysis (PCA) and Pearson's correlation coefficients were to study the relationship between the metabolites and fermentation time. Moreover, the metabolomics analysis in angkak was performed by using MetaboAnalyst 5.0. The amount of primary metabolites such as the organic acids (succinic acid, glycolic acid, oxalic acid, lactic acid, lactobionic acid, and pyruvic acid), amino acids (valine, isoleucine, and alanine), fatty acids (stearic acid, oleic acid, linoleic acid, palmitic acid, and linolenic acid), and, monosaccharides (i.e. fructose and glucose) increased gradually until 15 days of the fermentation and then would decrease (the death phase of *M. purpureus*). For the secondary metabolites, the levels of monacolin K, *Monascus* pigments, and citrinin (a mycotoxin)after day 10 of the fermentation increased more than 2 times as compared with the previous fermentation time of day 5, exposing to begin the stationary phase of *M. purpureus* growth. Similarly, according to the heat map of primary metabolites' production rates, there were their higher production rates of primary metabolites after 10 and 15 days of the fermentation; whereas, the higher production rates of secondary metabolites were found after 25 and 30 days of the fermentation. The PCA and Pearson's correlation coefficients explained that the primary and secondary metabolites were discriminated completely into different groups. Based on the data of 19 metabolites throughout 30 days of the fermentation, there were estimated 23 metabolisms, but outstanding 4 metabolisms were glycolysis, pyruvate metabolism, α-linolenic acid metabolism, and linoleic acid metabolism.

1. Introduction

Angkak or monascal rice is well known as red koji, Hung-Chu, Hong Qu, Ang-kak, Ankak rice, red mold rice, and Beni-Koji. Angkak is defined as one of fermented food products of Asia, which is prepared by *Monascus* fermentation with an incubation period of about 2 weeks, usually using polished rice or cereal corps as a *Monascus* substrate (Kraboun et al., 2019). During *Monascus* fermentation, *Monascus* mycelium would

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grow, cover the whole substrates, and then release the *Monascus* pigments to the substrates until becoming the whole red-pigmented substrates (Kraboun et al., 2013).

The secondary metabolites in terms of *Monascus* pigments, citrinin (a mycotoxin), monacolin K, dimerumic acid, antioxidants, etc. are simultaneously generated during *Monascus* fermentation. In particular, *Monascus* pigments have been appiled in a lots of the manufactures engaging in functional foods, natural food colorants, fabric dyes, etc. The consitients of *Monascus* pigments are comprised of not only yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubrin), and red (rubropunctamine and monascorubramine), but also the remarkable antioxidants, i.e. lovastatin or lactone-formed monacolin K, γ-aminobutyric acid (GABA), ergosterol, dimerumic acid, unsaturated fatty acids (USFA), and phenolic compounds (Babitha et al., 2007). It is well known that monacolin K possessess a powerful competitive inhibitor for HMG-CoA reductase since this reductase is able to generate mevalonate through cholesterol biosynthetic pathway (Suraiya et al., 2018). Hence, it can block the synthesis of total cholesterol (TC) and triglyceride (TG) in order to reduce their accumulation in mammalian blood, which it is benifical to suject to avoid the following risks, i.e. stroke, hypertension, heart disease, etc. For example, the previous study confirmed its ability like a potential medication (lovastatin) affecting raising high-density lipoprotein cholesterol (HDL-C) associated with diminishing significantly low-density lipoprotein cholesterol (LDL-C) (Gregory et al., 2012).

During angkak fermentation, *Monascus* sp. can gernerate the serveral hydrolyzing enzymes (e.g. amylase, protease, glucoamylase, maltase, pectinase, α-galactosidase, and ribonuclease) for depolymerizing the macrostructure of carbohydrate substrates to transform into the primary metabolites, i.e. reducing sugars, pyruvic acid, succinic acid, citric acid, gluconic acid, oxalic acid, ethanol, and ester compounds (Lin and Demain, 1991). These metabolites would be utilized as the essential nutrients for the formation of secondary metabolites blended with *Monascus* pigments. The dynamic variance of bioactive compounds from *Monascus* sp. may be altered independently depending on the differently environmental conditions of its fermentation. Therefore, the study on releasing the primary and secondary metabolites during angkak fermentation by *M. purpureus* to obtain more comprehensive knowledge about the metabolisms during *M. purpureus* growth on the broken jasmine rice substrate obtained from using metabolomics analysis should be considered as well as has never been reported. Interestingly, the metabolomics analysis in angkak would help to expect how the quality of finished angkak products as well as be a scientific source or a reference about the possible metabolism pathways in *Monascus*-related produces.

Metabolomics is a study on various metabolites produced from organism's metabolisms. The data of identified metabolites are used to analyze the corresponding metabolism pathways (Chen et al., 2022). There are the chromatographic techniques of gas chromatography time of flight mass spectrometry (GC-TOF-MS), gas chromatography-flame ionization detector (GC-FID), and high-performance liquid chromatography (HPLC) offering the metabolite analysis in previously several fermented products (Chayawat et al., 2009; Chen et al., 2023; Li et al., 2020; Son et al., 2018; Zhao et al., 2020). Chen et al. (2023) suggested that the application of GC-TOF-MS and GC-FID has monitored the production of metabolites throughout the corn wine fermentation (naturally fermented), which the collected metabolic products accompanied with natural microorganisms could be studied their relationship using chemometrics analysis. Li et al. (2020) explained that the metabolic pathway analysis from the metabolites during the fermentation of black waxy rice wine (fermented by Z-20 *Rhizopus*) was achieved by a combined analysis amongst GC-TOF-MS, GC-FID, and HPLC. Besides, Kongbangkerd et al. (2014) who analyzed to recover the secodary metabolites based on HPLC, especially monacolin K and citrinin in angkak produced by using 2-step fermentation.

In our experiment, the primary and secondary metabolites throughout angkak fermentation for 30 were observed and analyzed by using GC-TOF-MS, GC-FID, and HPLC in order to lead to metabolomics analysis. Furthermore, principal component analysis (PCA) was performed to understand the direction of relationship between the primary and secondary metabolites in angkak.

2. Material and methods

2.1. Raw material

Broken jasmine rice (*Oryza sativa* L.) was purchased from a local market in Sawankhalok district, Sukhothai, Thailand.

2.2. Preparation of PDA slants, Monascus purpureus TISTR 3090

M. purpureus TISTR 3090 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). The lyophilzed powder of *M. purpureus* was cultured on potato dextrose agar (PDA; HiMedia Laboratories, India) at ambient temperature for 12 days. The mycelium was again inoculated on PDA slants and incubated following the above condition. Ultimately, the PDA slants were comprised of $> 10^6$ *M. purpureus* spores/mL prior to used for angkak fermentation.

2.3. Preparation of angkak

Angkak fermentation was carried out following the protocol of Kraboun et al. (2013). 100 g of broken jasmine rice was washed with tap water. After drained through a colander, it was put into a flask 250 mL and added with 50 mL of distilled water. The sample flasks were sterilized at 121 °C for 15 min. After cooled down, 15 mL of 10^6 spores/mL of *M. purpureus* spore suspension (*M. purpureus* spores of each PDA slant suspended with 5 mL sterile water) was poured into the sterilized sample flasks and then tightly closed by using a cotton ball to remain the sterilized condition. The mixture was fermented in an incubator at 30 °C for 30 days with 5 intervals for monitoring the metabolites. Overall 21 sample flasks were separated into 7 groups which each group contained 3 flasks (equal to triplicate experiments) for the analysis in each time. Prior to the analysis, the angkak samples from 3 flasks were pooled by using a spatula, dried in a hot air oven until dryness, and ground into fine powder by a mill (Retsch ultracentrifugal mill, Germany) combined with a sieve 100 mesh. The fine powder was kept in a freezer at − 20°C prior to analysis.

2.4. Growth curve analysis of M. purpureus

1 g of angkak was transferred into a sterilized tube, mixed with 20 mL 1.0% (w/v) of sterilized NaCl solution, and then mixed homogeneously for 3 min at ambient temperature. The serial dilutions were carried out using 1.0% (w/v) NaCl solution for further steps. The viable count of *M. purpureus* TISTR 3090 was performed by using potato dextrose agar (PDA) (pH 6.0) (HiMedia Laboratories, India) with incubated under an aerobic condition at 30 °C for 2 days. The viable count of *M. purpureus* was enumerated in duplicate for each sample every 5 days and expressed as log CFU/g.

2.5. Sample preparation and GC-TOF-MS condition to determine metabolite profiles

The sample preparation and gas chromatography time of flight mass spectrometry (GC-TOF-MS) condition were described by Zhao et al. (2020), with a slight modification to adjust being an appropriate analysis condition for monitoring the metabolites in the angkak sample.

2.5.1. Sample preparation

1 g of the sample was extracted with 100 mL methanol and shaken at 200 rpm for 2 h. 200 μL of the extract was added to a test tube mixed

with 500 μL of methanol and 10 μL of 190.6 mM ribitol (as an internal standard; Sigma-Aldrich, USA). Then, the mixture was centrifuged at 15000 *g* for 10 min with controlled at 4 °C. 100 μL of the supernatant was blanded with a QC sample (total organic carbon sample QCI-731, Agilent Technologies, USA). The mixture was dried in a vacuum concentrator (2044 DryFast®, Welch, Germany) until being the dried metabolites, which were loaded with 100 μL of methoxyamine hydrochloride (50 mg/mL in pyridine; Sigma-Aldrich, USA) and then incubated at 82 °C for 35 min. The mixture was mixed with 200 μL of BSTFA (plus 1% TMCS; Sigma-Aldrich, USA) and then warmed at 72 °C for 2 h. Subsequently, the mixture was transferred into 10 μL FAMEs (in chloroform).

2.5.2. GC-TOF-MS condition

The system of GC-TOF-MS contained a gas chromatograph (Agilent 7890, Agilent Technologies, USA) connected to a time-of-flight mass spectrometer (Pegasus HT, Agilent Technologies, USA). A DB-5MS capillary column (30 m × 250 μm × 0.25 μm, Agilent Technologies, USA) was used to separate the metabolites. 1 μL aliquot of the sample was determined in a splitless mode. The flow rate of gas and front inlet purge were adjusted to 1 and 3 mL/min, respectively, which helium was a carrier gas. The temperature conditions were as follows; initial temperature was set to 55 °C for 2 min, risen to 320 °C at 15 °C/min, hold on 10 min at 320 °C. The temperatures of injection port, transfer line, and ion source were 300, 300, and 280 °C, respectively (Jiang et al., 2020). − 70 eV energy was applied in an electron impact mode. The full-scan mode from 40 to 600 *m/z* scanning 13 spectra/sec after a solvent delay of 7 min was operated to report mass spectrometry data.

2.6. Amount of organic acids, amino acids, fatty acids, and reducing sugars

About 1 g sample was soaked in 25 mL of methanol at 50 °C for 2 h, followed by filtration through a PTEE filter 0.45 μm (National Scientific, Rockwood, TN, USA) (Kongbangkerd et al., 2014) prior to observation for the primary metabolites.

For the assay of organic acids, the extract was determined by high performance liquid chromatography (HPLC) (Agilent-LC1260, Agilent Technologies, USA). The mobile phase, detector, and column temperature were 0.05 M KH_2PO_4 (pH = 3.0), G1315D, and 37 °C, respectively. ZORBAX SB-C18 column (4.6 ×150 mm, 5 μm) was directly connected to a 2 μL loop injector. The injection volume and flow rate were set at 20 μL and 2.0 mL/min, respectively. The wavelength at 220 nm was used to analyze the six organic acids in angkak. The content of six organic acids was obtained due to against the caribation curve of orgainic acid standard (Kongbangkerd et al., 2014).

Amino acid analysis was the procedure according to Bidlingmeyer et al. (1984). The phenylisothiocyanate (PITC) technique is appiled for the derivatiztion of amino acids, which was continuously described into two steps as follows: 1. protein or peptide hydrolyzed being free amino acids; 2. derivatiztion of amino acids using PITC (Sigma-Aldrich, USA) and analyzed by HPLC coupled to a UV detector at 254 nm. ZORBAX SB-C18 column (4.6 ×150 mm, 5 μm) was directly connected to a 2 μL loop injector. There were 2 eluents, i.e. (A) an aqueous buffer and (B) 65% acetonitrile in water. A gradient was done for separation, consisted of 10% B traversing to 51% B in 10 min using a convex curve (number 5). The standard of amino acids was taken to identify and calculated as the amount of amino acids.

The assay of fatty acids was described by the protocol via fatty acid methyl ester (FAME). The sample of 100 μL was dissloved in 1 mL hexane + 200 μL of 1.5 M sodium methoxide solution (Sigma-Aldrich, USA). The mixture was left for 15 min and then centrifuged to separate the FAME layer, which was analyzed by a gas chromatography-flame ionization detector (GC-FID) (Agilent 6890 N Network GC System, Agilent Technologies, Singapore). The column DBWAX (0.25 mm × 30 m, 0.25 μm) (Agilent Technologies, USA) was appiled. The conditions of temperatures was set up as the following: initial temperature was 130 °C for 2 min prior to risen to 220 °C at 6 °C/min for 15 min; final temperature was 250 °C for 10 min at 30 °C/min. The injector and detector temperatures were reached 220 °C during the analysis, which helium and hydrogen as the carrier gases were adjusted to a ratio of 100:2. The sample peaks were compared with the FAME standard peaks (Supelco, Bellefonte, PA). The percentage of fatty acids was calculated from the peak area of sample against the preak area of standard (Huang et al., 2017).

Sugar analysis was carried out via an HPLC system including as follows: 1. autosampler/injector (Varian Prostar model 400); 2. pump (Varian model 230); 3. column thermostat (DBS PCO-200 Peltier System); 4. detector (Varian RI-4 refractive index detector); 5. software (Varian Star Chromatography Workstation Version 6.00); 6. column (ZORBAX SB-C18 column, 4.6 ×150 mm, 5 μm). The mobile phase, flow rate, and injection volume were acetonitrile/water in a ratio of 100:20, 2 mL/min, and 20 μL, respectively. The study on sugar's kind and its amount were accomplished by the external standard. The external standard preparation was 200 mL of water:methanol (4:1) solution containing 2 g of each fructose and glucose. The results are expressed as mg/kg of each sugar (Kongbangkerd et al., 2014).

2.7. Amount of monacolin K and citrinin

About 1 g sample was immersed in 25 mL of methanol at 50 °C for 2 h, followed by filtration through a 0.2 μm membrane (Kongbangkerd et al., 2014).

The assay of monacolin K was explained according to the procedure of Chayawat et al. (2009), with a slight modification. 1 g filtrate was extracted with 50 mL of 80% ethyl alcohol at 55 °C for 12 h in darkness and then filtrated through a 0.45 μm PTEE filter unit (National Scientific, Rockwood, TN, USA). The supernatant was analyzed by HPLC. The HPLC system was as follows: (1) Shimadzu LC-10AT VP Liquid Chromatograph; (2) FCV-10AL VP pump; (3) LDC Analytical SpectroMonitor 3100 detector set to 240 nm; (4) LDC Analytical CI-4100 integrator. The column ZORBAX SB-C18 column (5 μm, 4.6 ×250 mm) was directly connected to a 20 μL loop injector. An isocratic mobile phase of acetonitrile:water was in the ratio of 65:35 (v:v). The flow rate and temperature were set at 2.0 mL/min and 25 °C, respectively. Monacolin K (Sigma-Aldrich, USA) dissolved in 80% ethyl alcohol was a standard.

Citrinin analysis was carried out by the method of Kraboun et al. (2013). 1 g filtrate was extracted with 100 mL of the solution (containing acetone and ethyl acetate (Sigma-Aldrich, USA) in the ratio of 2:2 (v:v)) using a shaker at 70 °C for 120 min. To get the supernatant, the mixture was centrifuged at 2000 *g* for 20 min and then filtrated through a 0.2 μm polytetrafluoroethylene (PTFE) syringe filter (CHM®, Ukraine). The HPLC system was connected to the ZORBAX SB-C18 chromatography column (5 μm, 4.6 ×250 mm). The mobile phase contained methyl alcohol: acetonitrile: 0.2% phosphoric acid in the ratio of 4:4:5 (v:v:v). The excitation and emission wavelengths of fluorescence detector were 330 and 500 nm, respectively. The sample was spiked to know the amount of citrinin (Sigma-Aldrich, USA) which the flow rate was 1 mL/min.

2.8. Intensity of Monascus pigments

The intensity of *Monascus* pigments was described by the procedure of Yongsmith et al. (2000). 10 g of the sample was dissolved in 200 mL methanol and then shaken at 180 rpm for 3 h. Then, it was filtered through Whatman no.4 filter paper. The supernatant was read at 500 nm by a spectrophotometer (model UV-1700 PharmaSpec, Shimadzu) against a blank. The pigment intensity was calculated following the below formula.

Monascus pigment intensity (Unit/g dry weight) = $\underline{A_{500}\ X\ \text{dilution factor}\ X\ \text{volume of methanol}}$ weight of sample (g)

Table 1
Mass spectrometry (MS) data of GC-TOF-MS from primary metabolites in angkak using EI mode.

Category	Metabolites	Retention time	Measure mass [M-H]⁻
Organic acids	Pyruvic acid	7.24	88.05
	Lactic acid	7.32	89.59
	Glycolic acid	7.53	75.05
	Oxalic acid	7.67	90.02
	Succinic acid	8.76	117.62
	Lactobionic acid	30.05	357.43
Amino acids	Alanine	8.00	88.59
	Valine	9.21	117.05
	Isoleucine	11.32	130.21
Fatty acid	Palmitic acid (C16:0)	20.56	255.32
	Linolenic acid (C18:3)	21.11	277.07
	Linoleic acid (C18:2)	22.12	279.49
	Oleic acid (C18:1)	24.13	281.05
	Stearic acid (C18:0)	24.56	283.15
Polyhydric alcohols	Ribitol	15.38	151.55
Carbohydrates	2-Deoxy-D-galactose	16.67	163.05
	Fructose	17.52	179.87
	Glucose	17.77	179.56
	Maltose	26.79	341.25
	Trehalose	27.88	341.34
	Isomaltose	28.17	341.78
Other	Glucose-6-phosphate	20.77	258.231
	Noradrenaline	16.84	168.21
	Purine riboside	19.45	251.56
	DL-dihydrosphingosine	25.99	300.22

2.9. Data analysis

The data were collected from triplicate experiments and expressed as mean ± SD. Statistical analysis for difference was carried out using SPSS 26.0 (SPSS Inc., USA) via Duncan's new multiple range test or DMRT ($p \leq 0.05$). Principal component analysis (PCA) and Pearson's correlation coefficients (r) were performed by using SPSS 26.0 (SPSS Inc., USA). Metabolomics analysis obtained from enrichment analysis from the metabolites released by *M. purpureus* was designed by using MetaboAnalyst 5.0 (https://metaboanalyst.ca/).

3. Results and discussion

3.1. Determination of lag, log, stationary, and death phases corresponded to primary and secondary metabolites throughout angkak fermentation

During angkak fermentation for 30 days, the primary and secondary metabolites found in angkak were produced by *M. purpureus* and detected by using GC-TOF-MS including 6 organic acids, 3 amino acids, 5 fatty acids, 1 polyhydric alcohol, 6 carbohydrates, and other metabolites (Table 1). The production patterns of primary and secondary metabolites and viable counts of *M. purpureus* are shown in Table 2 and Fig. 1, respectively throughout angkak fermentation. Obviously, the highest production of primary metabolites was explored after 10 and 15 days of the fermentation ($p > 0.05$) and then they would be gradually decreased. This was possible that the primary metabolites were highly released out in those periods implying the log (5–10 days of the fermentation) and stationary (10–15 days of the fermentation) phases of *M. purpureus* growth (Fig. 1). It was assumed that 0–5 days and after 20 days of the fermentation represented the lag and death phases of *M. purpureus* growth, respectively (Fig. 1). Differently, the amount of monacolin K, citrinin, and *Monascus* pigments simultaneously increased along with the fermentation (Table 2 and Fig. 1). They were produced highly after 10 days of the fermentation, which may be the stationary phase of *M. purpureus* growth due to commonly the secondary metabolic substances abundantly generated by *Monascus* sp. during the stationary phase of the fermentation (Kraboun, 2023; Kraboun et al., 2019). This indicated that the stationary phase of *M. purpureus* growth was shifted faster than that reported by the previous studies. For example, Kongbangkerd et al. (2014) and Kraboun et al. (2013) noted that most of the highest amount of the pigments, monacolin K, GABA, and other antioxidants in angkak prepared from the batch fermentation exhibited in the range of 14–16 days of the fermentation. This may result in dynamic changes in the significantly intrinsic and extrinsic factors affecting *M. purpureus* growth, i.e. nitrogen sources, carbon sources, pH, temperatures, initial moisture content, etc (Pattanagul et al., 2008). This experimental conditions seemed to be optimal since approximately 30% initial moisture content of the substrate (data not shown) and fermentation temperature and time set at 30 °C and 30 days, respectively were an appropriate combination for stimulating the activities of hydrolysing enzymes, i.e. glucoamylase and α-amylase in order to promote more rapid producing the bioactive compounds (Babitha et al., 2007;

Table 2
Dynamic changes of primary and secondary metabolites during angkak fermentation for 30 days.

Metabolites	Do	D5	D10	D15	D20	D25	D30
Primary metabolites							
Pyruvic acid (mg/kg)	200.32± 3.23^{a}	289.67± 4.32^{b}	389.11± 3.21^{c}	399.28± 3.27^{c}	356.98 + 5.32^{bc}	243.54± 5.34^{ab}	176.23± 5.88^{a}
Lactic acid (mg/kg)	135.11± 2.17^{a}	178.43± 2.45^{b}	233.89± 4.23^{c}	234.29± 5.34^{c}	190.23± 3.23^{b}	119.44± 4.34^{a}	95.12± 1.56^{a}
Glycolic acid (mg/kg)	23.13± 4.45^{a}	34.33± 1.67^{b}	56.23± 3.21^{c}	56.12± 2.12^{c}	42.11± 1.21^{b}	36.45± 3.02^{b}	18.45± 0.23^{a}
Oxalic acid (mg/kg)	79.13± 2.54^{a}	115.34± 1.33^{b}	189.90± 2.67^{d}	188.16± 2.56^{d}	178.99± 3.55^{d}	134.17± 6.45^{c}	109.21± 2.11^{ab}
Succinic acid (mg/kg)	200.44± 2.34^{a}	254± 3.10^{b}	389.09± 6.23^{d}	389.17± 4.23^{d}	301.12± 4.34^{c}	288.56± 8.34^{c}	202.90± 3.29^{a}
Lactobionic acid (mg/kg)	14.12± 1.25^{b}	28.13± 0.33^{c}	34.24± 0.32^{d}	34.09± 0.72^{d}	29.56± 1.16^{c}	16.23± 1.90^{b}	3.89± 0.01^{a}
Alanine (mg/kg)	167.12± 2.32^{b}	178.23± 1.03^{b}	209.13± 1.23^{c}	209.54± 1.98^{c}	189.29± 2.34^{b}	128.87± 1.44^{a}	112.13± 2.18^{a}
Valine (mg/kg)	78.98± 1.46^{a}	90.88± 2.56^{b}	138.21± 2.77^{c}	139.56± 2.79^{c}	125.99± 3.21^{c}	99.12± 1.33^{b}	67.23± 1.21^{a}
Isoleucine (mg/kg)	89.54± 3.21^{b}	145.99± 1.89^{c}	199.56± 2.56^{e}	199.28± 8.77^{e}	178.89± 6.23^{d}	62.89± 1.09^{a}	45.67± 1.21^{a}
Palmitic acid (C16:0) (%)	20.34± 1.65^{a}	36.19± 1.34^{b}	68.32± 4.23^{c}	68.77± 2.32^{c}	50.23± 1.89^{b}	42.12± 2.13^{b}	28.71± 0.23^{a}
Linolenic acid (C18:3) (%)	1.23± 0.01^{a}	2.45± 0.02^{b}	3.89± 0.04^{c}	3.85± 0.03^{c}	2.98± 0.02^{bc}	1.98± 0.04^{a}	0.94± 0.01^{a}
Linoleic acid (C18:2) (%)	7.21± 0.21^{a}	9.09± 0.01^{a}	34.98± 0.03^{d}	34.90 + 1.45^{d}	20.56± 2.12^{c}	16.24± 0.13^{b}	7.56± 0.02^{a}
Oleic acid (C18:1) (%)	5.67± 0.01^{a}	7.12± 0.02^{a}	23.90± 0.12^{c}	23.56± 1.09^{c}	15.89± 0.22^{b}	14.89± 0.02^{b}	9.90± 0.03^{a}
Stearic acid (C18:0) (%)	4.98± 0.01^{a}	7.89± 0.02^{b}	14.23± 0.02^{d}	15.44± 1.34^{d}	9.34± 0.01^{c}	7.32± 0.22^{b}	3.82± 0.01^{a}
Fructose (mg/kg)	200.67± 3.43^{b}	319.17± 2.87^{d}	399.21± 5.34^{f}	400.23± 2.34^{f}	356.56± 4.34^{e}	277.87± 3.89^{c}	122.98± 5.89^{a}
Glucose (mg/kg)	300.23± 5.32^{b}	389.89± 4.33^{c}	500.12± 4.23^{d}	499.55± 3.93^{d}	339.55± 3.21^{c}	289.21± 4.21^{b}	132.22± 5.34^{a}
Secondary metabolites							
Monascus pigments (unit/g)	n.d.	24.22± 3.23^{a}	321.22± 3.85^{b}	487.23± 2.34^{c}	500.98± 2.99^{d}	899.21± 4.55^{e}	977.55± 5.33^{f}
Monacolin K (mg/kg)	n.d.	56.23± 1.11^{a}	77.88± 2.12^{b}	112.32± 0.32^{c}	330.99± 2.34^{d}	700.33± 2.34^{e}	1400.55± 7.34^{f}
Citrinin (μg/kg)	n.d.	4.23± 0.01^{a}	10.98± 0.03^{b}	12.89± 0.21^{c}	15.11± 0.32^{d}	17.67± 0.32^{e}	18.67± 0.02^{f}

n.d. = not detected.
Different letters behind means within same row are significantly different ($p \leq 0.05$).

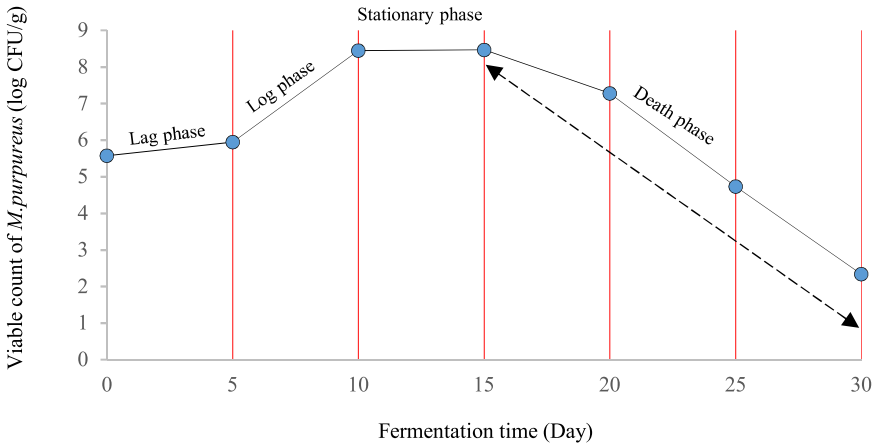

Fig. 1. Viable count of *M. purpureus* TISTR 3090 (log CFU/g) in angkak during fermentation time for 30 days.

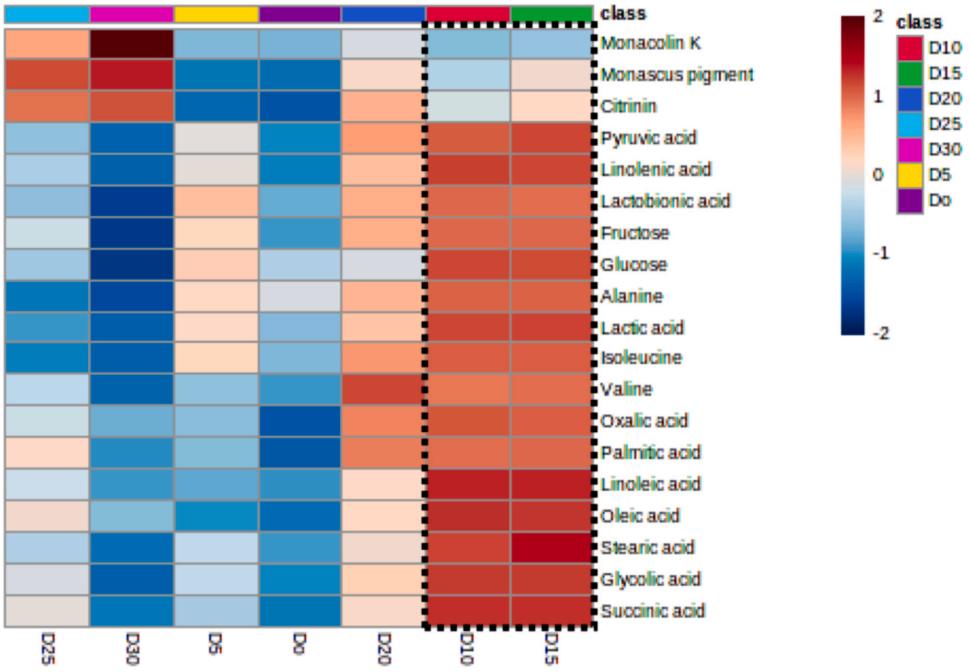

Fig. 2. Heat map of relative content between primary and secondary metabolites in angkak during fermentation time for 30 days.

Yongsmith et al., 2000).

Obviously, the data exhibited that the lag, log, stationary, and death phases of *M. purpureus* growth could imply to the behavior releasing the significant primary and secondary metabolites estimated during angkak fermentation using broken jasmine rice as a substrate. This may have an important role assessing or expecting accurately the relationship between their metabolites in angkak using rice substrate mainly which their correlations may not be because of using other substrates having the constituents unlike the rice substrate as well as other factors.

3.2. Multivariate analysis for correlation analysis between fermentation time combined with primary and secondary metabolites

The relative amount or production rate of primary and secondary metabolites observed following the fermentation time is illustrated in Fig. 2 using the heat map. The red and blue indicate higher and lower means of the metabolite content, respectively. The production rates of primary metabolites containing the organic acids (succinic acid, glycolic acid, oxalic acid, lactic acid, lactobionic acid, and pyruvic acid), amino acids (valine, isoleucine, and alanine), fatty acids (stearic acid, oleic acid, linoleic acid, palmitic acid, and linolenic acid), and monosaccharides (i.e. fructose and glucose) after 10 and 15 days of the fermentation were higher than those of other days, related to their amount in Table 2. Similarly, the growth of *M. purpureus* in that fermentation period corresponded to the log and stationary phases (Fig. 1), explaining possibility of the accumulation of reducing sugars such as glucose and fructose, fatty acids, and amino acids, and organic acids. After 20 days of the fermentation, the production rates of their metabolites decreased rapidly, indicating the death phase of *M. purpureus* growth (Fig. 1). This phenomenon could be explained that the glucose and fructose (as main components) were hydrolyzed under the glycolysis pathway to generate the pyruvic acid (as a finished product of glycolysis pathway), which was transferred into the tricarboxylic acid cycle (or the Krebs cycle) to produce the various kinds of organic acids during *M. purpureus* fermentation (Klosowski et al., 2015). Comparing the monitored acids, the production rates of succinic, pyruvic, and lactic acids were higher than those of other acids. This presumed that lactic acid formed by pyruvate (or pyruvic acid) via lactate dehydrogenase

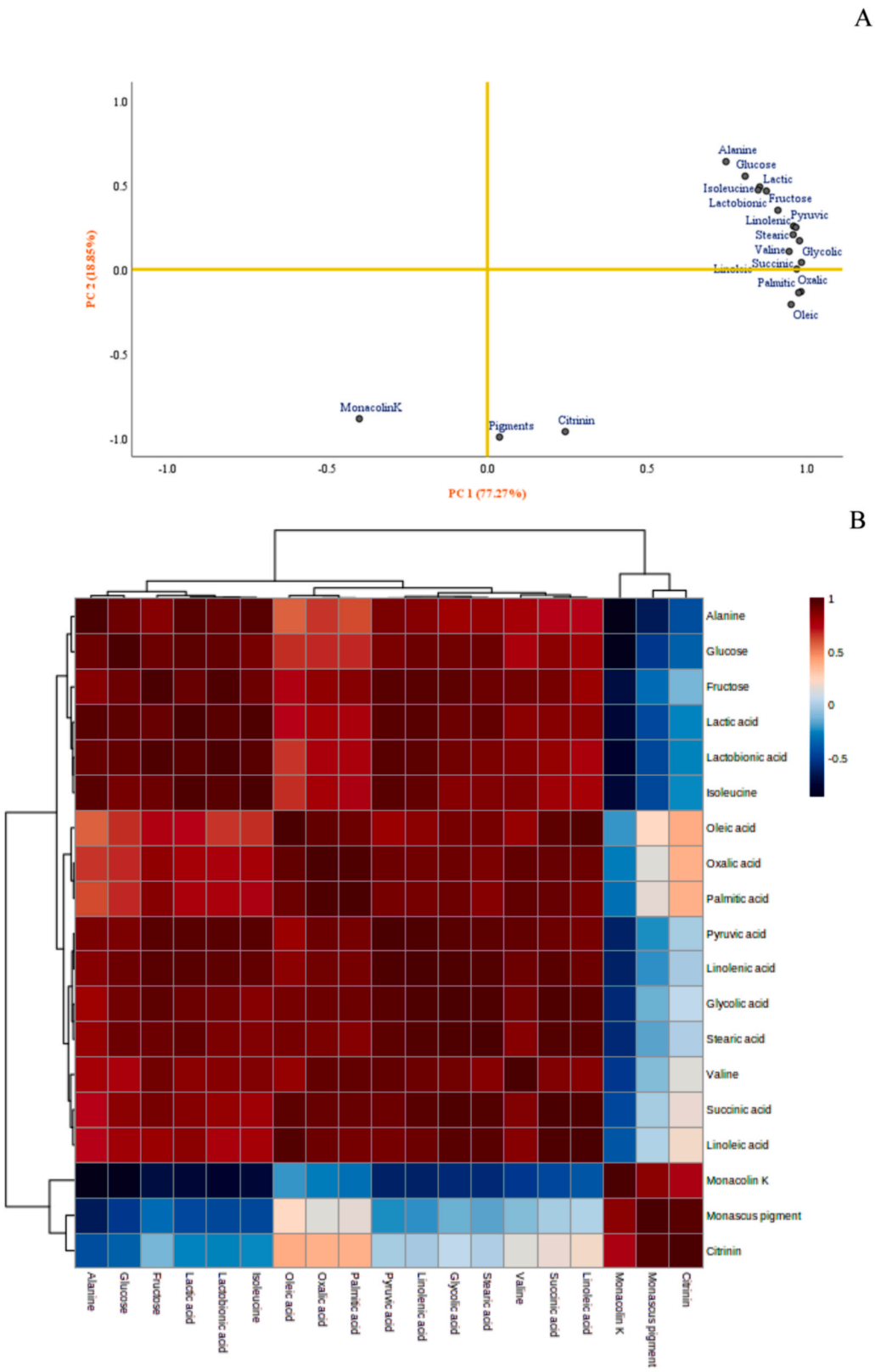

Fig. 3. Loading plot of principal component analysis (A) and heat map of Pearson's correlation coefficient (B) between primary and secondary metabolites during angkak fermentation for 30 days.

could react with alcohol to produce ethyl lactate (Suryanarayan, 2003). Generally during *Monascus* fermentation, phosphate as a distinguished intermediate induced the cellular signal transduction should be found in this experiment but was not found. The lack of phosphate may be against the phenomena of (1) adenosine diphosphate (ADP) phosphorylation formed in the glycolytic pathway and Krebs cycle and (2) phosphoric acid stimulating some proteins to control the cellular signal transduction in order to adjust in the various enviroments. After 10 and 15 days of the

fermentation, the production rate of linoleic acid was higher than that of other lipid acids, which was higher than 1.87 times as compared with its mean, equal to 18.65%. Besides, the occurrence of saturated fatty acids (palmitic acid and stearic acid) implied in the flavor of angkak. The valine, isoleucine, and alanine appeared in angkak represented as the precursors of alcohols, alehydes, esters, and ketones, which could explain the sensory attributes (Li et al., 2020). Obviously, there was the same trend between the heat map patterns of isoleucin and alanine which their intensity of heat maps were more than those of valine. Thus, this indicated an increase in the accumulation rates of isoleucin and alanine in *Monascus* cells. Fortunately, the higher production rate of alanine would help to support the physiological properties, i.e. neurotransmitter, hormone, precursors of coenzyme A, pantothenic acid, etc. in the cullular organisms (Hobson et al., 2012; White et al., 2001; Wu et al., 1993). In addition, Kraboun et al. (2013) have utilized the nitrogen sources such as peptone and monosodium glutamate (MSG) as the supplements in waxy corn (a solid substrate) for *M. purpureus* fermentation. Their supplements promoted the higher levels of pigments, monacolin K, and citrinin in monascal waxy corn.

For the production rates of monacolin K, *Monascus* pigments, and citrinin, their heat maps having more intensity (shown as higher mean) were explored after 25 days of the fermentation corresponded to the production rate of primary metabolites decreased after 20 days of the fermentation (Fig. 2). Interestingly, the remarkable formation rate of monacolin K after 30 days of the fermentation illustrating deep red of heat map was observed. In fact, this was supposed that the factors of amino acids and carbohydrates directly affected the secondary metabolites in angkak. This was in agreement with Kongbangkerd et al. (2014), who suggested that certain amino acids, i.e. isoleucin and alanine found in soybean substrate for angkak fermentation exhibited to enhance the accumulation of monacolin K. As well, Ajdari et al. (2011) and Miyake et al. (2006) reported that glucose and fructose affected the sporulation and enhancing the amount of monacolin K in angkak.

3.3. Multivariate analysis for correlation analysis between primary and secondary metabolites

To obtain the relationship between the primary and secondary metabolites in angkak throughout the fermentation time, principal component analysis (PCA) was performed to understand the trend or direction between depending variables (Li et al., 2020). The loading plot of PCA result obtained from the primary and secondary metabolites in angkak is shown in Fig. 3A. The PC1 and PC2 could account for 96.12% of total variance, which the eigenvalues of both PCs were quite different resulting in both groups of depending variables discriminated clearly. Based on PC1, it was the group having a high correlation and accounted for 77.27% of total variance. The PC1 contained the primary metabolites, i.e. organic acids (succinic acid, glycolic acid, oxalic acid, lactic acid, lactobionic acid, and pyruvic acid), amino acids (valine, isoleucine, and alanine), fatty acids (stearic acid, oleic acid, linoleic acid, palmitic acid, and linolenic acid), and monosaccharides (i.e. fructose and glucose). However, the secondary metabolites containing monacolin K, *Monascus* pigments, and citrinin were separated into PC2, which could describe 18.85% of total variance. The result of PCA indicated the depending variables within the same group representing a positive correlation but the different groups referring to a negative correlation. Hence, it was possible that change in the primary metabolites during angkak fermentation could be the potential indicators with opposite way for the secondary metabolites. This was confirmed by Fig. 3B, which was obtained from Pearson's correlation analysis with displaying the heat map. Pearson's correlation coefficients (r) inform the correlation level of couple variables, which are between -1 and $+1$ exhibiting negative and positive correlations, respectively (Wang et al., 2018). Deep red and blue report the degree of significantly positive and negative correlations, claimed by Table S1. Considering PC1, the positive correlations were found among all primary metabolites (r, 0.579–0.991) (Table S1). The significantly positive correlations ($p \leq 0.05$ and 0.01) were as follows: 1. between pyruvic acid and other primary metabolites; 2. between lactic acid and other primary metabolites, excepting oleic acid; 3. between glycolic acid and other primary metabolites; 4. between oxalic acid and other primary metabolites, excepting alanine and glucose; 5. between succinic acid and other primary metabolites, excepting alanine; 6. between lactobionic acid and other primary metabolites, excepting oleic acid; 7. between alanine and other primary metabolites, excepting palmitic, linoleic, and oleic acids; 8. between valine and other primary metabolites; 9. between isoleucine and other primary metabolites, excepting oleic acid; 10. between palmitic acid and other primary metabolites, excepting glucose; 11. between linolenic acid and other primary metabolites; 12. between linoleic acid and other primary metabolites; 13. between oleic and stearic acids; 14 between stearic acid and glucose or stearic acid and fructose; 15. between fructose and glucose. For PC2, *Monascus* pigments correlated with both monacolin K and citrinin were significantly positive (r, 0.859 and 0.966, respectively), excepting the relationship between monacolin K and citrinin were not found. To consider a combination between PC1 and PC2, some primary metabolites, i.e. lactobionic acid, alanine, and glucose could be the indices for monacolin K with r ranged from -0.768 to -0.849 ($p \leq 0.05$) (Table S1).

Table 3
Pathway names of metabolisms from primary and secondary metabolites during angkak fermentation for 30 days.

No.	Pathway name	*p*-value	Impact value
1	Biosynthesis of unsaturated fatty acids	8.7539E-6	0.0
2	Glycolysis / Gluconeogenesis	0.0013507	0.10065
3	Alanine, aspartate, and glutamate metabolism	0.0016838	0.0
4	Valine, leucine, and isoleucine biosynthesis	0.0020576	0.0
5	Aminoacyl-tRNA biosynthesis	0.0079837	0.0
6	Citrate cycle (TCA cycle)	0.013122	0.07907
7	Pyruvate metabolism	0.01579	0.20684
8	Linoleic acid metabolism	0.044409	1.0
9	Fatty acid degradation	0.046401	0.0
10	Valine, leucine, and isoleucine degradation	0.048593	0.0
11	α-Linolenic acid metabolism	0.11167	0.33333
12	Butanoate metabolism	0.12779	0.0
13	Pantothenate and CoA biosynthesis	0.1592	0.0
14	Selenocompound metabolism	0.16689	0.0
15	Propanoate metabolism	0.18957	0.0
16	Glyoxylate and dicarboxylate metabolism	0.2542	0.0
17	Glycine, serine, and threonine metabolism	0.26108	0.0
18	Cysteine and methionine metabolism	0.26108	0.0
19	Amino sugar and nucleotide sugar metabolism	0.28801	0.0
20	Arginine and proline metabolism	0.2946	0.0
21	Fatty acid elongation	0.30113	0.0
22	Tyrosine metabolism	0.32039	0.0
23	Fatty acid biosynthesis	0.3514	0.01473

3.4. Metabolism analysis

To study the expected metabolisms available in angkak during 30 days of the fermentation indeed, MetaboAnalyst 5.0 (https://metaboanalyst.ca/) was used to enrich 19 metabolites with significantly different which 23 metabolisms were possibly formed (Table 3). The required impact value and p-value were > 0.1 and < 0.05, respectively, to determine important metabolisms (Chen et al., 2018). Four notable metabolisms were as follows: 1. glycolysis; 2. pyruvate metabolism; 3. α-linolenic acid metabolism; 4. linoleic acid metabolism (Fig. 4). The fructose and glucose decreased after 15 days of the fermentation (as the death phase of *M. purpureus* growth) due to the effect of glycolysis. After the action of hexokinase, it was possible that glucose would be hydrolyzed into glucose-6-phosphate to become fructose-6-phosphate subsequently (Table 1). Also, fructose-6-phosphate would be oxidized or decarboxylated to change into acetyl CoA transferred into the Krebs cycle. At the same time, pyruvic acid, succinic acid, and lactic acid were formed to support the appearance of citrate cycle (TCA cycle or Krebs

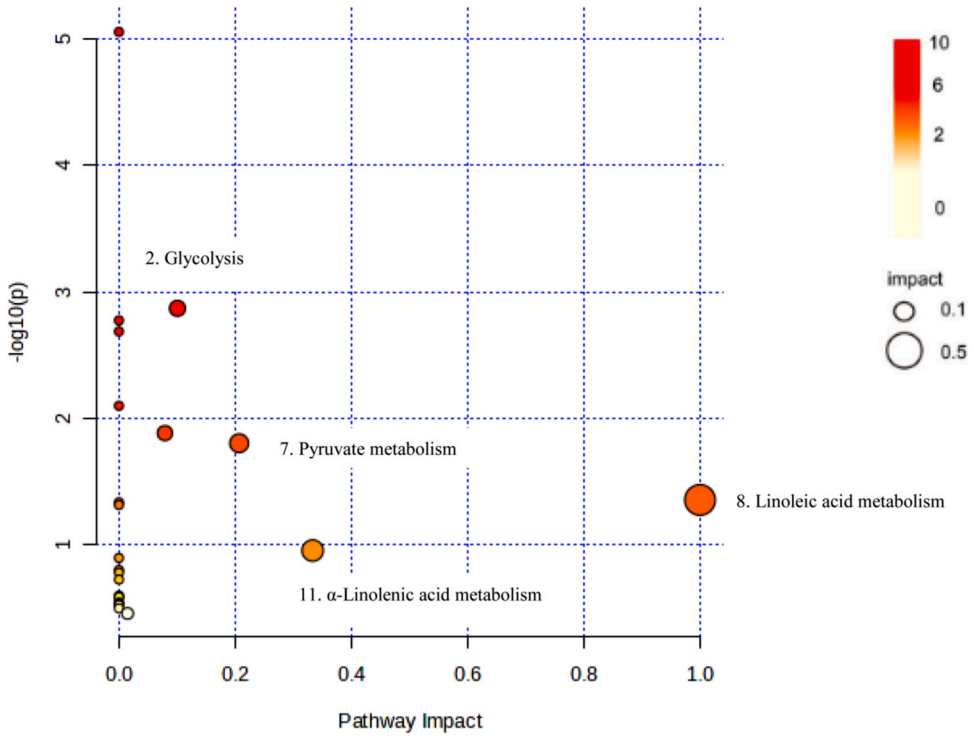

Fig. 4. Bubble diagram of metabolic pathways during angkak fermentation for 30 days (bubble size indicates the influential level of each metabolic pathway and intensity of color shows significant level).

cycle), but the *p*-value and impact value of this Krebs cycle (0.013122 and 0.07907, respectively) were not in the significant criteria to consider the remarkable metabolism during the fermentation. Besides, α-linolenic acid and linoleic acid metabolisms implied that these acids would be adjusted into the longer chain polyunsaturated fatty acids, i.e. docosahexaenoic acid (DHA) and arachidonic acid, respectively (Rahm and Holman, 1964). From metabolomics analysis, *M. purpureus* may be expected releasing DHA and arachidonic acid as the long chain polyunsaturated fatty acids during angkak fermentation that was significant data for angkak production and *Monascus*-related products. In conclusion, pyruvic acid, linolenic acid, and linoleic acid played a remarkable role in transformation into the secondary metabolites in angkak.

4. Conclusions

Based on metabolomics analysis, the primary and secondary metabolites in angkak were investigated by GC-TOF-MS, GC-FID, and HPLC to monitor changing their metabolites during the fermentation for 30 days and subsequently obtained data were studied the relationship between the metabolites and fermentation time as well as possible metabolism pathways. The lag, log, stationary, and death phases of *M. purpureus* growth were as follows: 1. 0–5 days; 5–10 days; 10–15 days; 15–30 days, respectively. This was confirmed by their higher production rates of primary metabolites after 10 and 15 days of the fermentation (see Fig. 2). The PCA and Pearson's correlation coefficients showed that lactobionic acid, alanine, and glucose were used as the indicators for monacolin K secreted by *M. purpureus*. In metabolism analysis using MetaboAnalyst 5.0, 19 metabolites containing the primary and secondary metabolites were converted into 4 metabolic pathways expected significantly, i.e. glycolysis, pyruvate metabolism, α-linolenic acid metabolism, and linoleic acid metabolism. It was expected that the pyruvic acid, linolenic acid, and linoleic acid were the important keys for producing *Monascus* pigments, monacolin K, and citrinin during angkak fermentation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2023.100006.

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