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Screening of Functional *Rhizopus stolonifer* for Alcohol Fermentation and Production of High Quality Korean Traditional Rice Wine

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Different strains of mold were screened for the production of high quality Korean traditional rice wine with anti-hypertension and good acceptability. We isolated 867 *nuruk* mold strains and selected 24 for further study based on measurement of amylase activity. Among them, mold No. 17 showed high ethanol production upon fermentation with *Saccharomyces cerevisiae* as well as anti-hypertensive properties. The No. 17 strain was therefore selected as the functional mold and later identified as *Rhizopus stolonifer* based on molecular biological characteristics. Optimal fermentation conditions for the brewing of anti-hypertensive traditional rice wine comprised the addition of *R. stolonifer* No. 17 koji at a concentration of 35 sp/g and a fermentation period of 10 days at 25°C using *S. cerevisiae*.

KEYWORDS : Anti-hypertensive traditional rice wine, Functional *Rhizopus stolonifer*

Recent demand for Makgeorly (Tagju), a type of Korean traditional rice wine, has significantly increased due to its taste and health-promoting effects [1]. In fact, its export market size has reached over 4.4 million US dollars as of 2008. Korean traditional rice wines and liquors have long been brewed using *nuruk* or koji, cooked rice, flour, yeast or medicinal plants or herbs. Additionally, there has been much research into quality improvement, for example, changes in microbe and enzyme activity during fermentation, nutrient content and acceptability, utilization of raw materials, standardization of manufacturing, improvement of storage, and so on [2-7]. Ever since the functionality of mold, such as chitooligosaccharide from koji, was confirmed, many functional traditional rice wines have been developed based on various medicinal plants [7]. However, problems that persist include lack of unique characteristics as well as inferior acceptability and functionality. Therefore, new Korean traditional rice wines with high acceptability and functionality must be developed. We previously reported the characterization and physiological functionalities of Korean traditional rice wines developed from dandelion, chamomile, acasia and *Paecilomyces japonica* [7-11]. The results of these studies suggest that medicinal plants or mushrooms are very useful for increasing the physiological functionality of Korean traditional rice wine. However, except for Indian millet koji [12] and barley koji [13], there is little to no information on how functional molds affect alcohol fermentation.

In this paper, we describe the screening of functional *Rhizopus stolonifer* and its use in the production of high quality as well as new Korean traditional rice wines.

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Materials and Methods

Materials and chemicals. Non-glutinous rice along with other cereals were purchased from a commercial market, cultivated at 2008. Angiotensin I-converting enzyme (ACE) (dipeptidyl carboxypeptidase I; EC 3.4.15.1) was extracted from rabbit lung acetone powder purchased from Sigma-Aldrich (St. Louis, MO, USA). ACE activity was determined using hippuric-histidine-leucine (Hip-His-Leu) (Sigma) as a substrate. *Saccharomyces cerevisiae* K-7 for the preparation of mash was obtained from the Laboratory of Biotechnology at Paichai University. Fibrin, pyrogallol and 1-diphenyl-2-picrylhydrazyl (DPPH) were also purchased from Sigma-Aldrich. Unless otherwise specified, all chemicals were of analytical grade. Molds isolated from various *nuruk* species were cultured in cereal at 25°C for 3 days, followed by the determination of amylase and protease activities. Finally, 25 types of mold were selected and used in the preparation of koji.

Identification of the selected mold. Identification of the selected molds was performed using internal transcribed spacer (ITS) 1 (CTTGGTCATTTAGAGGAGTAA) and ITS 4 (TCCTCCGCTTATTGATATGC) primers. Base sequences were analyzed by Macrogen Co. (Seoul, Korea).

Preparation of mash and fermentation. Preparation and fermentation of mash were performed according to previous methods [14]. Non-glutinous rice (600 g) was added into 1,500 mL of boiling water and then heated for 10

min. After cooling, koji (30 g) and *S. cerevisiae* (30 mL) cultured in YEPD medium at 30°C for 24 hr, were added to the mixture, followed by fermentation at 25°C for 7 days.

General analysis and sensory evaluation. The pH values were measured using a pH meter (Fisher Scientific Inc., Rockford, IL, USA), and the titratable acidity of the solution was estimated after titration with 0.1 N NaOH to pH 7.0. The succinic acid (%) was then calculated based on the titratable acidity. Ethanol was determined using an alcohol meter (Ceti Optical Instruments, Antwerp, Belgium) after water distillation [14].

Sensory evaluation of traditional rice wines were estimated by 50 trained sensory panels according to the method of quantitative descriptive analysis [14]. Subjects evaluated taste and odor of traditional rice wines on a scale of 1 to 5, with 5 being the highest score. The means were obtained and plotted as a polygonal graph. Overall acceptability according to taste and odor was evaluated using the mean value of a hedonic scale scored from 1 (dislike extremely) to 5 (like extremely).

Assay of enzyme activity and functionality. Dried koji powder (4 g) was added into 10 mL of distilled water, followed by shaking for 24 hr and filtration. Protease and amylase activities of water extracts were determined as follows [15]. The activities of acidic, neutral and alkaline proteases were determined by spectrophotometry using different buffer solutions (pH 3.0, 7.0, 10.0) containing 0.6% skim milk and folin reagents. α-Amylase activity was determined using 1% soluble starch.

Fifty microliter of traditional rice wine was concentrated to 5 mL, and its functionality was determined as follows [7]. ACE inhibition was assayed following a modified method of Cushman and Cheung [16]. A mixture containing 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 3 units of ACE and an appropriate amount of traditional rice wine was preincubated for 10 min at 37°C. The reaction was initiated by the addition of 50 μL of 5 mM Hip-His-Leu and was terminated after 30 min of incubation by the addition of 250 μL of 1 N HCl. Liberated hippuric acid was extracted with 1 mL of ethyl acetate, after which 0.8 mL of the extract was evaporated to dryness by a Speed Vac Concentrator (Eyela, Tokyo, Japan). The residue was then dissolved in 1 mL of sodium borate buffer. The absorbance at 228 nm was measured to provide an estimate of ACE inhibitory activity.

Fibrinolytic activity was assayed by the method of Fayek and El-Sayed [17]. Traditional rice wine (5 mL) was added to 3 mL of substrate solution (0.6% fibrin in 0.1 M McIlvaine buffer, pH 7.0) and incubated at 40°C for 10 min. The reaction was stopped by the addition of 3 mL of 0.4 M trichloroacetic acid for 30 min, followed by filtration with Whatman filter paper No 2. A reaction mixture comprising 1 mL of filtrate, 5 mL of 0.4 M Na_2CO_3 and 1 mL of 1 N Folin reagent was incubated at room temperature for 30 min. The amount of tyrosine released from fibrin was determined based on a tyrosine standard curve by measuring the absorbance at 660 nm. One unit of activity was defined as the production of 1 μg of tyrosine per minute by 1 mL of crude enzyme.

Superoxide dismutase (SOD)-like activity was assayed by the method of Marklund and Marklund [18]. A 20 mL sample was added to 20 mL of 55 mM tris-cacodylic acid butter (TCB, pH 8.2), after which the mixture was homogenized for 2 min and centrifuged at 4°C for 30 min at 12,000 rpm. The supernatant was adjusted to pH 8.2 and then increased in volume up to 50 mL (sample extracts). Five microliter of 24 mM pyrogallol containing 10 mM HCl (substrate) was added to 0.95 mL of the sample extracts, after which absorbance at 420 nm was measured for the first 2 min. SOD-like activity was calculated by the following equation:

$$\text{SOD-like activity (\%)} = [(A - B)/A] \times 100$$

where A is the increase in absorbance of TCB (blank) and B the increase in absorbance of sample.

Antioxidant activity was assayed by the method of Blois [19] using 1,1-diphenyl-2picrylhydrazyl (DPPH). A 0.8 mL aliquot of DPPH solution (12.5 mg of DPPH dissolved in 100 mL of ethanol) was added to 0.2 mL of sample, followed by shaking for 10 sec and incubation for 10 min, after which the absorbance was measured at 525 nm. Antioxidant activity was calculated as

$$[(\text{absorbance of reaction mixture} - \text{absorbance of sample alone})/\text{absorbance of blank}] \times 100$$

Acetylcholinesterase (AChE) inhibitory activity was measured using a spectrophotometer by the method of Ellman *et al*. [20]. A mixture comprising 110 μL of assay buffer (0.1 M sodium phosphate, pH 7.3), 30 μL of AChE (0.8 U/mL), 30 μL of substrate (acetylthiocholine chloride), 20 μL of DTNB and 10 μL of sample dissolved in assay buffer was incubated for 60 min at 37°C. The enzymatically produced reaction product, 5-thio-2-nitrobenzoate, was measured at 415 nm. The inhibition (%) was calculated using the equation:

$$\text{Inhibition (\%)} = [1 - \{(S - S_0)/(C - C_0)\}] \times 100$$

where C is the absorbance of control (enzyme, assay buffer, DTNB and substrate) after 60 min of incubation, C_0 is the absorbance of control at time zero, S is the absorbance of the tested samples (enzyme, sample solution, DTNB and substrate) after 60 min of incubation, and S_0 is the absorbance of the tested samples at time zero. To investigate the quenching effect of the sample, we added the sample solution to reaction mixture C and investi-

gated any reduction in absorbance. All data represent the mean of duplicate experiments.

Results and Discussion

Screening of functional *nuruk* molds. We collected several *nuruk* molds from provinces in Korea and isolated 867 strains on potato-dextrose agar media. Among them, 24 types of koji molds showing high amylase activity were initially selected. To select the most suitable koji mold for brewing, various rice wines were prepared using these molds along with cooked rice and *S. cerevisiae*, followed by measurement of ethanol production (Table 1). The koji molds produced 8.8% to 12.4% ethanol, but fermented broth containing No. 17 koji mold produced the largest percentage of ethanol content (12.4%). We selected 8 strains of koji showing high ethanol productivity.

Physiological functionalities were determined for rice wines made using 8 types of the 2nd level-screened koji molds (Table 2). All rice wines showed ACE inhibitory activity of over 70%. Specifically, rice wine No. 17 made using koji mold No. 17 showed the highest ACE inhibitory activity (83.8%). The antioxidant activity of rice wine No. 17 was 48.6%, whereas the SOD-like activities of rice wine No. 13 and 16 were about 63%. However, anti-dementia AChE inhibitory activity and fibrinolytic activity were not detected or were very weak.

Meanwhile, wheat bran cultures of 2nd level-selected koji molds were extracted with phosphate buffer (pH 6.5) for 12 hr at 10°C, followed by measurement of amylase and protease activities. As shown in Table 3, a high level of α-amylase activity (400 units/g koji) was observed for the extracts of No. 9, No. 13 and No. 2, and No. 8 koji showed 311 units/g koji. The other koji extracts showed α-amylase activities below 100 units/g koji except for extract No. 17 (135 units/g koji). The protease activities of the extracts under neutral and alkaline pH were not detected or were very weak. However, the acidic protease activity of No. 17 koji extract was high (203 units/g koji), and extracts No. 13 and No. 8 koji showed 98.6 units/g koji and 97.2 units/g koji, respectively. The high ACE inhibitory activity of No. 17 fermented broth (Table 2) was probably caused by the high protease activity of No. 17 koji mold since most ACE inhibitors are peptide compounds [12].

Physiological functionalities of the extracts of 2nd level-selected koji molds were determined (Table 4). Antioxidant activity was the highest for No. 11 koji extract (67.7%), and SOD-like activities were below 30% for all extracts. Anti-hypertensive ACE inhibitory activity was very high for the No. 16 (80.5%) and No. 17 (76.7%) koji extracts but was much lower for the No. 8 extract (53.6%). Fibrinolytic activity and anti-dementia AChE inhibitory were not detected or were below 1%.

From the analysis of ethanol production, enzyme activity and physiological functionality of koji molds, rice wine made from No. 17 koji mold had the highest ethanol content (12.4%) and anti-hypertensive ACE inhibitory activity (76.7%). It further showed high amylase and

Table 1. Ethanol production of twenty-four kinds of koji by first level-screened koji molds

Rice wines	Ethanol content (%)	Rice wines	Ethanol content (%)
No. 1 Rw	10.0	No. 14 Rw	10.8
No. 2 Rw	11.2	No. 15 Rw	12.0
No. 3 Rw	10.8	No. 16 Rw	11.2
No. 4 Rw	10.0	No. 17 Rw	12.4
No. 5 Rw	8.8	No. 18 Rw	10.0
No. 6 Rw	10.8	No. 19 Rw	9.6
No. 7 Rw	10.8	No. 20 Rw	9.6
No. 8 Rw	11.6	No. 21 Rw	9.6
No. 9 Rw	11.2	No. 22 Rw	8.8
No. 10 Rw	9.6	No. 23 Rw	10.8
No. 11 Rw	11.6	No. 24 Rw	10.8
No. 12 Rw	10.4	Commercial *koji* Rw	7.2
No. 13 Rw	12.0		

Rw, rice wines.

Table 2. Physiological functionalities of eight kinds of traditional rice wines made by 2nd level-screened koji molds

Rice wines[a]	Antioxidant activity (%)	SOD-like activity (%)	Fibrinolytic activity	ACE inhibitory activity (%)	AChE inhibitory activity (%)
No. 2 Rw	13.7 ± 0.9	54.1 ± 1.8	ND	80.3 ± 0.9	n.d
No. 8 Rw	19.4 ± 0.2	45.1 ± 1.2	ND	81.7 ± 1.9	2.6
No. 9 Rw	22.1 ± 4.5	52.7 ± 0.1	ND	77.8 ± 1.6	4.0
No. 11 Rw	23.3 ± 0.4	39.8 ± 2.7	ND	75.0 ± 0.3	3.6
No. 13 Rw	31.1 ± 1.8	63.2 ± 1.0	ND	79.9 ± 1.6	5.8
No. 15 Rw	32.1 ± 0.6	55.6 ± 0.0	ND	81.1 ± 0.9	0.7
No. 16 Rw	33.2 ± 1.6	63.4 ± 0.2	ND	79.8 ± 1.2	2.7
No. 17 Rw	48.6 ± 1.3	52.1 ± 1.6	ND	83.8 ± 1.4	1.7
Commercial *koji* Rw	5.7 ± 1.0	61.4 ± 3.6	ND	36.3 ± 1.2	6.1

Rw, rice wine; SOD, superoxide dismutase; ACE, angiotensin I-converting enzyme; AChE, acetylcholinesterase; ND, not detected.
[a]Rice wines which showed 10% over ethanol contents in Table 1.

Table 3. Activities of amylase and protease in water extracts of 2nd level-screened koji molds grown on wheat bran

Activity		No. 2 koji	No. 8 koji	No. 9 koji	No. 11 koji	No. 13 koji	No. 15 koji	No. 16 koji	No. 17 koji
α-Amylase (U/g)		401.7	311.0	433.1	124.2	414.3	78.4	36.9	135.2
Protease (U/g)	Acidic	84.1	97.2	17.0	55.2	98.6	15.6	9.9	203.1
	Neutral	ND	ND	ND	ND	ND	ND	ND	ND
	Alkaline	ND	ND	ND	ND	ND	ND	ND	ND

ND, not detected.

Table 4. Physiological functionalities of water extracts of 2nd level-screened koji molds grown on wheat bran

Koji extracts	Antioxidant activity (%)	SOD-likea ctivity (%)	Fibrinolytic activity	ACE inhbitory activity (%)[a]	AChE inhibitory activity (%)[a]
No. 2 Et	23.6 ± 3.6	ND	ND	36.8 ± 0.4	ND
No. 8 Et	36.5 ± 1.9	3.2 ± 2.2	ND	53.6 ± 2.4	ND
No. 9 Et	43.8 ± 0.6	ND	ND	40.1 ± 1.5	ND
No. 11 Et	67.7 ± 1.0	ND	ND	37.5 ± 1.8	ND
No. 13 Et	56.3 ± 1.0	ND	ND	22.5 ± 2.8	ND
No. 15 Et	48.6 ± 2.6	ND	ND	49.1 ± 1.7	2.0 ± 0.4
No. 16 Et	39.5 ± 0.2	29.1 ± 0.6	ND	80.5 ± 0.4	6.1 ± 2.6
No. 17 Et	46.0 ± 0.7	25.4 ± 2.3	ND	76.7 ± 2.6	ND

Et, ethanol extract; SOD, superoxide dismutase; ACE, angiotensin I-converting enzyme; AChE, acetylcholinesterase; ND, not detected.

protease activities in its water extracts. Therefore, we selected No. 17 koji mold as a functional mold for the brewing of new rice wine.

Identification of No. 17 koji mold. The No. 17 mold was identified as *R. stolonifer* using ITS 1 (CTTGGTCATTTAGAGGAGT-AA) and ITS 4 (TCCTCCGCTTATTGATATGC) primers. *R. stolonifer* is distributed in vegetables, fruits and flowers of postharvest, and sometimes it also causes plant soft rot [21]. Our report is the first to observe that *R. stolonifer* produces α-amylase and increases anti-hypertension during alcohol fermentation.

Optimal fermentation conditions for the brewing of functional traditional rice wine. The effect of fermentation period on the quality and functionality of traditional rice wine made from *R. stolonifer* No. 17 was investigated at 5 days and 10 days after fermentation. As shown in Table 5, ethanol contents were 11.2% to 12.8% 10 days after fermentation, which are not significantly different. However, anti-hypertensive ACE inhibitory activity was increased from 75.3% after 5 days of fermentation to 80.6% after 10 days. Antioxidant activity and SOD-like activity were also increased to 46.3% and 49.6%, respectively, 10 days after fermentation. Fibrinolytic activity and AChE inhibitory activity were below 3% or were not detected after 5 and 10 days of fermentation.

In order to increase anti-hypertension, cereals powders, which are known as anti-hypertensive materials [22], were added to mash containing cooked rice, *R. stolonifer* No. 17 and *S. cerevisiae*, followed by fermentation for 10 days at 25°C. Ethanol contents of rice wines were not significantly different between additive-treated rice wines (11.4%) and non-treated rice wine (13.0%) (Table 6). However, anti-hypertensive ACE inhibitory activity was increased by the addition of wheat bran, African millet, corn, red bean and especially wheat bran all experienced significant increases in ACE inhibitory activity. However, the acceptability of wheat bran-rice wine was very poor due to a strong vegetable off-flavor (Fig. 1). Even though ACE inhibitory activity was increased by addition cereals, the resulting sensory characteristics were very poor.

From these results, No. 17 rice wine brewed by the fermentation of *R. stolonifer* No. 17, cooked rice and *S. cer-*

Table 5. Effect of fermentation period on the ethanol content and physiological functionality of No. 17 traditional rice wine[a]

Fermentation period (days)	Ethanol contents (%)	Antioxidant activity (%)	SOD-like activity (%)	Fibrinolytic activity (clear zone:mm)	ACE inhibitory activity (%)	AChE inhibitory activity (%)
5	11.2	28.9 ± 1.1	44.2 ± 1.2	ND	75.3 ± 0.3	2.8 ± 0.3
10	12.8	46.3 ± 1.4	49.6 ± 3.0	ND	80.6 ± 0.5	2.6 ± 0.2
20	11.4	38.5 ± 1.7	49.4 ± 0.2	ND	80.4 ± 0.6	2.6 ± 0.5

[a]No. 17 traditional rice wine was brewed by *Rhizopus stolonifer* No. 17, cooked rice and *Saccharomyces cerevisiae* at 25°C.
SOD, superoxide dismutase; ACE, angiotensin I-converting enzyme; AChE, acetylcholinesterase;ND, not detected.

Table 6. Effects of additive materials on the physicochemical properties and ACE inhibitory activity of No. 17 traditional rice wine[a]

Additive materials	pH	Ethanol content (%)	Total acid content (%)	Volatile acid content (%)	Residual sugar content (mg/mL)	ACE inhibitory activity (IC_{50}: mg/mL)
Corn	3.64	11.6	0.325	0.0213	0.72	0.88
Wheat bran	3.80	12.0	0.556	0.0237	0.18	0.70
Brown rice	3.81	11.8	0.240	0.0124	0.48	1.03
Barley	3.84	12.4	0.249	0.0089	0.56	1.02
African millet	3.82	11.6	0.243	0.0154	0.25	0.74
Mung beans	3.89	11.8	0.260	0.0112	0.35	1.00
Red bean	3.98	13.0	0.246	0.0106	0.17	0.89
Soybean	3.98	12.6	0.266	0.0237	0.42	1.07
Kidney bean	3.91	11.4	0.237	0.0089	0.45	0.95
Control	3.89	12.6	0.237	0.0106	0.66	1.06

ACE, angiotensin I-converting enzyme.
[a]No. 17 traditional rice wine was brewed by *Rhizopus stolonifer* No. 17, cooked rice and *Saccharomyces cerevisiae* at 25°C.

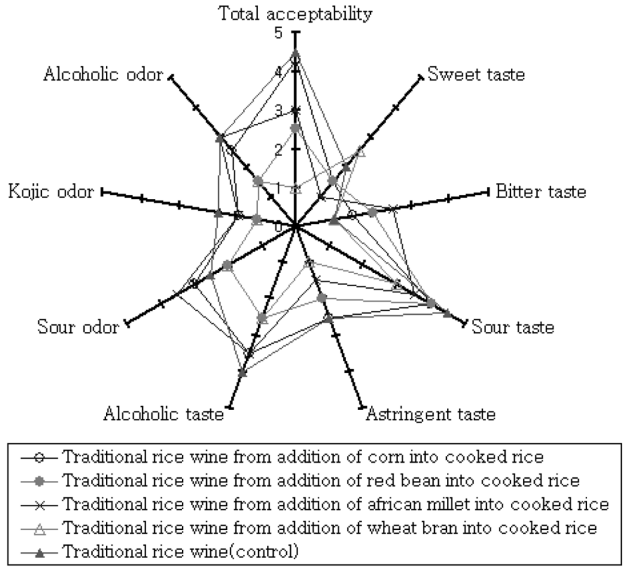

Fig. 1. The quantitative descriptive analysis profile for taste and flavor of various traditional rice wines from addition of additive meterials.

evisiae at 25°C for 10 days has potential as a new functional Korean tradition rice wine having high anti-hypertensive properties and good sensory characteristics.

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