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Effect of combination of Hom Nil or Riceberry broken rice with white rice on antioxidant properties of *Monascus*-fermented rice

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Abstract

The present work aimed to investigate the effects of combining by-products, namely Hom Nil broken rice (HNB) and Riceberry broken rice (RBB) with white rice (WR), in solid-state fermentation (SSF), by *Monascus purpureus* TISTR 3629. The HNB:WR and RBB:WR systems were varied in different ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (w/w). The secondary metabolites of *Monascus* such as pigments (yellow, orange, and red), monacolin K, and mycotoxin citrinin, as well as other bioactive compounds such as total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and their antioxidant activities were determined. Across these ratios, from 100:0 to 0:100 in HNB:WR and RBB:WR systems, significant increases were observed in pigments, monacolin K, TPC, TFC, and TAC, together with decreases in citrinin contents. Interestingly, strong DPPH and $ABTS^+$ radical scavenging activities (RSA) occurred in all *Monascus*-fermented rice samples. For the HNB:WR system, DPPH and $ABTS^+$ RSA were 78.2 - 92.5 and 98.0 - 99.7%, respectively, and for the RBB:WR system, they were 81.8 - 95.9 and 94.8 - 99.2%, respectively. Although HNB:WR or RBB:WR at a 0:100 (fermented WR) produced significant amounts of various bioactive compounds without citrinin, the HNB:WR or RBB:WR ratios of 25:75 yielded economic amounts of pigments, monacolin K, TPC, TFC, and TAC, along with low citrinin concentrations. The present work offered a valuable method by applying the co-substrate technique in SSF, for the conversion of low-cost agricultural by-products, like HNB and RBB, into high value-added products, which were rich in bioactive compounds and antioxidant activity.

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Introduction

Hom Nil and Riceberry rice are Thai pigmented rice (*Oryza sativa* L.) with black and purple-black grain, respectively. During husk removal and/or polishing, a by-product broken rice is generated, which is a fragment of the rice grain. In general, 20 - 30% of the total crop is broken, resulting in 1,200 - 1,800 tons of Riceberry broken rice per harvest season (Luang-In *et al*., 2018; Sapna *et al*., 2019; Eze *et al*., 2022). Often, broken rice is sold at low price for production of rice flour, snacks, and animal feed. However, pigmented broken rice such as Hom Nil broken rice (NHB) and Riceberry broken rice (RBB) contain various bioactive compounds such as oryzanols, phenolic acids, flavonoids, and anthocyanins, and also exhibit high antioxidant activity (Luang-In *et al*., 2018; Sapna *et al*., 2019). Therefore, NHB and RBB can be used to provide the functional ingredients for functional foods and/or nutraceuticals.

Solid-state fermentation (SSF) is one of the most effective methods for converting agricultural by-products into high value-added products containing higher amounts of bioactive compounds from microbial hydrolysis and biotransformation (Zhao *et al*., 2021). Filamentous fungi, *Monascus purpureus*, is attested as GRAS (generally regarded as safe) (Yang *et al*., 2022). Rice fermented by *M. purpureus* is well-known as *Monascus*-fermented

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rice or red yeast rice, which is generally used as a food colouring agent and in traditional Chinese medicine (Jirasatid *et al*., 2019).

Monascus species enhance bioactivity by synthesising secondary metabolites, namely monacolin K and pigments. Monacolin K (lovastatin) is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and contributes to the anti-hypercholesterolemic effect by inhibition of the key enzyme HMG-CoA reductase in a rate-limiting step of the cholesterol biosynthesis pathway in the human liver. *Monascus* pigments including yellow (monascin and ankaflavin), orange (rubrapunctatin and monascorubrine), and red (rubropunctamine and monascorubramine) possess HMG-CoA reductase inhibition, antioxidant, and anti-atherogenic properties. Nevertheless, the mycotoxin citrinin with nephrotoxic effect in animals and humans is also produced by *Monascus* spp. (Mohankumari *et al.*, 2021). Low levels of citrinin in *Monascus*-fermented rice assure the safety for consumption. Recently, a number of studies were carried out and confirmed that fermentation with *M. purpureus* improved total phenolic and/or total flavonoid contents as well as antioxidant activities of plant materials including oat (Bei *et al.*, 2017), corn kernel (Chen *et al*., 2021), rice bran (Razak *et al*., 2015; Cheng *et al.*, 2016), and quinoa (Yang *et al*., 2022).

Traditionally, *Monascus*-fermented rice was prepared from white rice (WR), a non-glutinous rice. However, fermentation of brown rice was investigated in some studies. Saithong *et al.* (2019) revealed that *M. purpureus* produced lower yields of monacolin K and pigments when grown on brown rice as compared to WR. However, Pengnoi *et al.* (2017) found that various purple rice varieties fermented by *M. purpureus* showed strong antioxidant activities due to the large amounts of antioxidants in the purple rice substrates. Therefore, in the present work, the combination of pigmented broken rice, including HNB or RBB with WR, was proposed for fermentation with *Monascus*. This approach may lead to the production of better bioactive compounds and/or enhance antioxidant activity. The aim of the present work was to investigate the effects of the ratios of HNB and WR, as well as RBB and WR in SSF using *M. purpureus* on bioactive compounds and antioxidant activity. It was expected that a high value-added product can be achieved from pigmented broken rice, an agricultural by-product, for utilisation as functional ingredients.

Materials and methods

Materials

Hom Nil broken rice (*Oryza sativa* L. cv. Hom Nil) was purchased from Green Net Cooperative, Thailand. Riceberry broken rice (*Oryza sativa* L. cv. Riceberry) and white rice Sao-Hai (*Oryza sativa* L. cv. Sao-Hai) were locally acquired in Chonburi and Bangkok provinces, Thailand, respectively.

Strain and preparation of culture

The fungus *Monascus purpureus* TISTR 3629 was purchased from the Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The strain was cultivated at 30°C on potato dextrose agar (PDA) (Himedia Laboratories Pvt. Ltd., India) for 15 d. The spore suspension was collected by submerging the fungal colonies with sterile distilled water (10 mL) and scraping them under aseptic condition, consequently obtaining approximately 1×10^6 spores/mL. Koji of *Monascus*-fermented rice was performed by SSF following the method described by Jirasatid *et al.* (2019). *M. purpureus* was cultivated on Sao-Hai rice at 30°C for 7 d, crushed, stored at 4°C, and then used as the inoculum.

Fermentation of Monascus-fermented rice and experimental design

The pigmented broken rice (HNB and RBB) and WR were separately ground and sieved through #20 mesh, and then used as the solid substrates for cultivation. Five treatments for each mixture were prepared by mixing of HNB or RBB with WR in different ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (w/w).

Ten gram of substrate were mixed with distilled water (10 mL) to give 50% moisture content. The substrate was sterilised by autoclaving at 121°C for 30 min, and left to cool down to room temperature before inoculation. Subsequently, 20% (w/w) of koji was smeared onto the surface of the substrate. After cultivation at 30°C for 15 d, *Monascus*-fermented rice was dried at 50°C until the moisture content was < 10% (wet basis). It was then ground and sieved through a #80 mesh for analysis of the functional properties.

Determination of pigments, monacolin K, and citrinin

The pigments of *Monascus*-fermented rice, including yellow, orange, and red, were analysed using a UV/vis spectrophotometer (Genesys 20, Thermo Scientific, USA) at 400, 470, and 500 nm, respectively, following the procedure of Jirasatid *et al.* (2019). Monacolin K and citrinin contents were assayed by high performance liquid chromatography (HPLC Model 600E, Waters, USA) in accordance with the method of Wang *et al*. (2004) and Jirasatid *et al*. (2019), respectively.

Extraction of phenolic compounds

Briefly, 1 g of *Monascus*-fermented rice was extracted with 80% (v/v) methanol (10 mL) (Kemaus, Elogo Enterprices Pty. Ltd., Australia) during ultrasonication (300 W, 45°C) for 60 min. The suspension was then centrifuged at 3,500 rpm for 15 min, and the supernatant was filtered with Whatman #1, and then kept at -18°C before further analysis (Huang *et al*., 2017; Chen *et al*., 2021).

Determination of total phenolic content

Total phenolic content (TPC) was quantified using the Folin-Ciocalteu's reagent (Loba Chemie Pvt. Ltd., India) as described by Huang *et al.* (2017). Briefly, 50 µL of phenolic extract or the standard solution of gallic acid (Sisco Research Laboratories Pvt. Ltd., India) was mixed with 2% sodium carbonate solution (2.0 mL), and incubated at ambient temperature for 2 min. Then, 100 µL of 50% Folin-Ciocalteu's phenol reagent was added, mixed well, and subsequently incubated at ambient temperature for 30 min in the dark before the absorbance at 720 nm was measured using a UV/vis spectrophotometer. TPC was reported as mg of gallic acid equivalent (GAE) per g of sample (mg GAE/g sample).

Determination of total flavonoid content

Total flavonoid content (TFC) was assessed using a colorimetric method previously described by Cheng *et al.* (2016). First, 1 mL aliquot of phenolic extract was mixed well with 10% aluminium nitrate (0.1 mL), 1 M potassium acetate (0.1 mL) (Kemaus, Elogo Enterprices Pty. Ltd., Australia), and 4.3 mL of ethanol (Liquor Distillery Organization, Thailand). The mixture was allowed to stand at room temperature for 40 min. The absorbance was measured immediately against a blank at 415 nm. A calibration curve was achieved using catechin, and TFC was presented as mg of catechin equivalent (CE) per gram of sample (mg CE/g sample).

Determination of total anthocyanin content

Total anthocyanin content (TAC) was determined by a pH-differential method using two buffer systems: 0.025 M potassium chloride buffer at pH 1.0, and 0.4 M sodium acetate buffer at pH 4.5 (Kemaus, Elogo Enterprices Pty. Ltd., Australia) (Kara and Ercelebi, 2013; Eze *et al.*, 2022). First, 0.2 mL aliquot of the extract was diluted with 1.8 mL of potassium chloride buffer or sodium acetate buffer. The mixture was vortexed and incubated in the dark for 15 min at room temperature. The absorbance was recorded at 520 and 700 nm, respectively, using a UV/vis spectrophotometer. The monomeric anthocyanin concentration was calculated as mg cyanidin-3-glucoside per gram of sample using Eq. 1:

$$\text{TAC (mg/g)} = \frac{\text{A}\times\text{Mw}\times\text{DF}\times\text{V}\times 1000}{\varepsilon\times\text{L}\times\text{W}} \qquad \text{(Eq. 1)}$$

where, A = (A_{520nm} - A_{700nm}) pH 1.0 - (A_{520nm} - A_{700nm}) pH 4.5; Mw = the molecular weight of cyanidin-3-glucoside (449.2 g/g mol); DF = dilution factor; V = extract volume (mL); ε = the molar extinction coefficient (26,900 L/g mol/cm) for cyanidin-3-glucoside; L = path length (1 cm); W = weight of sample powder (g); and 1,000 = conversion from g to mg.

HPLC determination of phenolic composition

The phenolic components of extract were measured using HPLC system (Model 600E, Waters, USA) equipped with a photodiode array detector (PAD, Waters, USA). Chromatographic separation was conducted on a Synergi^{TM} 4 µm Hydro-RP 80 A° column (5 µm, 250 × 4.6 mm; Phenomenex, USA). The mobile phase consisted of solvent A (acetic acid:water 20:980, v/v) and solvent B (acetonitrile). Different elution gradients were applied as follows: 0 - 3 min, 100% A; 3 - 15 min, 100 - 96% A; 15 - 30 min, 96 - 90% A; 30 - 50 min, 90 - 85% A; 50 - 60 min, 85 - 77% A; 60 - 66 min, 77 - 100% A; and 66 - 70 min, 100% A. The column temperature was set at 30°C, the flow rate was 1 mL/min, and the injection volume of sample was 10 µL. The PAD wavelength was set at 280 nm. The results were expressed as mg per kg of sample in each phenolic standard equivalent (Sigma-Aldrich, USA).

Determination of antioxidant activity

The antioxidant activity of phenolic extracts was determined by DPPH and $ABTS^+$ assay (Cheng *et al*., 2016; Bei *et al*., 2017; Huang *et al.*, 2017). For the measurement of the DPPH radical scavenging activity, the phenolic extract (0.2 mL) was mixed with DPPH solution (0.2 mM, 2.0 mL) (Sigma-Aldrich, USA) in methanol. After incubation in the dark for 30 min, the absorbance was read at 517 nm using a UV/vis spectrophotometer.

For $ABTS^+$ radical scavenging activity determination, $ABTS^+$ was generated by the oxidation of 7 mM ABTS (8 mL) (Sigma-Aldrich, USA) with 2.45 mM potassium persulphate (12 mL) (Kemaus, Elogo Enterprices Pty. Ltd., Australia), and then kept in the dark at ambient temperature for 16 h. The $ABTS^+$ solution was diluted with deionised water to obtain an OD_{734} of 0.7 before use. Subsequently, 20 µL of phenolic extract was mixed with 1 mL of $ABTS^+$ solution, vigorously vortexed, and incubated in the dark at room temperature for 30 min. The absorbance was then measured at 734 nm against a blank using a UV/vis spectrophotometer. The results were expressed as percentage free radical scavenging activities (RSA).

Statistical analysis

All the data were presented as mean ± standard deviation for each sample. The experiments were conducted following a completely randomised design (CRD). Data were statistically analysed for analysis of variance (ANOVA) using Tukey's multiple comparison test (Minitab version 18, Minitab Pty. Ltd., Australia) with the significance set at $p \leq 0.05$.

Results and discussion

Monacolin K, pigments, and citrinin

The effects of the different ratios of HNB:WR and RBB:WR substrates on the production of pigments, monacolin K, and citrinin by *M. purpureus* are presented in Table 1. The changes of HNB:WR ratios from 100:0 to 0:100 in SSF resulted in significant increases in pigment contents ($p \leq 0.05$); yellow (from 318.58 to 1297.15 OD unit/g), orange (from 201.81 to 738.42 OD unit/g), and red (from 221.87 to 738.38 OD unit/g) pigments, as well as monacolin K (103.39 to 5,988.61 mg/kg) ($p \leq 0.05$). In addition, there was a dramatic decrease in the mycotoxin citrinin (from 4.11 mg/kg to not detected (ND)) ($p \leq 0.05$). Similarly, as the proportion of RBB:WR varied from 100:0 to 0:100, pigments such as yellow, orange, and red significantly increased, with values ranging from 295.25 to 1297.15, 156.99 to 738.42, and 19.30 to 738.38 OD unit/g, respectively ($p \leq 0.05$), and monacolin K ranged from 190.20 to 5,988.61 mg/kg ($p \leq 0.05$), while low levels of citrinin were observed (from 2.18 mg/kg to ND). Thus, the highest yields of pigments and monacolin K, and also without citrinin, occurred in HNB:WR or

Table 1. Concentration of pigments including yellow, orange, red, monacolin K, and citrinin of *Monascus*-fermented rice cultivated on a mixture of Hom Nil broken rice and white rice (HNB:WR), and Riceberry broken rice and white rice (RBB:WR), at different ratios.

System	Ratio	Pigment (OD unit/g)			Monacolin K	Citrinin
		Yellow	Orange	Red	(mg/kg)	(mg/kg)
HNB:WR	100:0	318.58 ± 81.98^{b}	201.81 ± 75.12^{b}	221.87 ± 44.94^{b}	103.39 ± 3.56^{e}	4.11 ± 0.10^{a}
	75:25	398.88 ± 14.20^{b}	243.68 ± 69.11^{b}	259.33 ± 32.85^{b}	296.98 ± 8.63^{d}	1.22 ± 0.11^{b}
	50:50	479.71 ± 57.04^{b}	241.37 ± 27.37^{b}	282.93 ± 38.14^{b}	550.00 ± 11.06^{c}	0.82 ± 0.06^{c}
	25:75	843.46 ± 208.31^{ab}	372.90 ± 68.57^{ab}	407.64 ± 85.88^{ab}	1919.85 ± 24.94^{b}	ND
	0:100	1297.15 ± 258.59^{a}	738.42 ± 270.06^{a}	738.38 ± 188.56^{a}	5988.61 ± 11.39^{a}	ND
RBB:WR	100:0	306.40 ± 22.81^{b}	169.63 ± 8.33^{b}	206.84 ± 0.71^{b}	240.54 ± 5.59^{c}	1.65 ± 0.11^{b}
	75:25	295.25 ± 24.34^{b}	156.99 ± 4.47^{b}	193.02 ± 7.70^{b}	190.20 ± 14.72^{d}	2.12 ± 0.12^{a}
	50:50	426.04 ± 145.53^{b}	222.57 ± 70.23^{b}	254.39 ± 60.29^{b}	232.69 ± 4.56^{cd}	2.18 ± 0.06^{a}
	25:75	758.04 ± 272.65^{ab}	376.75 ± 110.77^{ab}	424.15 ± 104.99^{ab}	635.44 ± 30.87^{b}	1.03 ± 0.10^{c}
	0:100	1297.15 ± 258.59^{a}	738.42 ± 270.06^{a}	738.38 ± 188.56^{a}	5988.61 ± 11.39^{a}	ND

Values are mean ± standard deviation. Mean within column for each system with different lowercase superscripts are significantly different ($p \leq 0.05$). ND: not detected.

RBB:WR ratios of 0:100 (fermented WR). However, *Monascus* on both HNB:WR or RBB:WR at 25:75 yielded high amounts of pigments, which did not significantly differ from fermented WR ($p > 0.05$), together with low citrinin content. On the other hand, HNB:WR with a ratio of 100:0 (fermented HNB) yielded the lowest levels of pigments and monacolin K, along with the highest citrinin among the HNB:WR samples. Meanwhile, *Monascus* on RBB:WR at 75:25 yielded lower pigments and monacolin K than those of the other RBB:WR samples.

The results clearly showed that the combination of HNB and WR, as well as RBB and WR, significantly affected the production of secondary metabolites by *Monascus.* This variation resulted from various types and amounts of carbon and nitrogen sources, as well as trace elements present in the rice varieties. Previous studies reported that rice varieties, such as white-polished and pigmented rice, displayed differences in their chemical compositions including proteins, fibres, fatty acids, vitamins like vitamin B_1 and B_2, and minerals like iron and zinc (Fernando, 2013). Furthermore, pigmented rice contains bran, a hard outer layer, whereas white-polished rice lacks the rice bran layer, thereby facilitating the easier penetration of fungal hyphae into the substrate, and hence the release of enzymes (Yasui *et al*., 2020). This may enhance the absorption of nutrients, sustaining the growth of fungi and their metabolic activities.

It was obvious that the changes in pigments and monacolin K contents were inversely correlated with the citrinin content (Table 1). A probable mechanistic explanation is that the pathway shifted for the biosynthesis of pigments, monacolin K, and citrinin. Even though these compounds are produced from the polyketide pathway, pigments and monacolin K are synthesised from hexaketides, whereas citrinin is synthesised from tetraketides (Juzlova *et al.*, 1996). Hence, under high production of pigments and monacolin K, citrinin production may be reduced. Lin *et al.* (2008) demonstrated that the formation of pigments and citrinin were not necessarily associated. In the past, high yields of monacolin K and/or pigments with low levels of citrinin have been reported in many studies (Lee *et al*., 2006; Maric *et al*., 2019). This can be achieved by modification of environmental parameters, modification of substrate or nutritional parameters, and selection of a non-toxigenic *Monascus* strain (Maric *et al*., 2019). For example, Maric *et al*. (2019) found that the cultivation of *M. purpureus* on millet supported the formation of higher monacolin K and lower citrinin as compared to brown rice, however, brown rice was a better substrate for pigment formation.

From a toxicologic perspective, low concentration of the toxin citrinin in food supplements based on *Monascus*-fermented rice is an important factor in terms of food safety. In the European Union, the maximum allowance of citrinin content in food supplements based on rice fermented with *M. purpureus* is 2 mg/kg (EC, 2014). Taiwan recommended a citrinin limit of 2 mg/kg for functional foods. Japan specifies the presence of citrinin to be < 0.2 mg/kg in *Monascus* pigments (Shi and Pan, 2011). As seen in Table 1, citrinin contents of samples varied between ND - 4.11 mg/kg, and ND - 2.18 mg/kg for HNB:WR and RBB:WR systems, respectively. The present work suggested that all *Monascus*-fermented rice could be used as a functional ingredient because it is sparingly used in food products, therefore the contamination of citrinin in food supplements based on rice fermented with *M. purpureus* should be less than the recommended level (< 2 mg/kg food product according to European Union standard). Nonetheless, in the case of an application for a nutraceutical or dietary supplement, *Monascus*-fermented rice containing citrinin with < 2 mg/kg should be employed.

In the present work, WR proved to be a satisfactory substrate for the production of monacolin K and pigments, and without toxin citrinin by *M. purpureus*. However, the utilisation of broken pigmented rice such as HNB and RBB as functional ingredient could be performed by combining them with WR in order to enhance pigments and monacolin K, and achieve a low citrinin concentration. Notably, *Monascus* cultivated on HNB:WR or RBB:WR with a ratio of 25:75 produced economic yields of pigments with minimal citrinin. These samples showed the ability to increase pigment contents above those reported by Pengnoi *et al.* (2017) and Dajanta *et al*. (2019), as well as higher monacolin K concentrations than our previous study (Jirasatid *et al*., 2019). This might have been due to the differences in substrates, environmental factors including moisture content, pH, temperature, and inoculum size for cultivation, as well as the strains of *Monascus*.

Total phenolic, total flavonoid, and total anthocyanin contents

Significant improvements in TPC, TFC, and TAC were observed when the proportion of WR increased (or pigmented broken rice decreased) based on both HNB:WR and RBB:WR systems (Table 2). They showed a similar tendency with regard to pigments and monacolin K. The variation of HNB:WR ratios from 100:0 to 0:100 led to remarkable increases in TPC, TFC, and TAC, with values increasing from 23.44 to 40.14 mg GAE/g, 4.05 to 11.54 mg CE/g, and 0.09 to 2.48 mg/g, respectively ($p \leq 0.05$). In addition, among the RBB:WR samples, TPC, TFC, and TAC ranged from 10.78 - 33.66 mg GAE/g, 3.64 - 11.54 mg CE/g, and 0.14 - 2.48 mg/g, respectively ($p \leq 0.05$). In all *Monascus*-fermented rice samples, the maximum TPC (40.14 mg GAE/g) was observed in the HNB:WR ratio of 25:75, while the maximum TFC (11.54 mg CE/g) and TAC (2.48 mg/g) occurred in the HNB:WR or RBB:WR ratios of 0:100 (fermented WR).

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), DPPH radical scavenging activity (DPPH RSA), and $ABTS^+$ radical scavenging activity (ABTS RSA) of *Monascus*-fermented rice cultivated on a mixture of Hom Nil broken rice and white rice (HNB:WR), and Riceberry broken rice and white rice (RBB:WR), at different ratios.

System	Ratio	TPC (mg GAE/g dw)	TFC (mg CE/g)	TAC (mg/g)	DPPH RSA (%)	ABTS RSA (%)
HNB:WR	100:0	27.62 ± 0.28^{bc}	4.05 ± 0.75^{c}	0.09 ± 0.00^{c}	78.16 ± 5.87^{ns}	99.58 ± 0.06^{ns}
	75:25	23.44 ± 3.79^{c}	4.37 ± 0.55^{c}	0.11 ± 0.01^{c}	80.10 ± 8.14	99.72 ± 0.07
	50:50	34.53 ± 0.85^{ab}	4.75 ± 0.31^{c}	0.16 ± 0.01^{c}	89.35 ± 6.41	99.54 ± 0.26
	25:75	40.14 ± 1.34^{a}	7.26 ± 0.16^{b}	1.72 ± 0.40^{b}	92.50 ± 7.26	98.05 ± 2.17
	0:100	33.66 ± 0.91^{ab}	11.54 ± 0.42^{a}	2.48 ± 0.33^{a}	90.40 ± 0.48	98.07 ± 1.35
RBB:WR	100:0	12.20 ± 0.47^{bc}	4.21 ± 0.77^{c}	0.14 ± 0.03^{c}	87.29 ± 3.66^{ns}	94.85 ± 6.89^{ns}
	75:25	10.78 ± 0.32^{c}	3.64 ± 0.38^{c}	0.14 ± 0.10^{c}	81.76 ± 1.01	99.22 ± 0.72
	50:50	12.79 ± 1.00^{bc}	4.69 ± 0.97^{c}	0.22 ± 0.10^{c}	88.67 ± 5.89	98.13 ± 1.86
	25:75	14.85 ± 1.61^{b}	9.18 ± 0.22^{b}	1.08 ± 0.07^{b}	95.88 ± 1.09	96.55 ± 3.84
	0:100	33.66 ± 0.91^{a}	11.54 ± 0.42^{a}	2.48 ± 0.33^{a}	90.40 ± 0.48	98.07 ± 1.35

Values are mean ± standard deviation. Mean within column for each system with different lowercase superscripts are significantly different ($p \leq 0.05$).

From our preliminary test, the TPC, TFC, and TAC of the unfermented HNB were 1.73 mg GAE/g, 1.13 mg CE/g, and 0.02 mg/g, respectively. Similarly, TPC, TFC, and TAC of the unfermented RBB were 1.85 mg GAE/g, 1.10 mg CE/g, and 0.03 mg/g, respectively. After 15 days of fermentation, the TPC, TFC, and TAC of the fermented HNB (HNB:WR ratio 100:0) increased approximately 16.2-, 3.7-, and 4.1-fold, respectively. Likewise, for the fermented RBB (RBB:WR ratio 100:0), these values increase about 6.8-, 3.9-, and 6.1-fold, respectively. This result demonstrated that fermentation with *Monascus* effectively improved the TPC, TFC, and TAC of agricultural by-products including HNB and RBB. Interestingly, when HNB or RBB were combined with WR during fermentation, the concentrations of these functional components were higher compared to the fermented-HNB and/or -RBB. The TPC, TFC, and TAC of the HNB:WR at 25:75 were 1.4-, 1.8-, and 19.5-fold higher, respectively, than those of the fermented HNB. Similarly, the TPC, TFC, and TAC of the RBB:WR at 25:75 were 1.2-, 2.2-, and 7.5-fold higher, respectively, than those of the fermented RBB.

Besides, it was found that changes in the profile of pigments by the fermentation process in HNB:WR or RBB:WR systems showed a similar trend with regard to TAC and TFC. This was because the pigments of *Monascus*-fermented rice contained anthocyanin substances, which are flavonoids (Peranginangin *et al*., 2018). Therefore, as pigments increased, TAC and TFC also increased.

These results can be explained that the fungus *Monascus* has the capacity to produce a number of carbohydrate-hydrolysing enzymes including amylase, cellulase, xylanase, and β-glucosidase, as

well as proteases during its growth (Chen *et al*., 2021; Yang *et al*., 2022). Throughout fermentation, these enzymes hydrolysed the components of the plant cell wall like cellulose, starch, and protein, resulting in the release of phenolic and flavonoid compounds from the plant matrix, thereby contributing to an improvement of TPC, TFC, and TAC (Razak *et al*., 2019; Zhao *et al*., 2021). Moreover, phenolic compounds such as rutin and its glycosides that can be synthesised by genus *Monascus* are intermediates produced simultaneously through polyketide pathway (Bei *et al*., 2017; Yang *et al*., 2022). A strong interaction between the microorganisms and the substrates for the synthesis and release of polyphenols, such as phenolics and flavonoids, depends on physiological mechanisms (Chen *et al*., 2021).

Variation in phenolic composition

The phenolic acids in rice including white, black, and red rice have been identified in numerous studies as gallic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, and sinapic acids (Pang *et al*., 2018; Sapna *et al*., 2019). A high amount of chlorogenic acid is also present in black rice (Pereira-Caro *et al*., 2013). To investigate the shift in phenolic acid profile in rice substrates released from the HNB:WR or RBB:WR mixtures at different ratios, HPLC was used to determine the quantities of eight phenolic acids (gallic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, and sinapic acids). The contents of phenolic acids identified are summarised in Tables 3 and 4. It was found that chlorogenic acid was the major compound in the fermented HNB (95.95 mg/kg) and RBB (82.94 mg/kg), while sinapic

Table 3. Phenolic acid contents of *Monascus*-fermented rice cultivated on a mixture of Hom Nil broken rice and white rice (HNB:WR) at different ratios.

Phenolic acid (mg/kg)	HNB:WR				
	100:0	**75:25**	**50:50**	**25:75**	**0:100**
Gallic acid	39.05 ± 0.24^{d}	79.55 ± 1.48^{b}	105.2 ± 2.24^{a}	50.36 ± 1.90^{c}	36.55 ± 2.30^{d}
Chlorogenic acid	95.95 ± 5.94^{a}	70.17 ± 2.51^{b}	61.37 ± 3.11^{b}	8.79 ± 0.40^{d}	33.81 ± 2.25^{c}
Vanillic acid	8.87 ± 0.45^{d}	54.95 ± 0.41^{a}	50.79 ± 1.05^{b}	48.26 ± 1.60^{b}	46.56 ± 0.95^{c}
Caffeic acid	0.89 ± 0.04^{d}	0.35 ± 0.01^{e}	1.93 ± 0.27^{c}	9.54 ± 0.07^{b}	10.59 ± 0.10^{a}
Syringic acid	53.41 ± 1.24^{a}	35.38 ± 1.48^{b}	34.23 ± 1.23^{b}	33.04 ± 1.33^{b}	27.95 ± 1.33^{c}
p-Coumaric acid	2.23 ± 0.45^{d}	7.27 ± 0.32^{c}	10.68 ± 0.11^{b}	7.08 ± 0.12^{c}	24.32 ± 1.04^{a}
Ferulic acid	1.91 ± 0.30^{e}	3.70 ± 0.28^{d}	5.78 ± 0.28^{c}	11.42 ± 0.28^{b}	16.38 ± 1.04^{a}
Sinapic acid	32.06 ± 2.78^{e}	63.45 ± 2.36^{d}	91.44 ± 5.51^{c}	152.71 ± 11.97^{b}	188.60 ± 7.73^{a}

Values are mean ± standard deviation. Mean within row with different lowercase superscripts are significantly different ($p \leq 0.05$).

Table 4. Phenolic acid contents of *Monascus*-fermented rice cultivated on a mixture of Riceberry broken rice and white rice (RBB:WR) at different ratios.

Phenolic acid (mg/kg)	RBB:WR				
	100:0	**75:25**	**50:50**	**25:75**	**0:100**
Gallic acid	21.57 ± 0.13^{c}	38.86 ± 0.05^{b}	36.76 ± 0.05^{b}	48.29 ± 0.51^{a}	36.55 ± 2.30^{b}
Chlorogenic acid	82.94 ± 2.68^{a}	86.14 ± 2.49^{a}	74.82 ± 2.51^{b}	49.64 ± 1.38^{c}	33.81 ± 2.25^{d}
Vanillic acid	9.69 ± 0.70^{c}	10.26 ± 1.23^{c}	19.21 ± 1.21^{b}	10.61 ± 0.20^{c}	46.56 ± 0.95^{a}
Caffeic acid	0.77 ± 0.03^{c}	0.62 ± 0.01^{d}	1.01 ± 0.03^{b}	0.76 ± 0.00^{c}	10.59 ± 0.10^{a}
Syringic acid	8.94 ± 0.08^{c}	41.49 ± 3.49^{a}	49.64 ± 1.01^{a}	6.60 ± 0.47^{c}	27.95 ± 1.33^{b}
p-Coumaric acid	5.87 ± 0.17^{c}	3.35 ± 0.93^{d}	10.12 ± 0.16^{b}	3.51 ± 0.16^{d}	24.32 ± 1.04^{a}
Ferulic acid	2.41 ± 0.07^{bc}	1.79 ± 0.28^{c}	1.66 ± 0.03^{c}	3.73 ± 0.22^{b}	16.38 ± 1.04^{a}
Sinapic acid	33.49 ± 1.73^{d}	48.51 ± 2.51^{c}	37.18 ± 1.53^{d}	62.07 ± 1.49^{b}	188.60 ± 7.73^{a}

Values are mean ± standard deviation. Mean within row with different lowercase superscripts are significantly different ($p \leq 0.05$).

acid was the main compound in the fermented WR (188.60 mg/kg). Obviously, as the HNB:WR or RBB:WR ratios varied from 100:0 to 0:100, chlorogenic acid decreased significantly ($p \leq 0.05$), while ferulic and sinapic acids increased significantly ($p \leq 0.05$). Total phenolic acids (eight compounds) in HNB:WR and RBB:WR systems varied within the range of 234.38 - 384.75 and 165.67 - 384.75 mg/kg, respectively. As expected, the fermented WR gave the highest total phenolic acids (384.75 mg/kg). Nevertheless, the results confirmed increases in phenolic acids when HNB and WR, as well as RBB and WR, were incorporated and used as a substrate for fermentation compared to using HNB or RBB alone (HNB:WR or RBB:WR ratio of 100:0). This suggested that the changes in the phenolic profile resulted from the rice substrates and the metabolic activities of *Monascus*. Chen *et al.* (2020) demonstrated that the phenolic compounds in various forms could be transformed in SSF. This was due to the metabolic activities of *Monascus*, which contributed to the enzymatic degradation of the cell wall components and other macromolecules in the substrates, as well as the synthesis of micromolecular phenolic compounds during fermentation.

Antioxidant activity

Considering the antioxidant activity of the HNB:WR system in various ratios ranging from 100:0 to 0:100, strong DPPH and $ABTS^+$ radical scavenging activities were observed in *Monascus*-fermented rice samples. These activities ranged from 78.16 to 92.50% ($p > 0.05$) for DPPH, and from 98.05 to 99.72% ($p > 0.05$) for $ABTS^+$ (Table 2). In addition, all RBB:WR samples, ranging from ratios of 100:0 to 0:100, exhibited notable DPPH and $ABTS^+$ radical scavenging activities, with percentages ranging from 81.76 to 95.88% ($p > 0.05$) and 94.85 to 99.22% ($p > 0.05$), respectively (Table 2). Our preliminary test showed that the DPPH radical scavenging activities of the unfermented-HNB, -RBB, and -WR were 84.84, 86.77, and 3.35%, respectively. Meanwhile, the $ABTS^+$ radical scavenging activities of the unfermented -HNB, -RBB, and -WR were 47.82, 53.03, and 9.57%, respectively. Overall, these results reflected the potential of all *Monascus*-fermented rice samples to have radical scavenging activity, and clearly confirmed that SSF of *Monascus* significantly enhanced the antioxidant activities of rice substrates.

The results showed an obvious difference in substrates between two groups: the fermented-HNB and -RBB (HNB:WR or RBB:WR at 100:0) corresponded to low yields of pigments, monacolin K, TPC, TAC, and TFC, while the fermented WR (HNB:WR or RBB:WR at 0:100) corresponded to high yields of pigments, monacolin K, TPC, TAC, and TFC. However, excellent antioxidant activities were observed in all samples. This can be explained by the antioxidant activity in *Monascus*-fermented rice resulting from (1) antioxidants produced by metabolic activities of *Monascus* such as dihydromonacolins, dimerumic acid, phytosterol, and pigments, particularly rubropunctamine and monapilol B (Mohankumari *et al.*, 2021); and (2) antioxidants present in HNB and RBB substrates. In the case of WR, the significant improvement in antioxidant activity could have been due to the increases in pigments, phenolics, and flavonoids which originate from the metabolic activities of *Monascus* during fermentation. Meanwhile, the high antioxidant activity observed in fermented-HNB and -RBB might have been attributed to the presence of natural antioxidant compounds readily available in the substrates. This was supported by Pereira-Caro *et al*. (2013), who demonstrated that black rice contained high quantities of anthocyanins, flavone and flavonols, carotenoids, and gamma-oryzanols, which act as antioxidant compounds. Besides, Riceberry broken rice is a rich source of antioxidants like β-carotene, phenolic acids, flavones, anthocyanins, and gamma-oryzanols, and it also exhibits strong antioxidant activity (Luang-In *et al*., 2018; Eze *et al*., 2022).

These results agreed with Razak *et al.* (2019), in which the positive influence of fermentation on TPC, TFC, and antioxidant activities of broken rice was observed by *Aspergillus niger* and *Rhizopus oligosporus.* According to Chen *et al.* (2021), the increases in TPC and antioxidant activities of corn kernels after fermentation by *Monascus anka* could be attributed to α-amylase and cellulase. These enzymes destroy the cellular structure, and regulate the release of phenolics during fermentation.

Pearson correlation coefficient analysis was used to evaluate the relationship between bioactive compounds and antioxidant activity, and helped identify the major factors influencing the antioxidant activity of *Monascus*-fermented rice. Some specific compounds can be regarded as bioactive markers in

SSF (Chen *et al.*, 2021; Yang *et al.*, 2022). Correlation analysis is displayed in Table 5. With the exception of citrinin, the TPC, TFC, TAC, and pigments; yellow, orange, and red, were positively correlated with the DPPH radical scavenging activity of *Monascus*-fermented rice based on HNB:WR (r = 0.570 - 0.912) and RBB:WR (r = 0.238 - 0.710) systems. Only TPC was significantly correlated with the DPPH radical scavenging activity of *Monascus*-fermented rice in the HNB:WR system (r = 0.912, $p \leq 0.05$). In the RBB:WR system, a strong correlation coefficient between TFC and DPPH antioxidant activity was obtained, as shown by the high value of r (r = 0.710) (Schober *et al*., 2018). The results also revealed that *Monascus* pigments, particularly yellow pigment, were responsible for DPPH antioxidant activity, indicating a moderate to strong correlation (r = 0.495 - 0.722) due to the fact that *Monascus* pigments act as antioxidants (Mohankumari *et al.*, 2021). On the other hand, TPC, TFC, TAC, and pigments; yellow, orange, and red, were negatively or poorly correlated with the $ABTS^+$ radical scavenging activities of *Monascus*-fermented rice in both HNB:WR (r = -0.966 - -0.753) and RBB:WR (r = 0.018 - 0.211) systems. It may be that the different antioxidants can act through different mechanisms against oxidation reactions, thereby exhibiting different antioxidant activities (Bei *et al*., 2017). The antioxidant activities of phenolic compounds depend on their structure and the number of hydroxyl groups (Chen *et al.*, 2021). Similar to our results, TPC and TFC were found to have a strong correlation with DPPH antioxidant activity in fermented broken rice by *A. niger* and *R. oligosporus* (Razak *et al.*, 2019). As earlier mentioned, this result clearly confirmed that the presence of high levels of phenolic and flavonoid compounds was related to the high DPPH radical scavenging activity of *Monascus*-fermented rice based on HNB:WR and RBB:WR systems, respectively.

Based on the overall results, apart from fermented WR, the combination of pigmented broken rice (HNB or RBB) with WR, particularly HNB:WR and RBB:WR at a ratio of 25:75, showed potential for utilisation as functional ingredients. These products presented health benefits to humans due to their enrichment in pigments, monacolin K, TPC, TFC, TAC, and their strong antioxidant activity together with the low levels of toxin citrinin.

Table 5. Correlation coefficients between DPPH radical scavenging activity (DPPH RSA) and total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), yellow pigment, orange pigment, red pigment, monacolin K, and citrinin in *Monascus*-fermented rice.

	DPPH RSA of HNB:WR system	**DPPH RSA of RBB:WR system**
TPC	0.912*	0.324
TFC	0.649	0.710
TAC	0.715	0.499
Yellow pigment	0.722	0.559
Orange pigment	0.570	0.495
Red pigment	0.616	0.530
Monacolin K	0.581	0.238
Citrinin	-0.847	-0.560

HNB:WR: mixture of Hom Nil broken and white rice; and RBB:WR: mixture of Riceberry broken and white rice. *$p \leq 0.05$.

Conclusion

Solid state fermentation with *M. purpureus* was a useful method for acquiring functional ingredients from the by-products (HNB and RBB) of the rice milling industry. The amounts of bioactive compounds including pigments, monacolin K, TPC, TFC, and TAC, as well as the antioxidant activity of *Monascus*-fermented rice on HNB and/or RBB were enhanced by combining them with WR. However, the fermented WR generated large amounts of bioactive compounds without producing the toxin citrinin. Although each sample yielded different amounts of bioactive compounds, high antioxidant activities were observed in all *Monascus*-fermented rice samples. Therefore, *Monascus*-fermented rice on

HNB or RBB combined with WR might be considered as the choice for the production of functional ingredients to enhance the functional properties of nutraceuticals and functional foods.

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