Note

Composition and Antioxidant Activity of Rice Fermented with Saccharifying Organisms from Asian Countries

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In recent years, more effective use of rice has become important because of an annual increase in surplus rice. We fermented rice in pure cultures of eight organisms (*Aspergillus oryzae*, *Monascus pilosus*, *Absidia corymbifera*, *Mucor circinelloides*, *Mucor racemosus*, *Rhizopus oligosporus*, *Rhizopus oryzae*, and *Saccharomycopsis fibuligera*), which were isolated from molded rice and soybean products in Asian countries, and evaluated the composition and antioxidant activity of the products. Rice fermented with the two *Rhizopus* species had a high methanol extract yield, implying good fermentation properties. High saccharification and increased levels of total amino acids and total polyphenols were also found in *Rhizopus*-fermented rice samples. Ethyl acetate extracts of rice fermented with *Ab. corymbifera* and *Mu. circinelloides* had enhanced antioxidant activity compared to unfermented rice, and some fractions obtained from the extracts by high performance liquid chromatography exhibited high antioxidant activity. Based on these results, *Ab. corymbifera*, *Mu. circinelloides*, *R. oligosporus*, and *R. oryzae* are promising starter organisms for the development of new fermented rice products.

Keywords: surplus rice, fermented rice products, saccharifying organisms, composition, antioxidant activity

Introduction

Rice is a staple food in Japan and has been cultivated throughout the country for centuries. In recent years, however, approximately 200,000 tons of rice grain per year is surplus. Thus, increasing the consumption of rice is an important subject.

In addition to directly consumed steamed rice, numerous traditional Japanese foods, such as miso, soy sauce, and alcohol, are prepared using molded rice (*koji*), which is cultured with a mold known as *koji-kin*. While a granule type of *koji* called *bara-koji*, which is produced by a pure culture of an *Aspergillus* species, is commonly used in Japan, a cake type of *koji* called *mochi-koji*, which is produced by culturing with multiple organisms, including fungi, yeasts, and bacteria, is typically used in the broader Asian region. There are many types of *mochi-koji*, such as Korean *nuruk*, Bhutanese *chang poo*, and Vietnam *banh men*, and various saccharifying organisms, including fungi such as *Absidia*, *Mucor*, and *Rhizopus* species and yeasts such as *Saccharomycopsis* species, have reportedly been isolated from these cultures (Hesseltine *et al.*, 1988; Uchimura *et al.*, 1990a, 1990b; Lee *et al.*, 1999).

In Asia, several fermented soybean products are also consumed. One such product is Indonesian *tempeh*, prepared using the fungus *Rhizopus oligosporus*. Many reports focus on the health benefits of these foods; *tempeh* reportedly has physiological effects such as antioxidant and apoptosis-inducing properties (Matsuo *et al.*, 1997). Studies on molded rice produced using *Aspergillus oryzae* and *Monascus pilosus*, which are types of *bara-koji*, have also been actively conducted (Kim *et al.*, 2010; Cheng *et al.*, 2011). However, no reports have addressed the effects on human health of rice fermented in pure culture with organisms contained in various *mochi-koji*.

The purpose of this study was to promote the development of new fermented rice products by investigating the fermentability of rice by test organisms and assessing the

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nutritional properties and physiological effects of experimentally fermented rice. In this study, we prepared rice samples fermented in pure culture with *As. oryzae*, *Mo. pilosus* (these two organisms were used as references), *Absidia corymbifera*, *Mucor circinelloides*, *Mucor racemosus*, *R. oligosporus*, *Rhizopus oryzae*, and *Saccharomycopsis fibuligera* strains isolated from molded rice and soybean products in Asian countries, and evaluated their composition and antioxidant activity.

Materials and Methods

Materials and chemicals   Unpolished rice (*Oryza sativa*) was purchased from Maisen (Fukui, Japan). The microorganisms used were obtained from the National Institute of Technology and Evaluation Biological Resource Center (NBRC; Chiba, Japan) and the Japan Collection of Microorganisms, RIKEN Bioresource Center (JCM; Ibaraki, Japan). These included *As. oryzae* NBRC 4134, *Mo. pilosus* NBRC 4520, *Ab. corymbifera* JCM 5618, *Mu. circinelloides* NBRC 4554, *Mu. racemosus* NBRC 4581, *R. oryzae* NBRC 4706, and *S. fibuligera* NBRC 1665, which were derived from fermented rice resources and *R. oligosporus* NBRC 8631, which was derived from the fermented soybean product *tempeh*. Potato dextrose agar medium used for culturing fungal starters was from Kanto Kagaku (Tokyo, Japan), while Luria-Bertani medium used for culturing the yeast starter was from Nacalai Tesque (Kyoto, Japan). Ninhydrin, Somogyi and Nelson reagent, Folin-Ciocalteu phenol reagent, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were from Wako Chemical (Osaka, Japan). 2-Morpholinoethanesulfonic acid (MES) was from Dojindo (Kumamoto, Japan), and linoleic acid was from Nacalai Tesque. β-Carotene was from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade and were obtained from either Wako Chemical or Nacalai Tesque.

Cultivation of rice   To evaluate the fermentability of rice by each organism, we prepared a solid rice-based medium. First, 20 g of pulverized unpolished rice and 100 mL distilled water were added to an Erlenmeyer flask, which was then heated in an autoclave at 121°C for 40 min to steam-cook the rice. The cooled rice medium was evenly inoculated with a 1.0 mL spore suspension (10^6 spores/mL) of each test organism and then placed in an incubator at the optimal temperature for each organism for 1 week, with agitation once a day. The test organisms able to liquefy the solid rice medium were considered suitable starters for rice fermentation.

Preparation of extracts with solvents   Samples (with mycelium removed) were freeze-dried and ground into powder using a mill. Of this powder, 5 g was soaked in 200 mL methanol with shaking at ambient temperature for 24 h.

Extracted solid matter was removed by filtration, and a fresh 200 mL of methanol was added to the solid matter, followed by shaking at ambient temperature for 24 h; these procedures were repeated two more times. The methanol extract was concentrated by evaporation of the combined solvent. Methanol extract yield (MEY) was calculated using the following equation:

$$MEY\ (\%) = [\text{content of methanol extract (g)} / \text{content of freeze-dried sample (g)}] \times 100$$

Methanol extracts were used for measurements of reducing sugar, total amino acid, and total polyphenol content, because solutions of freeze-dried rice samples in water were quite viscous due to the presence of rice starch; the content of each component in dried rice samples was calculated on the basis of the content in methanol extracts and the MEY value. After removal of a portion of the methanol extract, distilled water was added to the remaining methanol extract in a separating funnel, and an equal volume of ethyl acetate was added to the funnel. After the resulting mixture was shaken using a funnel shaker (Yamato Scientific, Tokyo, Japan) for 10 min at room temperature, the ethyl acetate layer was transferred to an Erlenmeyer flask and fresh ethyl acetate was added to the funnel; these procedures were repeated two more times. A final ethyl acetate extract was obtained by evaporation of the combined solvent. For preparation of test samples, each extract was dissolved in dimethyl sulfoxide (DMSO).

Reducing sugar content   The reducing sugar content of the rice samples was determined by the method of Somogyi and Nelson (Nelson, 1944). The methanol extract dissolved in DMSO (5 μL), which was prepared at an appropriate concentration, distilled water (245 μL), and Somogyi reagent (250 μL) were added to a glass test tube. The test tube was boiled in water for 20 min and then cooled. Nelson reagent (250 μL) was added to the test tube, which was incubated for 15 min at ambient temperature. Absorbance of the reaction mixture at 540 nm was measured using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The measurements were compared to a standard curve generated using glucose solutions of known concentration, and the reducing sugar content was expressed as milligrams of glucose equivalents per gram of dried rice sample.

Total amino acid content   The total amino acid content of the rice samples was determined by the method of Moore and Stein (1954). The methanol extract dissolved in DMSO (50 μL), distilled water (450 μL), and ninhydrin reagent (500 μL) were added to a glass test tube. The test tube was boiled in water for 15 min and then cooled. Absorbance of the reaction mixture at 570 nm was measured using a spectrophotometer. The measurements were compared to a standard

curve generated using L-alanine solutions of known concentration, and the total amino acid content was expressed as milligrams of L-alanine equivalents per gram of dried rice sample.

Total polyphenol content   The total polyphenol content of the rice samples was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965), with modifications for 96-well microplates. The methanol extract dissolved in DMSO (5 μL), distilled water (45 μL), and Folin-Ciocalteu phenol reagent (10 μL) were pipetted into wells of a 96-well microplate. After 3 min of incubation, 10% Na_2CO_3 (25 μL) solution was added to each well, and the solution was further incubated for 30 min at ambient temperature. Absorbance at 650 nm was measured using an iMark plate reader (Bio-Rad Laboratories). The measurements were compared to a standard curve generated using caffeic acid solutions of known concentration, and the total polyphenol content was expressed as milligrams of caffeic acid equivalents per gram of dried rice sample.

DPPH radical-scavenging activity   DPPH radical-scavenging activity was determined by the method described by Yoshida *et al.* (2010). The ethyl acetate extract dissolved in DMSO (50 μL), ethanol (50 μL), and MES buffer (pH 6.0, 50 μL) were pipetted into wells of a 96-well microplate. As a control, DMSO (50 μL) was added to one well instead of the ethyl acetate extract. The assay reaction was initiated by addition of 0.5 mg/ml DPPH solution (50 μL) in ethanol. After 20 min of incubation at ambient temperature, absorbance was measured at 520 nm using the iMark plate reader. The scavenging rate (S) of the DPPH radical was calculated using the following equation:

$$S\ (\%) = [(A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$$

where A_{sample} represents absorbance using the ethyl acetate extract, $A_{control}$ represents absorbance using DMSO, and A_{blank} represents absorbance without the addition of 0.5 mg/mL DPPH solution. The sample concentration representing a 50% scavenging rate was expressed as EC_{50}.

Measurement of antioxidant activity by the β-carotene bleaching method   Prevention of autooxidation of linoleic acid was determined by a β-carotene bleaching method (Uchida *et al.*, 2001). Briefly, 1 mg/mL β-carotene (0.25 mL), 0.1 g/mL linoleic acid (0.1 mL), and 1 g/mL Tween 40 (0.5 mL), all dissolved in chloroform, were mixed in an eggplant flask, and the chloroform was evaporated. The residual was dissolved in a solution of distilled water (45 mL) and 0.2 M phosphate buffer (pH 6.8, 5 mL), and the resulting solution was ultrasonicated to prepare an emulsion of linoleic acid and β-carotene. The ethyl acetate extract, dissolved to 10 mg/mL in DMSO (0.1 mL), and the linoleic acid-β-

carotene emulsion (4.9 mL) were added to a glass test tube. As a control, DMSO (0.1 mL) was added to the tube instead of the ethyl acetate extract. The test tubes were incubated at 50°C in a water bath for 120 min, and absorbance of the reaction mixture was measured at 470 nm using a spectrophotometer. The inhibitory ratio (I) was calculated using the following equation:

$$I\ (\%) = [(E_{120\ min} - C_{120\ min}) / (C_{0\ min} - C_{120\ min})] \times 100$$

where $E_{120\ min}$ represents absorbance at 120 min with the ethyl acetate extract, $C_{120\ min}$ represents absorbance at 120 min with DMSO, and $C_{0\ min}$ represents absorbance at 0 min with DMSO.

High performance liquid chromatography (HPLC) analysis and preparation   Ethyl acetate extracts of fermented and unfermented rice samples dissolved in DMSO were filtered through a 0.45 μm DISMIC-25C cellulose acetate membrane (Advantec, Tokyo, Japan) and separated by an ACQUITY UPLC system (Waters, Milford, MA, USA) using a COSMO-SIL $5C_{18}$-MS-II column (4.6 × 250 mm, 5 μm particle size; Nacalai Tesque). The analysis for phenolic compounds with HPLC was carried out according to the method described by Tian *et al.* (2004). The solutions used for the mobile phase had the following composition: (A) H_2O (0.1% TFA); (B) CH_3CN (0.1% TFA). Elution was performed under the following conditions: 0 − 5 min, isocratic elution with 100% A; 5 − 10 min, a linear gradient of 0 to 9% B; 10 − 20 min, isocratic elution with 9% B; 20 − 27 min, a linear gradient of 9 to 11% B; 27 − 40 min, a linear gradient of 11 to 18% B; 40 − 50 min, isocratic elution with 18% B; 50 − 60 min, a linear gradient of 18 to 100% B; 60 − 65 min, a linear gradient of 100 to 0% B. The flow rate was 0.8 mL/min. Absorbance at 270 nm was monitored using a photodiode array detector, and the column temperature was maintained at 40°C. Phenolic compounds in the samples were identified by comparing their relative retention times and UV spectra with authentic compounds. Fractions collected by preparative HPLC were lyophilized with an evaporator and a freeze-dryer, and finally dissolved in DMSO. The antioxidant activity of each fraction was measured in the same manner as described above.

Statistical analysis   Values are shown as means ± standard error (SEM) (except for Table 1). The difference between groups was evaluated by a two-sided Student's *t* test. Differences were considered significant at $p < 0.05$.

Results and Discussion

Extract yield   Table 1 shows the MEY from the fermented and unfermented rice samples. Unfermented rice showed the lowest MEY, with a value of 3.5%. In contrast, rice fermented with the four fungal starters exhibited a high MEY

Table 1. MEY of fermented and unfermented rice samples.

Organism	MEY (%)
Unfermented	3.5
As. oryzae	62.4
Mo. pilosus	55.1
Ab. corymbifera	17.1
Mu. circinelloides	21.6
Mu. racemosus	7.6
R. oligosporus	62.6
R. oryzae	86.9
S. fibuligera	17.5

value, ranging from 55.1% for *Mo. pilosus* to 86.9% for *R. oryzae*. At the end of the 1-week fermentation period, the fungi cultured on solid rice medium had liquefied it. Given that liquefaction of rice media (i.e. suggesting degradation of rice starch) is an index of fermentation progress, these organisms appear to be suitable starters for rice fermentation. It was reported that the growth rate of *Rhizopus* species on steamed polished rice grain was low, and was caused by the lack of nitrogen compounds in the rice medium (Tanaka *et al.*, 1982). The good fermentation property of *Rhizopus* species in our research may be due to the use of unpolished rice containing rice protein (i.e. nitrogen compounds). The MEY of rice fermented with *Mu. racemosus* was low and the medium was not liquefied. Moreover, propagating mycelia were observed, indicating that fermentation did not progress.

Although *Mu. racemosus* was reportedly isolated from *murcha*, a common type of molded rice in Nepal (Nikkuni *et al.*, 1996), this fungus might not have participated in the fermentation process. The MEY of rice fermented with two other fungi, *Ab. corymbifera* and *Mu. circinelloides*, and the yeast *S. fibuligera* showed intermediate values, with the rice medium showing a paste-like texture after fermentation. Because the MEY appears to reflect the extent of rice fermentation, we concluded that the two *Rhizopus* species exhibiting a high MEY value may be superior starter organisms suitable for the production of molded rice, similar to *As. oryzae*.

Saccharification of rice by fermentation   The reducing sugar content of the rice samples was measured as an index of the extent of saccharification (Fig. 1). Higher reducing sugar content was observed in rice fermented with *As. oryzae*, *Mo. pilosus*, *Ab. corymbifera*, *R. oligosporus*, and *R. oryzae* than in unfermented rice. In particular, the reducing sugar content of rice fermented with either of the *Rhizopus* species was approximately two-fold higher than that of rice fermented with *As. oryzae*, indicating that these two *Rhizopus* species have an extremely high ability to produce saccharification enzymes such as glucoamylase. In contrast, the reducing sugar content of rice fermented with *Mu. circinelloides*, *Mu. racemosus*, and *S. fibuligera* was almost the same as that of unfermented rice. Based on this result, it would be difficult to use any of these three organisms in pure culture for producing molded rice, as a high glucose content
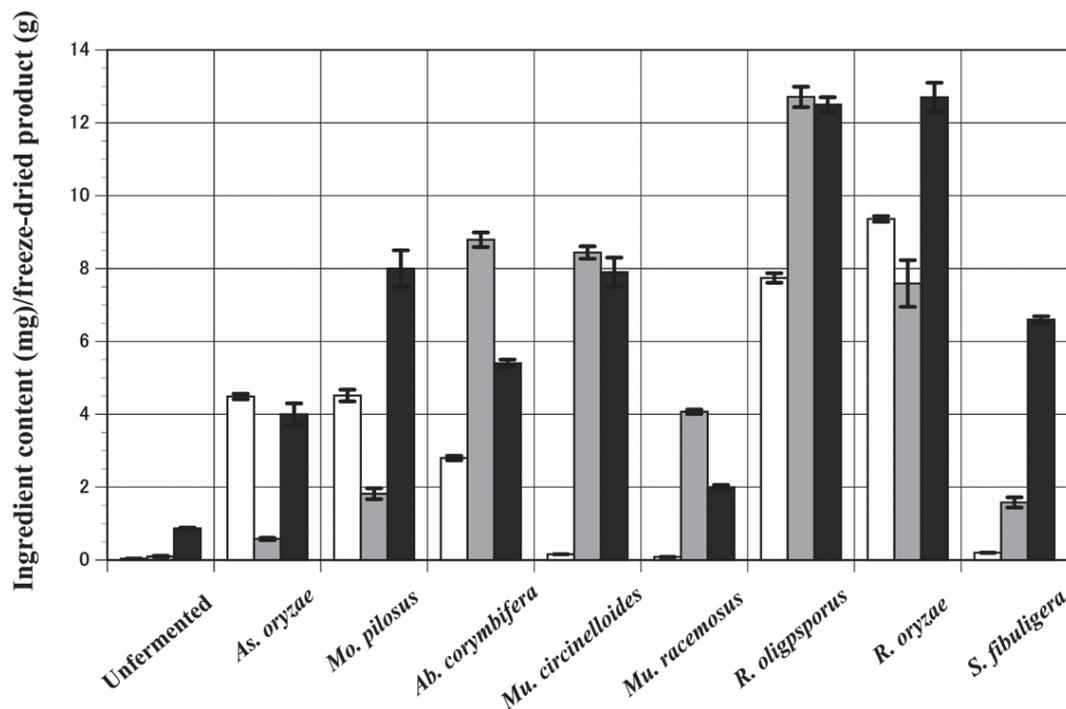

Fig. 1. Reducing sugar, total amino acid, and total polyphenol contents of fermented and unfermented rice samples. Values are means (± SEM) of triplicate experiments. White bars, reducing sugar content; gray bars, total amino acid content; black bars, total polyphenol content.

Table 2. Antioxidant activity of ethyl acetate extracts from fermented and unfermented rice samples.

Organism	EC_{50} of ethyl acetate extract (mg/mL)	Inhibition ratio (%)
Unfermented	17.4 ± 1.1	27.1 ± 1.7
As. oryzae	17.8 ± 0.92	16.3 ± 1.4
Mo. pilosus	7.0 ± 0.55**	47.8 ± 1.1**
Ab. corymbifera	9.7 ± 0.69**	52.0 ± 2.9**
Mu. circinelloides	9.2 ± 0.71**	59.4 ± 2.2**
Mu. racemosus	18.8 ± 1.1	27.1 ± 1.0
R. oligosporus	13.3 ± 0.58*	21.7 ± 1.9
R. oryzae	15.5 ± 0.38*	30.9 ± 2.0
S. fibuligera	20.5 ± 1.3	18.5 ± 1.4

Values are means (± SEM) of triplicate experiments. *$p < 0.05$, **$p < 0.01$ (unfermented versus fermented rice samples).

is needed for subsequent steps, such as ethanol fermentation for alcoholic beverage production. However, utilizing these organisms in blended culture with other saccharifying organisms may be useful for adding their characteristic properties.

Total amino acid and polyphenol contents As shown in Fig. 1, the total amino acid content of rice fermented with *Ab. corymbifera*, *Mu. circinelloides*, *R. oligosporus*, and *R. oryzae* was significantly higher than the amino acid content obtained with *As. oryzae* and *Mo. pilosus*. The fermentation of rice by *koji* mold increases the flavor called *umami* or *teimi* due to the amino acids resulting from degradation of rice proteins (Ito *et al.*, 2009). Thus, the use of these four organisms for the production of molded rice may contribute to good flavor. The total polyphenol content of all fermented rice samples was higher than that of unfermented rice (Fig. 1). In particular, rice fermented with the two *Rhizopus* species had extremely high polyphenol content. Chemical compounds such as sesaminol glucosides, which are potent antioxidants, are extracted from sesame at higher levels in organic solvents following fermentation, a phenomenon that may be attributable to the increased fragility of the cellular tissue (Ohtsuki *et al.*, 2003). We suggest that the increase in the total polyphenol content following rice fermentation is attributable to the same property. Because the increases in total amino acid and total polyphenol content appear to depend on the progress of fermentation, based on the results of Fig. 1 and the MEY values in Table 1, we concluded that fermentation can contribute to increasing the nutritional value of rice.

Antioxidant activity of ethyl acetate extracts of fermented and unfermented rice samples DPPH radical-scavenging activity and inhibition of lipid autooxidation were measured to evaluate the antioxidant activity of ethyl acetate extracts from rice samples (Table 2). Both activities were significantly higher in rice fermented with *Mo. pilosus*, *Ab. corymbifera*, and *Mu. circinelloides* than in unfermented rice. The high activity with *Mo. pilosus* may reflect the previous finding

that *Monascus*-fermented red yeast rice attenuates oxidative stress (Cheng *et al.*, 2011). HPLC analysis showed that the composition of ethyl acetate extracts from rice fermented in pure cultures of *Mo. pilosus*, *Ab. corymbifera*, and *Mu. circinelloides* was very different from that of unfermented rice (Fig. 2). Phenolic compounds, which are contained in the rice grain, particularly its bran layer, are potent antioxidants (Shashidi *et al.*, 1992). We assumed that the increased antioxidant activity following fermentation with these three organisms is partially attributable to phenolic compounds; however, although the amounts of hydroxybenzoic acid and vanillic acid were slightly increased, no significant increase in the amount of phenolic compounds was observed. On the other hand, several peaks, with retention times ranging from 10 min to 20 min, were observed in the fermented samples, but not in unfermented samples. In all three fractions, higher antioxidant activities were observed than in the ethyl acetate extracts from which they are derived. (Tables 2 and 3). These results suggest that the substances in these fractions are mainly responsible for the increased antioxidant activity of these samples. Kojic acid and Monacolin K were reportedly isolated from Japanese rice *koji* and Chinese *beni-koji* as second metabolites of *As. oryzae* and *Mo. pilosus*, respectively (Friedemann, 1934; Endo, 1979). Antioxidant compounds contained in these fractions may be also secondary metabolites produced by the starter organisms. We are currently attempting to identify the antioxidants in these fractions.

Table 3. Antioxidant activity of fractions collected by preparative HPLC.

	EC_{50}	Inhibition ratio (%)
a	5.3 ± 0.45	62.6 ± 4.7
b	4.2 ± 0.72	72.2 ± 2.8
c	3.7 ± 0.61	74.8 ± 3.2

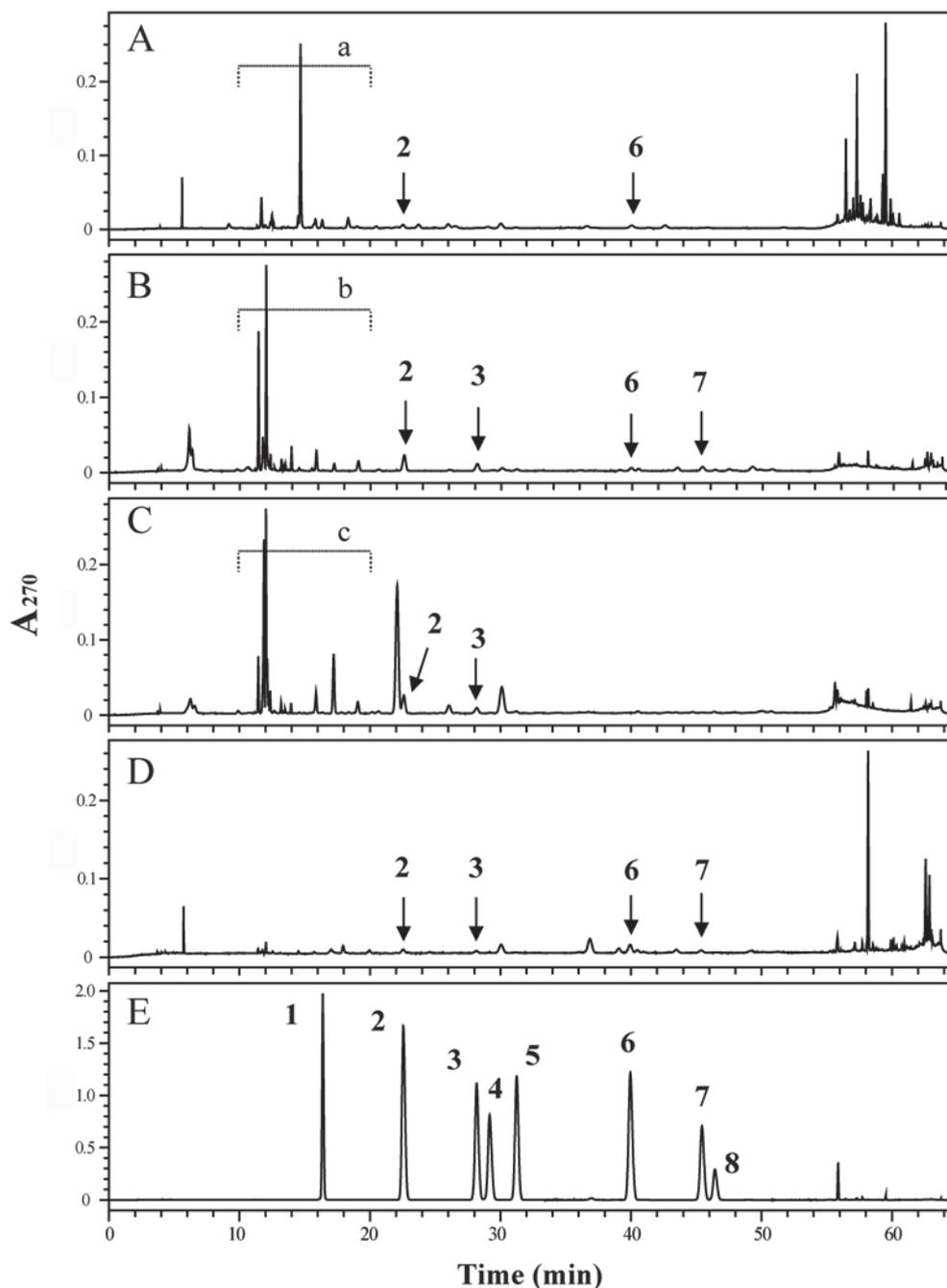Values are means (± SEM) of triplicate experiments.

Fig. 2. High performance liquid chromatograms of ethyl acetate extracts of rice fermented with *Mo. pilosus* (A), *Ab. corymbifera* (B), and *Mu. circinelloides* (C), of unfermented rice (D), and of a mixture of eight phenolic compounds at a concentration of 1 mg/mL each (E): 1, protocatechuic acid; 2, hydroxybenzoic acid; 3, vanillic acid; 4, caffeic acid; 5, syringic acid; 6, *p*-coumaric acid; 7, ferulic acid; 8, sinapinic acid. Detection was at 270 nm. Arrows indicate peaks that correspond to each phenolic compound. The bracketed lines (a, b, and c) represent the boundaries of the fractions collected by preparative HPLC with retention times ranging from 10 to 20 min.

Conclusion

We fermented rice in pure cultures with various organisms found in Asian countries, and revealed that rice fermented with two *Rhizopus* species had a remarkably high MEY value and increased reducing sugar, total amino acid, and total polyphenol contents, while ethyl acetate extracts of rice fermented with *Mo. pilosus*, *Ab. corymbifera* and *Mu.*

circinelloides exerted enhanced antioxidant activity. In the aforementioned five organisms, *Mo. pilosus* is well known as a starter for red yeast rice, and the other four organisms may be candidates for starter organisms in pure or blended cultures for the development of fermented rice products with good fermentation properties and antioxidant activity. To our knowledge, this is the first report comparing the fermentabil-

ity of rice by saccharifying organisms isolated from various molded rice and fermented soybean products in Asian countries, and examining the composition and antioxidant activity of the fermented products. Even though further research to determine the optimal processing conditions is needed for practical use, our findings should lead to the development of new fermented rice products.

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