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Comparative study of red yeast rice with high monacolin K, low citrinin concentration and pigments in white rice and brown rice

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Abstract: Growth pigments and metabolites of monacolin K and citrinin were compared for *Monascus purpureus* during 14-day solid-state fermentation on white rice and brown rice (Chai-Nart cultivar). *Monascus purpureus* IFRPD 4046 was selected as the target strain which produced the highest monacolin K content and the lowest citrinin content. Optimum fermentation conditions regarding moisture content, temperature and fermentation time were determined. A comparative study showed that monacolin K production in white rice was about twice higher than in brown rice. At the optimum conditions, concentrations of monacolin K dried at 55°C to constant weight were 132.98 and 66.48 mg/100 g in white rice and brown rice, respectively while citrinin was not detected. Results revealed that the IFRPD 4046 strain has a potential to produce red yeast rice with higher monacolin K in white rice than in brown rice with low citrinin content.

Keywords: Chai-Nart rice; functional food; moisture content; *Monascus purpureus*; toxin

Red yeast rice is a fermented food product produced by inoculating *Monascus purpureus* into steamed rice (Shi & Pan 2011). It has traditionally been used for colouring, flavouring and preserving food for over one thousand years in East Asia and is still commonly used in Chinese cuisine. Rice is the common substrate for Monascus solid-state fermentation, and Monascus-fermented rice has been widely consumed by people in China, Japan and South East Asia countries (Dufosse *et al.* 2005; Feng *et al.* 2012)

During solid-state fermentation, *M. purpureus* produces various secondary metabolites, mainly pigments and other products including monacolin K and γ-aminobutyric acid (GABA). Monacolin K, commercially known as Mevacor, Cholestin, and Lovastatin may be effective in decreasing blood pressure and lowering plasma cholesterol level (Endo 1979, 1985; Kennedy *et al.* 1999). Monacolin K in red yeast rice can inhibit and lower cholesterol biosynthesis in both humans and animals (Martinkova *et al.* 1995; Wang *et al.* 2004).

However, some *Monascus* strains could produce the mycotoxin citrinin as a secondary toxic metabolite that was previously found mainly in the genera *Aspergillus* and *Penicillium* (Blanc *et al.* 1995). Citrinin naturally occurs in stored food commodities such

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as rice, maize, wheat and barley, and recent research has investigated the control of citrinin concentration in red yeast rice (Hu & Chen 2003). In 2000, research on the methods for citrinin determination was listed in fifteen national projects. After that, a number of studies were carried out on the method for citrinin determination and the current situation of citrinin in the Monascus products was introduced by Xu *et al.* (2003, 2004) and Li *et al.* (2003, 2005a). In Japan, the maximum allowed level of citrinin in red yeast rice is set at 200 ng/g (Srianta *et al.* 2014). In 2011, the European Food Safety Authority (EFSA) provided a scientific opinion on the health claim related to monacolin K from red yeast rice based on human intervention studies. The EFSA did not classify such products as food or medicine. The EFSA's opinion should not be interpreted as an approval of red yeast rice for food or medicinal use (Lachenmeier *et al.* 2012).

The previous research revealed that rice varieties influenced the red yeast rice qualities (Chairote *et al.* 2009). Besides rice varieties, rice characteristics were also an important factor for the quality of red yeast rice. High amylose rice (15–30% amylose) was preferred to use as a raw material of red yeast rice production, especially the polished rice (white rice). However, the polishing process leads to a loss of nutritional compounds as rice bran has been removed. Brown rice (non-polished rice) was reported to contain a high amount of antioxidative and anti-cancer components including polyphenols (Panlasigui & Thompson 2006). Thus using brown rice as a raw material for red yeast rice is an interesting issue which might lead to the use of non-polished rice or other pigment rice.

This study was focused on screening selected strains of *M. purpureus* with high monacolin K and non-producing/low-producing citrinin in white rice and brown rice. Results may increase understanding regarding the application and comparison of red yeast rice using agricultural raw material (white rice and brown rice) as the carrier to produce useful microbial metabolites.

MATERIAL AND METHODS

Microorganism cultivation. In total, eight strains of *Monascus purpureus* (Table 1) were cultured. Four strains were obtained from the culture collection of Institute of Food Research and Product Development (IFRPD) (Kasetsart University, Thailand); three strains by isolation from rice and one strain was obtained from Thailand Institute of Scientific and Technological Research (TISTR). Stock culture was maintained on SDA (Sabouraud – 2% dextrose agar) slant. After inoculation from the original slant, the cultures were incubated at 30°C for 5–7 days. A suspension of spores was obtained by washing the slant cultures with sterile water, and approximately 108 spores were inoculated into 500 ml Erlenmeyer flasks containing 100 ml SDA medium. The seed culture was incubated at 30°C for 4 days in a rotary shaker at 200 rpm.

Solid-state fermentation of Monascus sp. A local source of white rice and brown rice (Chai-Nart cultivar) was used throughout the experiments. White rice and brown rice were soaked in tap water for 3 and 4 h, respectively. After the water had been removed, the soaked rice was drained for 5–10 min and then a 500-ml flask containing 100 g of rice was autoclaved at 121°C for 20 min and cooled to room temperature. After cooling, the initial moisture content was adjusted with sterilized water in the range of 29–44%, the substrates were inoculated with 5 ml of the spore suspension culture, mixed well and incubated at 30°C for 14–15 days. The fermented rice was then dried to constant weight at 55°C until the moisture content was 8–10% and analysed for monacolin K, citrinin and pigments.

Extraction and analysis of monacolin K. A total of 0.5 g of fermented red yeast rice powder was accurately weighed and transferred into a 20 ml plugged centrifuge tube. Triplicate preparations were extracted with 8 ml of 75% ethanol for 30 min in an ultrasonic bath and subsequently centrifuged for 10 min at 3000 rpm. This extraction procedure was repeated three times; the supernatants were combined and transferred into a 25 ml volumetric flask, adding 75% ethanol to exactly 25 ml. The final solution was left to stand for 30 min, and then filtered through a 0.45 µm membrane before being placed in vials for HPLC analysis.

The chromatographic condition was analysed by high performance liquid chromatography (Model Agilent 1200, USA). A C_{18} column of water symmetry (150 nm × 3.9 nm i.d., 5 µm) was chosen as the stationary phase with a gradient of acetonitrile (eluent A) and 0.1% TFA (eluent B) as the mobile phase. Linear gradient elution was carried out at 1 ml per minute from 35% to 75% of elution A in 20 min, retaining 75% elution A from 20 min to 28 min-

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utes. Total analysis time was 35 min, and the chromatograph of the baseline was stabilized with the separation system reaching equilibrium. The photodiode array (PDA) detector was set at 210–350 nm and the chromatogram was detected at 237 nm. Column temperature was set at 30°C with injection volume at 20 µl. Methods for the determination of monacolin K followed Li *et al.* (2005b).

Determination of citrinin. A volume of 20 ml of extraction solvent (methanol) was added to 1 ± 0.02 g of ground sample in 30 ml screw capped glass bottles. The samples were vortexed for 1 min and then heated in a water bath at 70°C for 30 minutes. After cooling to room temperature, the samples were vortexed for another minute and filtered through 13 mm × 0.22 µm Nylon syringe filters directly into an autosampler vial. HPLC-fluorescence analysis was performed by an Agilent 1200 HPLC system equipped with pump, autosampler, column oven and fluorescence detector at wavelengths of 330 and 500 nm for excitation and emission, respectively. Citrinin was determined by HPLC on a C_{18} column using the mobile phase, with the composition of acetonitrile-water-trifluoroacetate (55 + 45 + 0.05 v/v). Flow rate was set at 1 ml/min and fluorescence detection was used. Citrinin determination followed the method of Lee *et al.* (2006).

Measurement of pigment concentration. An accurate weight of 1 g of fermented rice powder was transferred into a flask and mixed with 40 ml of 75% ethanol. The mixture was agitated on a rotary shaker (200 rpm) for 1 hour. The extract was then centrifuged at 5000 *g* for 20 min and the supernatant analysed by the spectrophotometer against 75% ethanol blank with absorbance at 470 nm. Results were expressed in absorbance units at the corresponding wavelength per gram (AU/g) according to Bussaba *et al.* (2000) and Ignatius *et al.* (2016).

Statistical analysis. The experiments were performed in triplicate. The results were reported as mean with standard deviation. The data were subjected to analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Screening of Monascus sp. strains. The productivity of monacolin K, citrinin and pigments was examined using solid-state culture on eight strains of *Monascus* sp. (Tables 1 and 2). Table 1 shows that *M. purpureus* IFRPD 4046 produced the highest monacolin K amount in both white rice and brown rice with yields of 118.64 mg/100 g and 67.77 mg/100 g, respectively. The lowest yield was recorded by S-1 strain in both white and brown rice at 14.85 and 2.48 mg/100 g, respectively. *M. purpureus* strains IFRPD 4044 and IFRPD 4046 did not produce any citrinin, while R strain gave the highest yield at 0.15 and 0.22 µg/g of citrinin in white rice and brown rice, respectively. Different strains of the fungus resulted in diverse monacolin K and citrinin production (Chen 2005). Monacolin K recorded in our study was higher than in other reports; for example Rajasekaran & Kalaivani (2012) reported monacolin K at 37 mg/100 g using *M. purpureus* MTCC1090 on Indian rice, while Xu *et al.* (2005) recorded mo-

Table 1. Screening for Monacolin K and citrinin contents in red yeast rice with various *M. purpureus* after fermentation at 30°C for 14 days

Strain	Source	White rice		Brown rice	
		monacolin K (mg/100g)	citrinin (µg/g)	monacolin K (mg/100g)	citrinin (µg/g)
IFRPD 4044	IFRPD (Thailand)	56.01 ± 0.21^{e}	nd	22.14 ± 0.51^{k}	nd
IFRPD 4045		87.54 ± 1.24^{c}	0.12 ± 0.01^{b}	34.15 ± 1.34^{i}	0.11 ± 0.02^{b}
IFRPD 4046		118.64 ± 0.03^{a}	nd	67.77 ± 0.66^{d}	nd
IFRPD 4047		86.85 ± 0.14^{c}	0.09 ± 0.04^{b}	32.08 ± 0.60^{j}	0.08 ± 0.05^{b}
TISTR 3090	TISTR (Thailand)	21.40 ± 1.22^{k}	0.10 ± 0.05^{b}	2.34 ± 0.32^{n}	0.14 ± 0.07^{b}
No. R	Isolated from rice	100.84 ± 0.83^{b}	0.15 ± 0.02^{b}	52.20 ± 1.48^{f}	0.22 ± 0.01^{a}
No. S-1		14.85 ± 2.11^{l}	0.09 ± 0.07^{b}	2.48 ± 1.54^{n}	0.12 ± 0.02^{b}
No. CH		32.08 ± 0.60^{j}	0.05 ± 0.01^{c}	4.68 ± 0.60^{m}	0.07 ± 0.03^{b}

Different superscript letter within columns of Monacolin K and Citrinin expressed significant different ($P \leq 0.05$); nd – not detected

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Table 2. Pigments concentration of red yeast rice producing by various *M. purpureus* strains after fermentation at 30°C for 14 days in white rice and brown rice

Monascus strain	Pigment concentration (AU/g) at 470 nm	
	white rice	brown rice
IFRPD 4044	198.20 ± 0.36[b]	187.25 ± 0.25[d]
IFRPD 4045	165.51 ± 0.12[f]	157.24 ± 0.30[f]
IFRPD 4046	222.20 ± 0.03[a]	194.90 ± 0.15[c]
IFRPD 4047	101.25 ± 0.19[j]	98.65 ± 0.13[k]
TISTR 3090	50.37 ± 1.34[m]	23.60 ± 0.98[n]
No. R	179.60 ± 0.04[e]	157.20 ± 0.16[f]
No. S-1	83.60 ± 0.76[l]	143.50 ± 1.50[h]
No. CH	147.80 ± 0.61[g]	137.30 ± 0.07[i]

Different letters within the columns different significantly ($P \leq 0.05$)

nacolin K production by *M. ruber* at 344.6 mg/100 g on rice added to soybean flour. In addition, some factors affected the yield of monacolin K such as fermentation method, temperature and raw material. Chiu *et al.* (2006) studied monacolin K produced by liquid state fermentation on rice material that showed a lower yield of 46.5–53.5 mg/100 g, while Su *et al.* (2003) recorded the highest yield of monacolin K by solid state fermentation at 30°C.

Results of pigment concentration (Table 2) indicated that *M. purpureus* IFRPD 4046 presented the highest value of absorbance unit per g (AU/g) at 470 nm in both white rice and brown rice as 222.20 and 194.90 AU/g, respectively. This strain was selected as the target for further study.

Optimization of solid-state fermentation conditions of M. purpureus IFRPD 4046. To further improve the capability of IFRPD 4046 strain to produce monacolin K, solid-state fermentation conditions (including initial moisture content, incubation temperature and time) were optimized. Results in Table 3 show that with the initial moisture content of white rice in the flasks between 32% and 38%, IFRPD 4046 strain produced higher concentrations of monacolin K. The maximum of 132.98 mg/100 g was produced at 35% moisture content, with citrinin not detected. Growth of *M. purpureus* IFRPD 4046 as a solid-state culture in brown rice with initial moisture of 38% was clearly the highest with the concentration of monacolin K at 66.48 mg/100 g, less than half the maximum in white rice, whereas citrinin concentration produced by this strain was not detected.

Production of monacolin K by *M. purpureus* IFRPD 4046 strain was modified by temperature at 25, 30°C and room temperature (RT) and time (0–24 days) (Figure 1). Higher room temperature at 32–35°C and lower at 25°C could decrease monacolin K concentration. Japakaset *et al.* (2009) also supported and reported that monacolin K concentration from *M. purpureus* IFRPD 4046 decreased under cultivation at 35°C. Chen & Hu (2005) revealed that monacolin K produced lower concentration at 20°C than at 25°C with culture temperature of 30°C, monacolin K reached a maximum in white rice after 14-day fermentation and in 16 days in brown rice. Thus, the optimization condition of solid-state fermentation of *M. purpureus* IFRPD 4046 strain was 100 g pre-soaked white rice with 35% initial moisture content and incubation for 14 days at 30°C. Furthermore, the optimization condition of brown rice giving the highest yield of monacolin K was achieved at initial moisture content of 38% and incubation time 16–18 days at 30°C. Citrinin, a nephrotoxic agent,

Table 3. Effect of moisture content in white rice and brown rice on monacolin K and citrinin produced by *M. purpureus* IFRPD 4046

Moisture content (%)	White rice		Brown rice	
	monacolin K (mg/100g)	citrinin (μg/g)	monacolin K (mg/100g)	citrinin (μg/g)
29	98.76 ± 0.07[c]	nd	22.55 ± 0.12[i]	nd
32	115.20 ± 0.12[b]	nd	31.59 ± 0.14[h]	nd
35	132.98 ± 0.16[a]	nd	45.94 ± 0.25[f]	nd
38	70.99 ± 0.09[d]	nd	66.48 ± 0.02[e]	nd
41	24.20 ± 0.23[i]	nd	38.23 ± 0.17[g]	nd
44	2.78 ± 1.28[k]	nd	8.42 ± 0.46[j]	nd

Different letters within the columns different significantly ($P \leq 0.05$)

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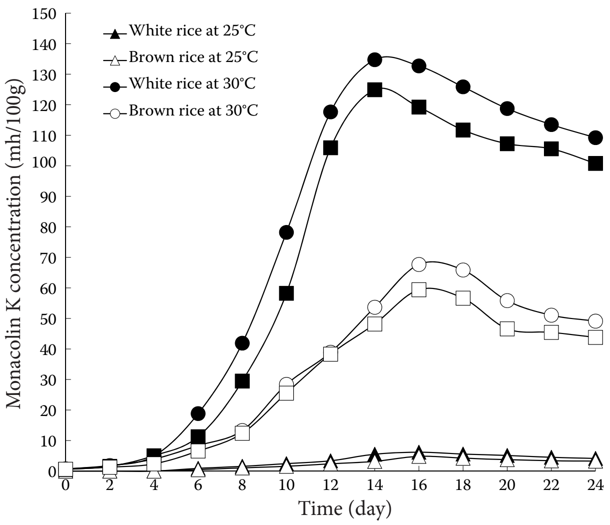

Figure 1. Effect of temperature and time on the production of monacolin K by *M. purpureus* IFRPD 4046. Values are presented as mean ± SD (n = 3)

is produced by *M. purpureus* or other strains in both submerged and solid-state cultures. According to Blanc *et al.* (1995), there is some risk of citrinin contamination in the fermentation process. However, this can be avoided either by using a strain of *M. purpureus* that does not produce any citrinin or by adjusting the fermentation conditions for citrinin-free production. However, no citrinin was detected. Therefore, both fermented rice products passed the standards of Japan, Taiwan and EU which have imposed maximum concentration of citrinin at 2000 ppb (Chung *et al.* 2009; European Commission 2014).

CONCLUSIONS

Agricultural raw materials of white rice and brown rice (Chai-Nart cultivar) were used as solid carriers for efficient conversion of high monacolin K, red pigments and absent citrinin by *M. purpureus* IFRPD 4046. The yield of monacolin K on white rice was about twice higher than on brown rice at 132.98 and 66.48 mg/100 g, respectively while citrinin was not detected. The optimum temperature of cultivation was 30°C after 14-day fermentation with 35% initial moisture when monacolin K reached a maximum in white rice. The strain of *M. purpureus* and the rice cultivar have a major effect on growth. A detailed mechanistic study on the differences between white rice and brown rice regarding physical properties and activity of key enzymes is in progress.

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