Functional Golden Brown Rice Fermentation by *Monascus* sp. Yellow, White Parental Strains and Their Protoplast Mutants and Fusants

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ABSTRACT

This report points out the possibility of producing value-added fermented products from broken-milled rice using yellow pigment-producing molds: *Monascus kaoliang* TISTR 3642 and its developed strains, 2 protoplast mutants (U2.3 and U2.5) and 3 fusants (F7, F10 and F43). All strains could produce yellow pigments with a single absorption peak at 370 nm, and the amount of pigment produced in depended on the pH and medium formula. Various factors affecting the growth rate, glucoamylase and yellow pigment activities of *Monascus* in golden-brown rice fermentation were investigated and compared among the parental strains and the five developed strains. Conditions of 38% weight per weight initial moisture content with an initial pH 5 of rice substrate, and room temperature (28–32 °C) incubation were found optimal for golden brown rice fermentation of all strains. Furthermore, their noteworthy functional properties of antioxidant activities, total phenolic compounds as well as antichoresterol agents (monacolin K) were observed and discussed.

Keywords: *Monascus* mutant, fusant, antioxidant, functional golden brown rice, monacolin K

INTRODUCTION

Monascus fermented red rice has been generally used as a traditional alternative medicine, food colorant and fermentation starter for more than 1,000 years, especially in China, Japan, and Southeast Asian countries (Li *et al*., 2010; Shi and Pan, 2011).

Rice is also one of Thailand's major agricultural exports of economic importance (Titapiwatanakun, 2012). After milling, the rice is polished, resulting in a seed with a bright, white, shiny appearance, while the seeds that are broken during the drying and milling process are obtained as a byproduct. Broken-milled rice could serve as a sustainable raw material for value-added secondary products through fermentation with *Monascus* molds (Tiyasiwaporn, 2008; Chayawat *et al*., 2009). These molds can produce glucoamylase enzyme which hydrolyzes starch to fermentable carbohydrate substrate for producing pigment and other metabolites (Iizuka and Mineki, 1977; Iizuka and Lin, 1981; Yongsmith *et al*., 1990, 2000).

Monascus red rice, also known as red yeast rice or in Thai as *angkak* (*ang* = red, *kak* = seed), is a fermented rice product whose pigments are characterized by multiabsorption peaks at λ

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420 nm and λ 500 nm of red, orange and yellow. The minor yellow pigments can only be obtained through a multistep chemical extraction of *angkak* (Sweeny *et al*., 1981). A secondary mutant of *Monascus* sp. TISTR 3642 (previously known as KB20M10.2) which is capable of producing yellow pigments at a single peak (λmax 370 nm) was first reported by Yongsmith *et al*. (1990). This strain could be used for the production of innovative golden brown rice products containing exclusive yellow pigments (Yongsmith *et al*., 2013). This slow-growing and low-glucoamylase-producing strain has now been developed through protoplast mutation and protoplast fusion techniques (Klinsupa *et al*., 2013) to be more efficient. The present study has continued to determine the optimal conditions for growth, glucoamylase and yellow pigment production of *Monascus* sp. TISTR 3642 and its selected protoplast mutants and fusants on rice solid culture. In addition, their potent functional metabolites were also measured, compared and discussed.

MATERIALS AND METHODS

Microorganisms and cultivation

A white-producing mutant strain, TISTR 3644, selected through ultraviolet (UV) primary mutation and a yellow producing mutant strain, TISTR 3642, selected through UV secondary mutation (both generated from *Monascus* sp. TISTR 3179) were compared with their subsequently developed strains. The yellow-producing protoplast mutants, U2.3 and U2.5, were earlier selected through protoplast mutation of the strain TISTR 3642 by UV mutagenesis (Klinsupa *et al*., 2013). Yellow-producing fusants, F7, F10 and F43, were selected through protoplast fusion between strains TISTR 3642 and TISTR 3644. All seven *Monascus* strains were used throughout the experiment. The stock cultures were maintained on malt-yeast agar and C-medium (Hiroi *et al*., 1979) for spore preparation.

Solid-state fermentation of *Monascus* sp.

A local source of broken-milled rice (Hom Mali cultivar) was used throughout the experiments. Broken-milled rice was soaked in tap water for 3 hr with the initial pH adjusted to 3, 5, 7, 9 and 11 by the addition of 10% hydrochloric acid or 10% sodium hydroxide. After the water had been removed, the soaked rice was drained for 5–10 min and then a 500 mL flask containing 100 g rice was autoclaved at 121 °C for 15 min and cooled to room temperature. Amounts of sterile distilled water (0, 5, 10 and 15 mL) were added into the flasks to adjust the initial rice moisture content to 32, 35, 38 and 41%, respectively. Each flask was inoculated with 2 mL of 1×10^6 spores.mL^{-1} of *Monascus* and incubated at various temperatures (20, 25, 28–32, 37, 40 and 45 °C) for 20 d. Fermented rice (1 g) was extracted with 9 mL of sterile water at room temperature for 2 hr and then the pH of each fermented sample was measured using a pH meter (e30; Hach; Loveland, CO, USA).

Preparation of fermented rice powder

Fermented-rice powder was prepared by autoclaving the fermented rice at 100 °C for 20 min and drying at 60 °C overnight, then crushing to a coarse powder using a household grinder. Fermented-rice powder was kept in the dark at room temperature for further study.

Glucosamine content

The glucosamine content was evaluated using the Morgan-Elson method (Van de Loo, 1976). Fermented rice (0.25 g) was extracted with 5 mL of HCl at room temperature for 20 hr. The mixture was centrifuged at 10,000 revolutions per minute (rpm) for10 min. Two milliliters of supernatant was mixed with 1 mL of distilled water. The mixture was boiled at 100 °C for 2 hr and neutralized to pH 7 with 30% NaOH. The mixture was filled to 50 mL volume with distilled water and filtered through a No.4 Whatman filter. The supernatant was assayed for glucosamine.

One milliliter of supernatant was mixed with 1 mL of acetyl acetone and held at 100 °C for 20 min. Next, 10 mL of 95% ethanol and 1 mL of Ehrlich's reagent were added. The mixture was mixed well under static conditions for 30 min. The glucosamine content was determined using a UV-1700 spectrophotometer (Shimadzu; Kyoto, Japan) at 530 nm absorbance. The glucosamine concentration was estimated from a standard curve with 10–100 μg.mL^{-1} glucosamine HCl.

Glucoamylase activity

Fermented rice (1 g) was extracted with 9 mL of acetate buffer at pH 5.5 using a laboratory blender (stomacher) and kept at 4 °C for 20 hr. The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was further analyzed for its glucoamylase content. The glucoamylase activity was measured as follows. A reaction mixture containing 0.5 mL of 1% soluble starch in 0.1 M acetate buffer at pH 5.5 and 0.5 mL of suitably diluted enzyme solution were incubated in a water bath at 55 °C for 20 min. The amount of reducing sugar produced was determined by the modified method of Nelson-Somogyi (Nelson, 1944). One unit of glucoamylase activity was defined as the amount of enzyme which liberates reducing sugar equivalent to 1 μg of glucose per 1 min under the above conditions.

Measurement of pigment

The pigment concentration was measured using a spectrophotometer (UV-240; Shimadzu; Kyoto, Japan) at 370 nm for yellow pigment. Pigment in fermented rice samples corresponding to 1 g of initial rice substrate was extracted with 39 mL of 50% ethanol for 3 hr on a rotary shaker (300 rpm). The extract was then centrifuged at 7,000×g for 15 min and the supernatant was analyzed using the spectrophotometer against a 50% ethanol blank. The moisture content of the rice samples was determined by heating the fermented rice in a hot air oven overnight at 105 °C and the weight loss was measured. The extracted, yellow pigment yield was expressed as its λ_{max} unit per gram dry weight of fermented matter (A_{370}U.gdw^{-1}).

Determination of antioxidant activity

Monascus strain*s* were cultured on steamed rice at 30 °C for 14 d. The crushed and dried rice with the mold was used for the experiments. One gram of fermented rice powder was mixed with 4 mL of 95% (volume per volume) ethanol and kept at 37 °C for 60 min and then centrifuged at 3000 rpm for 15 min. The supernatant was stored at -20 °C and used as the mold extract. The antioxidative activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH, a stable free radical with a violet color) scavenging action. The reaction mixture consisted of 1 mL of 0.1 mM DPPH in ethanol and 1 mL of mold extract or 1 mL of 95% ethanol (the control). The mixture was shaken vigorously and left to stand for 30 min in the dark at room temperature. The absorbance was then measured at 517 nm against a blank. The absorbance of the mold extract alone was subtracted as the blank from that of the reaction mixture. If free radicals have been scavenged, DPPH will change in color from purple to yellow (Piao *et al*., 2004). The DPPH radical scavenging activity of the extract is expressed as the EC50 value (the effective concentration at which DPPH radicals were scavenged by 50%) and the EC50 of fermented rice product from each *Monascus* strain was obtained by interpolation from linear regression analysis (Lee *et al*., 2007).

Determination of total phenolic compound

The total phenolic content was determined using the method of Singleton and Rossi (1965) with slight modifications. Approximately 0.25 mL of Folin–Ciocalteau reagent was added into 0.05 mL of sample extract and the mixture was allowed to stand at room temperature for 5 min. Later, 0.25 mL of 20% sodium carbonate solution was added into the mixture and incubated at room temperature for 30 min. The absorbance was measured at 725 nm using the spectrophotometer and the results

were expressed as gallic acid equivalents (GAE) in milligrams per 100 g wet weight.

Anti-cholesterol agent or monacolin K

The fermented rice powder in portions of 0.5 g was extracted with 25 mL 70% ethanol. Extraction was performed at 50 °C for 2 hr. Samples were then filtered through a 0.22 μm filter membrane and measured. The high performance liquid chromatography instrument consisted of an LC-10AT VP liquid chromatograph (Shimadzu; Kyoto, Japan), FCV-10AL VP pump, LDC analytical spectro monitor 3100 detector set at 238 nm and an LDC analytical CI-4100 integrator. The chromatography columns consisted of Persuit C18, 5μm, 250×4.6 mm connected to a guard column (Meta Guard Pursuit 4.6 mm 5μC18) and a 20 μL loop injector. An isocratic mobile phase of acetonitrile:water at the ratio of 65:35 (by volume) was used and the flow rate was 1.0 mL.min^{-1}; T= 28 °C (Ganrong *et al*., 2003). Monacolin K (Sigma; Singapore) dissolved in 70% ethanol to prepare the standard solutions for the calibration curve in a range of 0, 1, 2, 3 and 4 μg.mL^{-1} and a 20 μL aliquot were injected. The amount of monacolin K was thus calculated using the standard curve of monacolin K.

RESULTS

Solid state fermentation time course

Analysis was done of the 20 d-course of the growth, glucoamylase activity, yellow pigment, moisture content and pH of broken-milled rice solid cultures fermented with all seven test *Monascus* strains starting with 32% (weight per weight; w/w) initial moisture content and incubation at room temperature (28–32 °C) in order to compare the properties of the different strains. Figures 1a and 1b show that both *Monascus* yellow (TISTR 3642) and white (TISTR 3644) parental strains began to grow at 3 d of fermentation and grew quickly up to the eighth day of cultivation and slowed down after that. Brown pigmentation

was not seen during the first 2 d of cultivation, but appeared later after 3 d of cultivation and it increased rapidly after 5 d of cultivation along with increased humidity. Protoplast mutants and fusants showed similar patterns of growth, glucoamylase activity, pigment production, pH and moisture content to those of the yellow parental strain (Figures 1c, 1d, 1e, 1f, 1g). All protoplast mutants and fusants showed higher glucosamine content and glucoamylase activity than the yellow parental strain, but such amounts were lower than the white parental strain. Protoplast mutant isolates U2.3 and U2.5, and protoplast fusant isolates F7 and F10 produced more pigment than their yellow parental strain did at 19.88, 29.11, 23.97 and 31.46%, respectively. However, only one protoplast fusant isolate (F43) could produce the highest glucoamylase but demonstrated the lowest yellow pigment production amongst the selected developed strains.

Factors affecting growth, glucoamylase and yellow pigment production on rice solid culture

Key factors that affected growth, glucoamylase and yellow pigment production by the strains TISTR 3642, TISTR 3644, U2.3, U2.5, F7, F10 and F43 in rice solid culture were studied. First, the initial moisture content of broken-milled rice samples of 32, 35, 38 and 41% (w/w) gave remarkably different results from individual strains (Figure 2). Thirty-eight percent initial moisture content was found to be suitable for growth and the production of metabolites while a lower or higher moisture content resulted in lower growth, glucoamylase and pigmentation. Second, the effects of the incubation temperature ranging from 25 to 35 °C were determined and showed that an incubation temperature of 35 °C gave the best production while the lowest yields were obtained at a temperature of 25 °C. A yellowish-brown rice tone was produced with a single peak at 370 nm (Figure 3) of yellow pigmentation at 30 °C and 35 °C incubation temperatures from
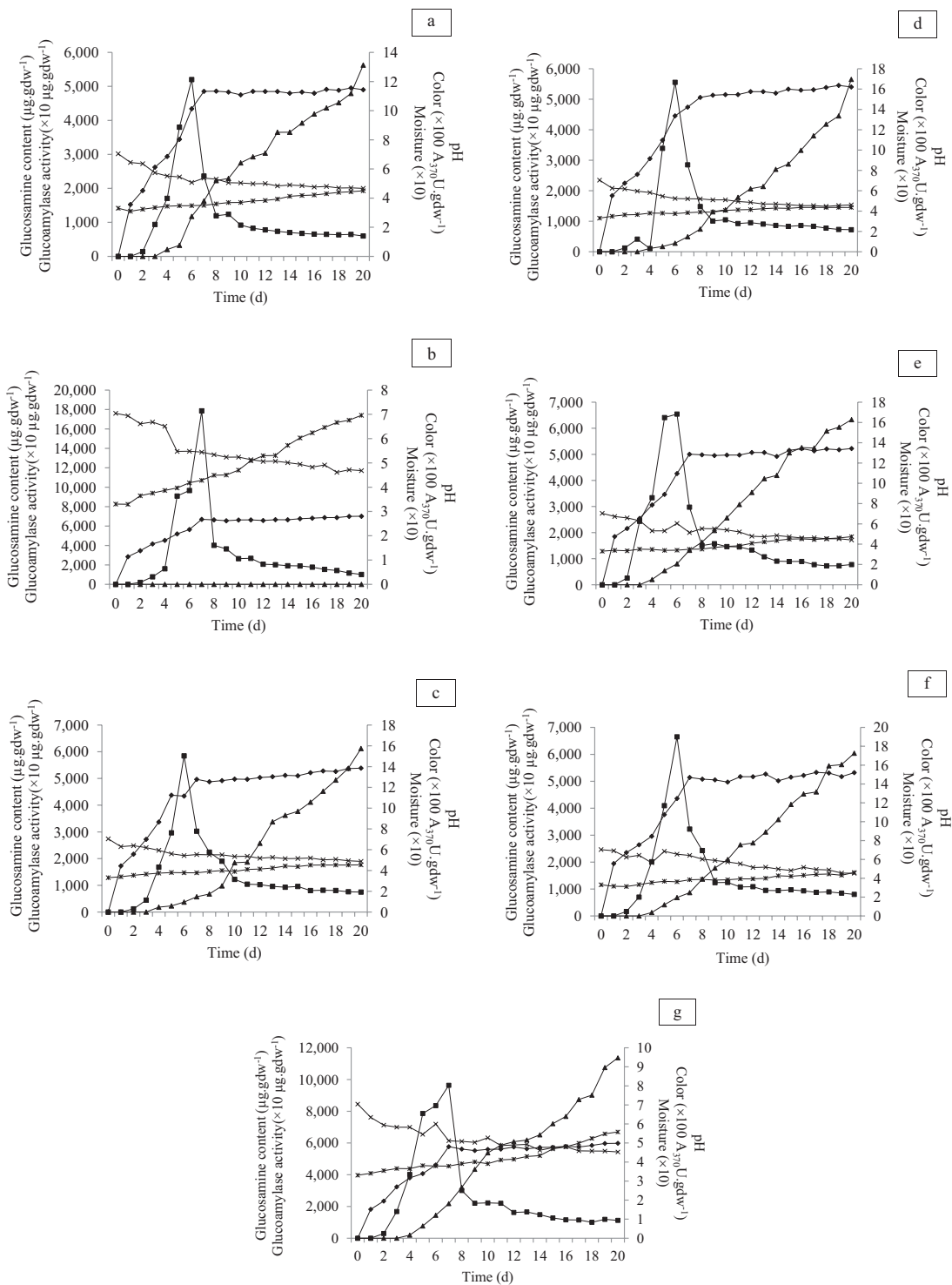
Figure 1 Broken-milled rice solid fermentation time course of glucosamine content, glucoamylase activity, color, pH and moisture content in *Monascus*-fermented rice at room temperature (28–32 ℃) for 20 d: (a) TISTR 3642; (b) TISTR 3644; (c) U2.3; (d) U2.5; (e) F7; (f) F10; (g) F43. Glucosamine content (♦); Glucoamylase activity (■); Color (▲); pH (✖); Moisture content (✱).

all strains tested, whereas, a reddish-brown rice tone was obtained at 25°C incubation temperature. Following this finding, incubation at a lower temperature of 23–26 °C, produced a reddish-brown pigment with multiple peaks at 320, 370 and 500 nm, and this phenomenon could be observed throughout the 20 d incubation period (Figure 4). All developed strains could produce more pigment than their parent (TISTR 3642) at 30 °C and 35 °C incubation temperatures. Third, when the initial pH ranged from 3 to 11, the results showed that an initial pH of 5 promoted the greatest growth, glucoamylase and pigment production. A brown rice tone appeared after 3 d of incubation at room temperature at various initial pH values (pH 5–9). The production of yellow pigment concentrations in TISTR 3642, U2.3, U2.5, F7, F10 and F43 were maximized at pH 5 at 1,552.33, 1,978.95, 1,737.65, 2,099.19, 2,194.36 and 967.14 A_{370}U.gdw^{-1} (Figure 5).
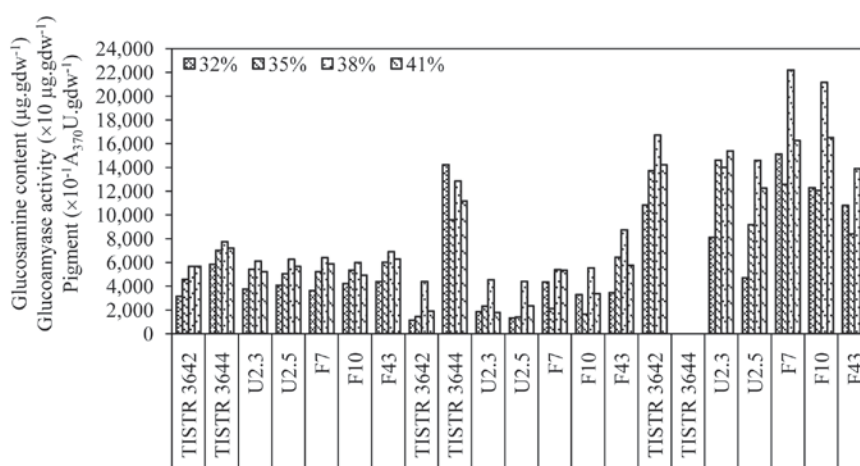

Figure 2 Effect of initial moisture on glucosamine content, glucoamylase activity and pigment production in rice solid cultures by *Monascus* strains at room temperature (28–32 ℃) for 20 d.
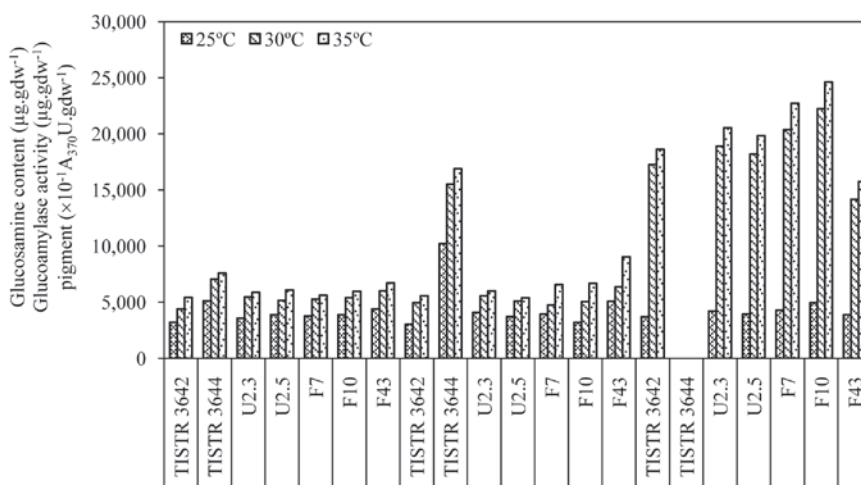

Figure 3 Effect of incubation temperature on glucosamine content, glucoamylase activity and pigment production in rice solid cultures by *Monascus* strains for 20 d.

Determination of some functional metabolites from selected *Monascus* strains

Determination of monacolin K, the antioxidant activity and total phenolic compounds were subsequently carried out. The results showed that for all strains, the parental yellow and white mutants, the two protoplast mutants and the three fusants produced metabolites from 4 d fermentation onward and steadily increased production until the end of fermentation. The protoplast mutants U2.3 and U2.5 could produce monacolin K at a rate higher than the yellow parental strain with 2,266 and 2,390 mg.kg^{-1} dry weight, respectively (Figure 6). It is noteworthy that the white mutant which

produce neither color nor monacolin K, whereas its protoplast fusants (F7, F10 and F43) could produce both functional products.

The antioxidant activity and total phenolic compounds were also examined. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable free radical and is often used to evaluate the antioxidant activity of several natural compounds. The antioxidant activity is indicated by the EC50 value (the effective concentration at which DPPH radicals were scavenged by 50% of the initial DPPH concentration). The ethanol extract of F10 cultured rice showed the highest scavenging ability on DPPH radicals compared to those of U2.3, F7,
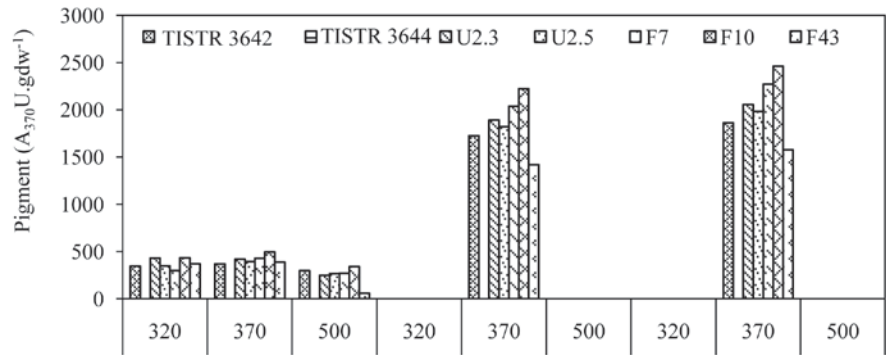

Figure 4 Effect of incubation temperature on pigment production in rice solid cultures by *Monascus* strains for 20 d.
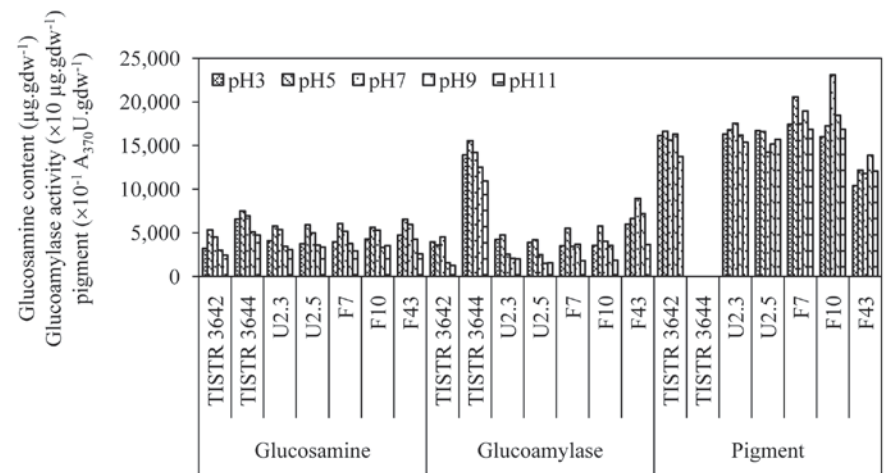

Figure 5 Effect of initial pH on glucosamine content, glucoamylase activity and pigment production in rice solid cultures by *Monascus* strains at room temperature (28–32 ºC) for 20 d.

U2.5, the yellow parental strain, F43 and the white parental strain at 0.55, 0.576, 0.743, 0.836, 0.931, 1.43 and 2.163 mg.mL^{-1}, respectively, while their total phenolic compounds were 17.6, 18.7, 14.9, 15.0, 12.3, 8.3 and 6.8 mg.gdw^{-1}, respectively (Figure 6).

DISCUSSION

Monascus molds have been commercially employed for the production of major red pigments and orange pigments or both (Hesseltine1965; Sweeny *et al*., 1981). Their chemical structure has been elucidated in many reports. *Monascus* can produce six pigment compounds (Lin and Iizuka, 1982; Jůzlová *et al*., 1996) consisting of two yellow—monascin (Salomon and Karrer 1932; Chen *et al*., 1969) and ankaflavin (Manchand and Whalley, 1973); two orange—rubropunctatin (Chen *et al*., 1969) and monascorubrin (Manchand and Whalley, 1973) and two red—rubropunctamine and monascorubramine (Kumasaki *et al*., 1962; Sweeny *et al*., 1981). Only orange pigments are produced biosynthetically while the other pigments (yellow and red) are formed from these by chemical transformations. The red pigments are produced by N-substitution. The reduction of the orange pigment gives rise to the yellow pigment ankaflavin from monascorubrin, or monascin from rubropunctatin (Hajjaj *et al*., 2000). In order to obtain the yellow fraction (which is the minor component of the pigment mixture) multiple steps of solvent extraction and chemical modification are needed (Sweeny *et al*., 1981). In contrast, the current studies have led to the production of exclusive yellow pigments by a specific strain of *Monascus* sp. (TISTR 3642) using either submerged fermentation (Yongsmith *et al*., 1994) or rice solid culture (Yongsmith *et al*., 2013). This strain, which is a second-generation mutant derived from a red wild type strain (KB9), produced yellow pigment with the predominating single absorption maximum at 370 nm independent of the medium formula and pH. Its crude CH_2Cl_2 extract from rice solid culture, sequentially subjected to Sephadex LH-20 and silica gel chromatography, yielded two innovative yellows—monacusone A and B—together with two known yellow compounds—monascin and FK17-P2b2 (Jongrungruangchok *et al*., 2004). Subsequently, improvement of *Monascus* sp. TISTR 3642 by protoplast UV mutagenesis and protoplast fusion was completed, fusing a slow-growing, low-glucoamylase-producing, but yellow-pigment-forming yellow mutant (TISTR 3642) with a fast-growing, high-glucoamylase-
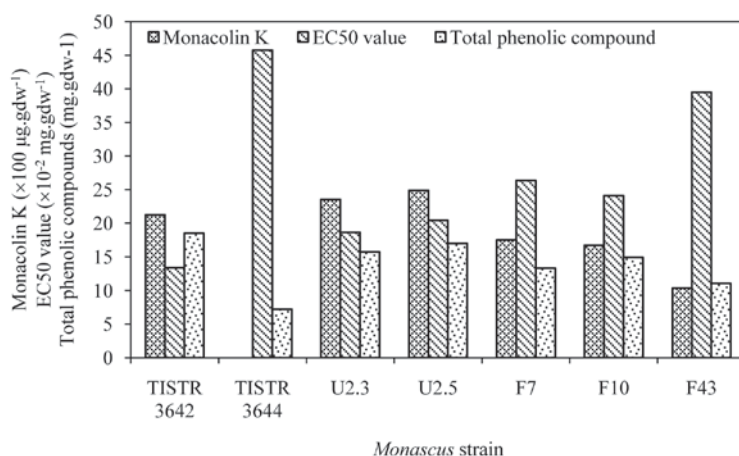

Figure 6 Monacolin K, antioxidant activity and total phenolic compounds of fermented-rice by *Monascus* strains at room temperature (28–32 ºC) for 20 d.

producing, white mutant (TISTR 3644) for improved yellow pigment and glucoamylase production (Klinsupa *et al.*, 2013). Selected potent isolates of protoplast mutation (U2.3 and U2.5) and protoplast fusion (F7, F10 and F43) showed a similar fermentation time-course to their parental strains. Growth (glucosamine content) as well as glucoamylase activity reached their maxima at 7 d cultivation while yellow pigments appeared after 3 d cultivation and continuously increased until 20 d cultivation. The current research showed that the optimal conditions for yellow pigment fermentation of all the improved strains are the same: 38% initial moisture content, initial pH 5.0 and 35 °C incubation temperature. Under optimized conditions, all improved isolates except the F43 isolate could produce increased yellow pigmentation compared to the parental strains. It is interesting that the lower yellow-pigment-producing fusant F43 conversely exhibited the highest growth and glucoamylase activity among the improved isolates.

Furthermore, *Monascus* pigments possess a range of biological activities, such as anti-mutagenic and anticancer properties (Izawa *et al.*, 1997; Akihisa *et al.*, 2005; Su *et al.*, 2005), antimicrobial activities (Wong and Bau, 1977; Wong and Koehler, 1981; Lee *et al.*, 1995; Martínková *et al.*, 1995, 1999; Kim *et al.*, 2006), hypolipidemic properties (Chun *et al.*, 2010; Lee *et al.*, 2010), anti-obesity properties (Kim *et al.*, 2007; Lee *et al.*, 2013) and antidiabetic and antioxidative stress properties (Shi *et al.*, 2012). Beside biopigments, the *Monascus* molds can produce other secondary metabolites including dimerumic acid, tannin and phenol, which provide anti-inflammatory and antioxidative stress responses (Aniya *et al.*, 1998, 1999, 2000; Taira *et al.*, 2002; Zöchling *et al.*, 2002; Lee *et al.*, 2006; Kuo *et al.*, 2009). *Monascus* molds have been identified as a source of the cholesterol-lowering agent known as monacolin K (Endo, 1979, 1985; Endo *et al.*, 1985, 1986; Negishi *et al.*, 1986; Komagata *et al.*, 1989; Schneweis *et al.*, 2001) as

well as containing γ-aminobutyric acid (GABA) with neurotransmitting, hypotensive and diuretic effects (Keisuke *et al.*, 1992; Ueno *et al.*, 1997).

The present study also found that some functional activities and antioxidant and phenolic compounds could be detected in all the tested strains, while monacolin K could be detected in most strains except the white parental strain (TISTR 3644). However, the amounts of functional metabolites produced by the developed strains were not remarkably high, being only in between the values produced by their parental strains. In addition, previous studies found that *Monascus* brown rice could act as a strong antimutagenic agent, based on results using the prokaryote test (Kruawan *et al.*, 2005) or eukaryote test (Siriwan, 2005). *Monascus* white rice could produce significant amounts of GABA, which is beneficial for health in the reduction of hypertension and stress (Tiyasiwaporn, 2008). Thus, it is believed that these additional functional metabolites (antimutagenic substances as well as GABA) could be contained in the *Monascus* golden brown rice product fermented with the improved strains developed through protoplast mutation and protoplast fusion.

CONCLUSION

Overall, the findings have demonstrated that among all the *Monascus* strains studied, the developed strains, protoplast mutants (U2.3, U2.5) as well as protoplast fusants (F7, F10) could produce higher amounts of yellow pigments than their yellow-producing parents under optimized conditions. Even though the white parent could not produce monacolins (an anticholesterol product), its protoplast fusants could produce monacolins to some extent. Both the developed strains, protoplast mutants as well as protoplast fusants could thus be used to produce an innovative functional fermented golden brown rice product containing at least monacolins, antioxidant and phenolic compounds as an alternative to the well-known

red *Monascus* rice. However, the possible presence of more diversified functional metabolites of these developed strains remains to be determined in further investigations.

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