Monacolin K, pigments and citrinin of rice pasta by-products fermented by *Monascus purpureus*

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Abstract

Monacolin K, an anti-hypercholesterolaemic agent, is produced by the fungi *Monascus* spp. along with the bioactive pigment compounds. However, the mycotoxin citrinin is also synthesised by *Monascus* spp. In the present work, low-cost agricultural by-products (rice pasta by-products) were used as substrates for growth and production of pigments, monacolin K and citrinin by *Monascus purpureus* TISTR 3541 and *M. purpureus* TISTR 3629 in solid-state fermentation. Substrate sources were by-products from the production of white rice pasta, brown rice pasta, red jasmine rice pasta, black jasmine rice pasta and mixed rice pasta. *M. purpureus* TISTR 3629 on white rice pasta by-product produced the most economic yields of pigments including yellow (880.35 OD units/g substrate dry weight (sdw)), orange (519.56 OD units/g sdw) and red (586.64 OD units/g sdw), monacolin K (117.69 mg/kg), and low citrinin concentration (4.11 mg/kg).

Introduction

Rice fermented with the red moulds *Monascus* spp. is known as angkak, anka or red yeast rice, and is commonly used to colour food as well as traditional medicine in Asia (Pattanagul *et al.*, 2008; Srianta *et al.*, 2012). *Monascus*-fermented products exhibit anti-hypercholesterolaemic and anti-atherosclerosis properties. Such anti-hypercholesterolaemic effect is contributed by monacolin K or lovastatin as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyses the reduction of HMG-CoA to mevalonate, a rate-limiting reaction of cholesterol biosynthesis pathway in liver (Kennedy *et al.*, 1999; Manzoni and Rollini, 2002). Monacolin K was approved by the United States Food and Drug Administration (FDA) for the treatment of hypercholesterolemia (Manzoni and Rollini, 2002; Jirasatid *et al.*, 2013). *Monascus* spp. also produce pigments that have anti-inflammation properties (Hsu *et al.*, 2013). The yellow pigments (monascin and ankaflavin) possess anti-hyperlipidaemic, anti-atherosclerosis and anti-oxidation functions (Lee *et al.*, 2010; 2012). However, *Monascus* spp. also synthesise citrinin (monascidin A) that is a mycotoxin with nephrotoxic effect in animals and humans (Jirasatid *et al.*, 2013; Samsudin and Abdullah, 2013). The LD_{50} values of citrinin were 35 - 58 mg/kg for mouse and 50 mg/kg for rat (Shi and Pan, 2011). Red yeast rice should consist of low citrinin to ensure the safety for consumption (Samsudin and Abdullah, 2013). The maximum levels of citrinin in food supplements based on rice fermented with red yeast *M. purpureus* are 2 mg/kg in the European Union (European Commission, 2014). Taiwan also has a recommended citrinin limit of 2 mg/kg for functional food products (Jirasatid *et al.*, 2013). The United States Food and Drug Administration (FDA) action level on citrinin in agricultural products for sale is 0.2 mg/kg, while Japan has also prescribed 0.2 mg/kg citrinin limit in *Monascus* pigments (Shi and Pan, 2011; Wang *et al.*, 2017).

The demand for nutraceutical compounds and/or safe natural pigments has increased, especially those produced at reasonable cost. Considering *Monascus* fermentation, solid-state fermentation (SSF) has various advantages over submerged fermentation

(SmF) such as higher pigments productivity, lower cost production and fewer requirements for downstream processing (Jirasatid *et al.*, 2013). In addition, previous studies revealed that the utilisation of cheap available substrates such as agricultural residues through SSF could be economical in the production of monacolin K and/or pigments from *Monascus* (Srianta *et al.*, 2012; Srianta and Harijono, 2015; Jirasatid and Nopharatana, 2017).

Many agricultural residues such as jackfruit seed (Babitha *et al.*, 2006; 2007), corn meal, peanut meal, coconut residue, soybean meal (Nimnoi and Lumyong, 2011), corn cob (Velmurugan *et al.*, 2011), durian seed (Srianta *et al.*, 2012), whole sorghum grain, dehulled sorghum grain, sorghum bran (Srianta and Harijono, 2015) and rice bran (Jirasatid and Nopharatana, 2017) have been used as substrates for pigment production by *Monascus* spp. in SSF. Agricultural residues such as soybean residue (Japakaset *et al.*, 2009), durian seed (Srianta *et al.*, 2012), whole sorghum grain, dehulled sorghum grain and sorghum bran (Srianta and Harijono, 2015) have also been used as substrate for monacolin K production.

However, to our knowledge, no effort has been made to employ rice pasta by-product (RPBP) for the production of secondary metabolites by *Monascus* spp. RPBP includes white rice pasta by-product (white RPBP), brown rice pasta by-product (brown RPBP), red jasmine rice pasta by-product (red jasmine RPBP) and black jasmine rice pasta by-product (black jasmine RPBP), which are by-products obtained from rice pasta by extrusion process. Many studies have indicated that rice varieties as well as *Monascus* strains could influence the formation of secondary metabolites such as pigments and monacolin K (Lee *et al.*, 2006; Chairote *et al.*, 2007; Pattanagul *et al.*, 2008; Pengnoi *et al.*, 2017). Therefore, the present work was aimed to investigate the effect of RPBP (white RPBP, brown RPBP, red jasmine RPBP, black jasmine RPBP and mixed RPBP) on growth and production of pigments, monacolin K and citrinin by *M. purpureus* TISTR 3541 and *M. purpureus* TISTR 3629 with the aims to produce high yield of pigments and monacolin K and low yield of citrinin.

Materials and methods

Cultures

Monascus purpureus TISTR 3541 and TISTR 3629 were purchased from Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The strains were preserved at 4°C on potato dextrose agar (PDA) and sub-cultured once in every 4 w.

Inoculum preparation

Sterile distilled water (10 mL) was added to active cultures at 14 - 15 d. Spores were scraped under aseptic conditions and used as inoculum (1×10^6 spores/mL) (Jirasatid *et al.*, 2013).

Solid-state fermentation

Rice pasta by-product (RPBP) from four rice varieties, white, brown, red, black and mixed from equal portions of all four varieties were obtained from Family Tree Foods Co. Ltd., Thailand. RPBP was defective pasta in shape or broken after extrusion. RPBP (moisture content < 10%) was ground and used as solid-substrate for cultivation. 10 g was transferred to Petri dish. The distilled water was added and well mixed to adjust the moisture content to approximately 50%. The substrate was steamed at 121°C for 30 min, cooled to room temperature and inoculated with 5% (v/w) spore suspension of either *M. purpureus* TISTR 3541 or TISTR 3629, and then cultivated at 30°C for 14 d. *Monascus*-fermented products were dried at 50°C to moisture content < 10% (wet basis), ground and sieved through #80 mesh (Jirasatid *et al.*, 2013). Moisture content and pH were measured every 2 d during cultivation. Following cultivation, the biomass and concentration for each pigment, monacolin K and citrinin were determined.

Moisture content and pH determination

Moisture content and pH were determined as described in Association of Official Analytical Chemists (AOAC, 1995) and Johns and Stuart (1991), respectively.

Biomass determination

Monascus growth was represented by glucosamine content, in form of chitin, present in cell walls of most fungi. Glucosamine was estimated from N-acetylglucosamine released by acid hydrolysis of chitin (Aidoo *et al.*, 1981) Glucosamine content was calculated to fungal biomass through the conversion factor determined using membrane culture technique (Jirasatid *et al.*, 2013). Biomass content was expressed as mg biomass per gram substrate dry weight (mg/g sdw).

Pigment determination

Monascus-fermented product (0.1 g) was extracted in 70% ethanol (50 mL) in a rotary shaker (100 rpm for 1 h) and filtered through Whatman #1 filtrate paper. The optical density (OD) of the supernatant was measured by UV/vis spectrophotometer (Genesys 20, Thermo Scientific, USA) at 400, 470 and 500 nm for yellow, orange and red pigments, respectively against

a 70% ethanol blank (Johns and Stuart, 1991). The pigment concentration (OD units per gram substrate dry weight) was calculated as:

$$\text{pigment concentration} = \frac{\text{OD x dilution x v}}{\text{w}}$$

where OD = absorbance, v = volume of alcohol (50 mL), w = weight of sample, dilution factor = amount of dilution fold.

Monacolin K determination

Monacolin K was measured by high performance liquid chromatography (HPLC Model 600E, Waters, USA) as described by Wang *et al.* (2004).

Citrinin determination

Citrinin was analysed according to Wang *et al.* (2014) with some modification. *Monascus* fermented-product (1 g) was extracted with 30 mL of EW solution (ethanol:water, 70:30, v/v) by shaking on rotary shaker at 200 rpm for 30 min at 40°C, exposing to ultrasonication at 40°C for 30 min, and again shaking on rotary shaker at 200 rpm for 1.5 h at 40°C. All of the solution was filtered with Whatman #1 filtrate paper. Subsequently, the supernatant liquid was filtered with a 0.45 μm pore size filter into HPLC vial for HPLC determination (Model 600E, Waters, USA). Chromatagraphic separation was conducted on an Atlantis C18 column with particle size of 5 μm, 100 A and 150 × 4.6 mm I.D. (Atlantis T3, Waters, USA). Acetonitrile-water (pH was adjusted to 2.5 with H_3PO_4; 50:50) was used as the mobile phase. Eluent was pumped at 1.0 mL/min, and citrinin was measured with a fluorescence detector (model Alliance e2695, Water, USA) at excitation and emission wavelengths of 331 and 500 nm, respectively.

Statistical analysis

A completely randomised design (CRD) was developed for five treatments. Statistical analysis was performed by analysis of variance (ANOVA). Significance of mean values at 95% confidence levels was based Duncan's multiple range tests (DMRT).

Results and discussion

The initial moisture content of RPBP substrates ranged from 49 - 52% and 48 - 53% for the cultivation of *M. purpureus* TISTR 3541 and TISTR 3629, respectively, and was appropriate for growth and pigments production by the genus *Monascus* (Johns and Stuart, 1991; Babitha *et al.*, 2007). The moisture content continuously increased during cultivation (data not shown). Final moisture contents of RPBP varied between 72 – 79% and 73 - 80% for *M. purpureus* TISTR 3541 and TISTR 3629, respectively. The increase in moisture contents were attributed to microbial metabolism for hydrolysis of the nutrients such as carbon and nitrogen sources into biomass and other products including organic acids, carbon dioxide and water (Rosenblitt *et al.*, 2000; Pattanagul *et al.*, 2008).

The pH of samples slightly decreased in the early cultivation period (0 to 8 or 10 d), and then slightly increased (data not shown). Thus, the initial pH of RPBP substrates from *M. purpureus* TISTR 3541 and TISTR 3629 varied from 5.5 - 6.9 and 5.2 - 6.9, respectively with final values between 5.2 - 7.2 and 6.2 - 8.0, respectively. Changes of pH profiles observed in the present work are in accord with those reported by Teng and Felheim (2001). The decrease in pH early in cultivation may be resulted from proliferation of organic acids, produced via the tricarboxylic acid cycle (TCA cycle). Subsequently, the increase in pH may be resulted from ammonium production, a consequence of deamination (Deacon, 2006). Carvalho *et al.* (2003) suggested that while *Monascus* growth occurred within pH 2.5 - 8.0, optimum was 4.0 - 7.0, consistent with those observed in the present work.

Both *Monascus* strains used in the present work grew and produced secondary metabolites consisting of yellow, orange and red pigments and monacolin K, but citrinin was also produced in various quantities on RPBP substrates (Table 1 and 2). Citrinin contents in all samples were higher than the Japanese regulatory limit (< 0.2 mg/kg in *Monascus* pigment). In addition, the contents of biomass and these secondary metabolites were significantly obtained from the different RPBP substrates. Growth of both *Monascus* strains was the highest on red jasmine RPBP. The maximum biomass contents of *M. purpureus* TISTR 3541 and TISTR 3629 were 724.11 and 1667.40 mg/g sdw, respectively (p < 0.05) (Table 1 and 2). The highest yields of yellow, orange and red pigments, and monacolin K for *M. purpureus* TISTR 3541 were obtained on white RPBP (*p* < 0.05), while the highest amount of citrinin occurred on red jasmine RPBP (*p* < 0.05) (Table 1). Solid-state fermentation of *M. purpureus* TISTR 3629 on white RPBP yielded the highest pigments and monacolin K along with citrinin (*p* < 0.05) (Table 2). The results showed obvious difference of substrate into two groups for both *Monascus* strains: brown RPBP corresponded to high biomass content, but low yields of secondary metabolites including pigments,

Table 1. The concentrations of biomass, pigments, monacolin K and citrinin in different rice pasta by-products following cultivation with *Monascus purpureus* TISTR 3541 for 14 days.

Rice pasta by-products (RPBP)	Biomass (mg/g sdw)	Pigment (OD units/g sdw)			Monacolin K (mg/kg)	Citrinin (mg/kg)
		Yellow	Orange	Red		
White RPBP	449.40 ± 92.14[c]	383.06 ± 2.47[a]	160.02 ± 22.06[a]	180.89 ± 22.51[a]	82.93 ± 7.05[a]	33.57 ± 0.49[a]
Brown RPBP	717.44 ± 19.85[a]	141.89 ± 12.09[d]	67.14 ± 12.04[b]	95.62 ± 9.38[b]	13.66 ± 0.35[cd]	17.47 ± 2.78[b]
Red jasmine RPBP	724.11 ± 13.32[a]	319.00 ± 12.12[b]	112.93 ± 28.69[ab]	122.49 ± 34.91[ab]	44.18 ± 7.00[b]	40.30 ± 0.71[a]
Black jasmine RPBP	604.95 ± 0.27[abc]	157.97 ± 16.99[cd]	70.08 ± 2.59[b]	96.47 ± 29.94[b]	21.49 ± 6.61[c]	17.02 ± 1.34[b]
Mixed RPBP	620.55 ± 41.05[ab]	184.61 ± 2.41[c]	85.44 ± 1.12[b]	99.04 ± 2.17[b]	21.72 ± 2.76[c]	18.10 ± 4.31[b]

Mean within columns with different superscripts are significantly different ($p < 0.05$). Data are means ± standard deviation.

monacolin K and citrinin. In contrary, white RPBP corresponded to low biomass content, but high yields of secondary metabolites such as pigments, monacolin K and citrinin (Table 1 and 2). These results implied that the production rate of secondary metabolites did not correlate with the fungal growth rate. This can be explained by the fact that pigments, monacolin K and citrinin are secondary metabolites of *Monascus* spp. The secondary metabolism is usually suppressed by high specific growth rate of cultures. However, in medium supporting the slow growth rates, some nutrient factors may limit growth and favour secondary metabolite production instead (Demain, 1986). Moreover, pigments, monacolin K and citrinin are synthesised from the same polyketide pathway, thus their production could be interrelated (Lee *et al.*, 2006).

The results indicated clearly that substrate significantly influenced growth and production of secondary metabolites by *M. purpureus*, thus reflecting the differences in type and content of carbon and nitrogen sources as well as trace elements in rice varieties. Previous studies reported that rice varieties including white-polished, brown, red and black rice contained different chemical compositions such as protein, fibre, fatty acids, vitamins and minerals (Ha *et al.*, 1999; Fernando, 2013). In addition, the environmental conditions such as pH also affected the growth and formation of secondary metabolites by *Monascus* (Johns and Stuart, 1991; Carvalho *et al.*, 2003). This is in accord with Pengnoi *et al.* (2017), who reported that purple rice varieties influenced the production of red pigment, monacolin K and citrinin by *M. purpureus*.

The biomass contents of *M. purpureus* TISTR 3629 on various RPBP substrates were approximately 1.8 to 2.5 times greater than those from *M. purpureus* TISTR 3541. Yields of yellow, orange, red pigments and monacolin K from *M. purpureus* TISTR 3629 were about 1.0 - 2.3, 1.4 - 3.2, 1.5 - 3.2 and 1.2 - 2.2 times, respectively higher than those from *M. purpureus* TISTR 3541. Moreover, low yields of citrinin (1.63 - 4.11 mg/kg) were also found for *M. purpureus* TISTR 3629, which were 8.2 - 12.6 times

Table 2. The concentrations of biomass, pigments, monacolin K and citrinin in different rice pasta by-products following cultivation with *Monascus purpureus* TISTR 3629 for 14 days.

Rice pasta by-products (RPBP)	Biomass (mg/g sdw)	Pigment (OD units/g sdw)			Monacolin K (mg/kg)	Citrinin (mg/kg)
		Yellow	Orange	Red		
White RPBP	1112.20 ± 139.17[b]	880.35 ± 21.99[a]	519.56 ± 84.91[a]	586.64 ± 7.57[a]	117.69 ± 14.30[a]	4.11 ± 0.66[a]
Brown RPBP	1374.00 ± 54.89[ab]	314.76 ± 58.25[b]	186.59 ± 15.97[b]	235.64 ± 19.19[b]	16.61 ± 0.53[c]	1.66 ± 0.47[b]
Red jasmine RPBP	1667.40 ± 76.99[a]	332.24 ± 3.65[b]	154.74 ± 3.02[b]	184.04 ± 6.85[b]	57.76 ± 2.66[b]	3.20 ± 0.86[ab]
Black jasmine RPBP	1141.80 ± 56.80[b]	371.40 ± 80.36[b]	208.59 ± 76.13[b]	258.93 ± 88.41[b]	34.31 ± 5.76[b]	1.63 ± 0.06[b]
Mixed RPBP	1105.70 ± 102.20[b]	414.62 ± 56.59[b]	248.36 ± 42.43[b]	303.81 ± 66.16[b]	47.56 ± 7.26[b]	2.06 ± 0.89[b]

Mean within columns with different superscripts are significantly different ($p < 0.05$). Data are means ± standard deviation

less than *M. purpureus* TISTR 3541. The result revealed that *M. purpureus* TISTR 3629 was a more suitable strain in terms of pigments and monacolin K yield with lower yield of citrinin.

M. purpureus TISTR 3629 cultivated at 30°C, 50% of initial moisture content for 14 d in the presence of oxygen on white RPBP produced relatively large amounts of pigments including yellow (880.35 OD units/g sdw), orange (519.56 units/g sdw) and red (586.64 units/g sdw), and monacolin K (117.69 mg/kg sdw) along with low amounts of citrinin (4.11 mg/kg sdw). Therefore, the present work demonstrated and suggested that white RPBP fermented by *M. purpureus* TISTR 3629 could be used as functional ingredient. In the present work, white RPBP fermented by *M. purpureus* TISTR 3629 showed the potential to increase the pigments yields higher than those previously studied by Babitha *et al.* (2007) and Nimnoi and Lumyong (2011), and monacolin K concentration higher than that reported by Patcharee *et al.* (2007) and Srianta *et al.* (2012). This might be due to the difference in substrates, environmental conditions such as moisture content, pH and temperature for culture, as well as *Monascus* strains.

Conclusion

This is the first report that demonstrates the utilisation of RPBP for growth and secondary metabolites production by *M. purpureus*. White RPBP fermented by *M. purpureus* TISTR 3629 was a good alternative to enhance amounts of pigments and monacolin K along with a reduction in citrinin. The obtained results thus provide an economically efficient procedure to produce functional ingredient from *Monascus* for industrial applications.

Acknowledgement

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