Food Sci. Biotechnol. 22(2): 425-432 (2013)
DOI 10.1007/s10068-013-0097-6

RESEARCH ARTICLE

Aspergillus oryzae Strains Isolated from Traditional Korean *Nuruk*: Fermentation Properties and Influence on Rice Wine Quality

Siyoung Yang, Seung Jun Choi, Jungki Kwak, Kihyun Kim, Minjae Seo, Tae Wha Moon, and Yin-Won Lee

Received: 27 July 2012 / Revised: 26 September 2012 / Accepted: 27 September 2012 / Published Online: 30 April 2013

Abstract Forty-seven strains of *Aspergillus oryzae* isolates from Korean *nuruks* were compared for their brewing characteristics. *A. oryzae* YI-A6 and YI-A7 showed the highest acid α-amylase, glucoamylase, and carboxypeptidase activities, respectively. Sixteen isolates with high amylolytic or proteolytic enzyme activities were selected for investigation of their rice wine fermentation characteristics. After 12 days of brewing at 15°C, ethanol concentrations were 10.2-14.3% for *A. oryzae* strains. Fermentation rates were the highest for YI-A7. Most rice wine samples fermented with *nuruk* strains had lower concentrations of off-flavor compounds than the control did. All mean sensory attribute values significantly differed among samples. Pearson correlation coefficients showed that glucoamylase activity was positively correlated to both ethanol productivity and overall harmony ($p<0.01$). Thus, glucoamylase activity was identified as the best factor for screening *Aspergillus* strains for use in rice wine brewing.

Keywords: amylolytic enzyme activity, *Aspergillus oryzae*, *nuruk*, proteolytic enzyme activity, rice wine

Siyoung Yang, Yin-Won Lee (✉)
Department of Agricultural Biotechnology and Center for Fungal Pathogenesis, Seoul National University, Seoul 151-921, Korea
Tel: +82-2-880-4671; Fax: +82-2-873-2317
E-mail: lee2443@snu.ac.kr

Siyoung Yang, Jungki Kwak, Kihyun Kim, Minjae Seo
Lotte R&D Center, Seoul 150-104, Korea

Seung Jun Choi
Department of Food Science and Technology, Seoul National University of Science and Technology, Seoul 139-743, Korea

Tae Wha Moon
Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea

Introduction

Several saccharifying agents have been used to assist yeast alcohol fermentation during the production of alcoholic beverages from raw grains (1,2). Various types of indigenous alcoholic starters are used in many Asian countries to produce traditional alcoholic drinks (3,4). A traditional starter culture used for brewing alcoholic beverages in Korea is *nuruk*, prepared from uncooked dough made from coarsely ground grains and water. *Nuruk* contains filamentous fungi, yeasts, and lactic acid bacteria obtained by natural inoculation. It functions as both a saccharifying agent, by hydrolyzing starch into sugars, and a fermenting agent, by producing ethanol during the alcoholic fermentation process. Some of the diverse fungi species, of the order Mucorales and the genus *Aspergillus*, present in *nuruk* have amylolytic activity (5-7).

Aspergilli are recognized as important amylolytic enzyme producers in several Asian starters, including *nuruk* and *koji*. In addition, aspergilli produce proteolytic enzymes, such as acid protease and acid carboxypeptidase, during alcohol fermentation. These enzymes generate peptides or amino acids that are used as yeast nutrients and flavor compound precursors (6-10). However, a number of *Aspergillus* species, including *A. flavus* and *A. parasiticus*, produce the highly toxic and carcinogenic metabolites known as aflatoxins (11). Two closely related species, *A. oryzae* and *A. sojae*, commonly used in alcohol and food fermentation in Asia, do not produce aflatoxins (12).

Among Korean alcoholic beverages, *cheongju* is made from the *Aspergillus*-containing rice *koji* alcoholic starter, which is also used to produce other rice wines (e.g., *takju*/*makgeolli* and *yakju*). Several studies identified *Aspergillus* strains with high amylolytic activities in *nuruk* (5,6,8, 13,14). However, a full examination of *nuruk* enzyme production, ethanol productivity, flavor profile, and quality

characteristics to determine its suitability for commercialization is lacking. In our previous study it was reported that the presence of mycobiota in *nuruk* and isolated filamentous fungal strains from *nuruk* (15). In this study, the fermentation properties of previously isolated *A. oryzae* strains were evaluated, including enzyme activity analysis, rice wine fermentation, color analysis, sensory evaluation, and safety verification.

Materials and Methods

Strains, media, and cultivation In total, 47 *Aspergillus oryzae* isolates from Korean *nuruk* were used. The isolates were grown on potato dextrose agar (BD Bioscience, Franklin Lakes, NJ, USA) slants and incubated at 25°C for 7 days. Fungal spores were then resuspended in 5 mL sterile 0.85%(w/v) NaCl containing 0.05%(w/v) Tween 80, and 2-3.5 mL of this suspension was used to inoculate the rice *koji* preparation. The Japonica rice 'Chucheong' harvested in Korea in the autumn of 2009 was used for *koji* preparation and rice wine fermentation.

Preparation of rice *koji* Rice *koji* was prepared using a polishing rate of approximately 92%. Rice (5 kg) was washed and soaked in 5 L tap water for 1 h at ambient temperature. The soaked rice was steamed for approximately 90 min at 100°C, cooled to 35°C, then divided into 200 g portions, placed in sterilized plastic bottles, and inoculated with fungal spore suspension (5×10^5 spores/g rice). The inoculated rice was incubated at 38°C for 44 h at a relative humidity of >85%. Rice *koji* samples were stored at −70°C before use.

Measurement of enzymatic activity Extracts were prepared from 20 g rice *koji* by shaking in 50 mL of 0.2 M sodium acetate buffer, pH 5.0, containing 0.5%(w/w) NaCl, for 3 h at room temperature, followed by filtration through Whatman no. 1 paper. Enzyme activities of the filtrate were determined according to the official method of the National Tax Administration Agency of Japan, with slight modifications.

For the measurement of α-amylase activity, 0.1 mL enzyme solution was added to 2 mL of 0.04 M sodium acetate buffer, pH 5.0, containing 1% soluble starch, at 40°C. After 10 min of incubation, 0.1 mL of reaction products was added to 10 mL of 0.25 mM iodine solution, and the percentage transmission of the resulting starch-iodine color (Tt, %) was measured at 670 nm against distilled water. α-Amylase activity (units/g *koji*) was calculated using the equation of Shinoki *et al*. (16).

For the measurement of glucoamylase activity, 1 mL of 1%(w/v) soluble starch solution was added to 0.2 mL of 200 mM sodium acetate buffer, pH 5.0, and incubated at 40°C for 5 min. The enzyme solution (0.1 mL) was then added to this mixture and incubated at 40°C for 20 min, and then, the reaction was stopped by addition of 0.1 mL of 1 M NaOH. An enzyme blank for each sample was prepared by adding 1 M NaOH before the enzyme solution. The amount of glucose produced by fungal enzymes was measured in both samples and blanks by using a glucose assay kit (Megazyme, Bray, Ireland), and net glucose production was calculated by subtracting the glucose concentration in the reaction mixture from that in the blank solution. Glucoamylase activity (units/g *koji*) was calculated from the net glucose production. One unit of glucoamylase activity was defined as the amount of enzyme required to catalyze the release of 1 mg glucose/h under normal assay conditions.

For the measurement of acid carboxypeptidase activity, 1 mL of 0.5 mM carbobenzoxy-glutamyl-tyrosine (Cbz-Glu-Tyr) in 0.5 M sodium acetate buffer, pH 3.0, was pre-heated at 30°C for 5 min, and then, enzyme solution (0.1 mL) was added and the mixture was incubated at 30°C for 20 min. The reaction was stopped by the addition of 0.5 mL ninhydrin solution and heating to 100°C for 20 min. The reaction mixture was then cooled quickly and diluted with 5 mL of 60%(v/v) ethanol. After equilibration for 5 min at room temperature, the absorbance at 570 nm was measured against distilled water. Blanks for each sample were prepared by adding ninhydrin solution without enzyme. Ninhydrin solution was prepared by mixing 2 solutions together immediately before use: solution A, comprising 1 g ninhydrin dissolved in 25 mL 2-methoxyethanol (methyl cellosolve); and solution B, consisting of 0.5 mL of 10 mM KCN, 4.5 mL of 2-methoxyethanol, and 20 mL of 2 M sodium acetate buffer (pH 5.0). A calibration curve was constructed using L-tyrosine standard solutions (0-50 μg/mL). One unit of acid carboxypeptidase activity was defined as the amount of enzyme required to catalyze the release of 1 μg L-tyrosine/h under normal assay conditions.

For the measurement of acid protease activity, 1.5 mL of 2% casein and 1 mL of 0.1 M McIlvaine buffer, pH 3.0, were incubated with enzyme solution (0.5 mL) at 38°C for 1 h, and the reaction was stopped by the addition of 3 mL of 0.4 M trichloroacetic acid (TCA). Reaction products were clarified by filtration, and the quantity of TCA-soluble peptides in 1 mL filtrate was measured by incubation with 5 mL of 0.4 M sodium carbonate solution and 1 mL Folin-Ciocalteu's phenol reagent at 38°C for 30 min. The absorbance at 660 nm was measured against distilled water. Blanks consisted of reaction mixtures containing TCA solution and lacking enzyme. A calibration curve was constructed using L-tyrosine standard solutions (0-100 μg/mL). One unit of acid protease activity was defined as the amount of enzyme required to catalyze the release of 1 μg

tyrosine/h under normal assay conditions.

Rice wine fermentation *Koji* extract broth was prepared as follows. Rice *koji* was prepared and inoculated with an *A. oryzae* strain and then mixed with tap water and incubated at 65°C for 6 h. The digested extract was prepared by centrifugation at 4,300×*g* for 15 min and filtration through a gauze sheet. The filtrate was adjusted to a Brix value of 10 with distilled water and autoclaved. Yeast (*Saccharomyces cerevisiae* Kyokai no. 1001 [K1001] of the Brewing Society of Japan) starter cultures made by inoculating 5 mL of *koji* extract broth with yeast and incubating at 25°C for 48 h without shaking were added to 100 mL of the same medium and incubated under the same conditions. Rice wine fermentation was performed in a 500-mL Erlenmeyer flask with a silicone sponge plug to allow the CO_2 gas product to escape. Rice wine mash consisted of 20 g rice *koji*, 80 g steamed rice, 140 mL tap water, and 0.55 mL of 90%(w/v) lactic acid, and the initial yeast concentration was 1.0×10^5 cells/g mash. This mash was incubated at 15°C for 12 days without shaking and weighed daily to estimate CO_2 production. Thereafter, the mash was clarified by centrifugation at 4,300×*g* for 30 min and filtering the supernatant through a cellulose acetate membrane of 5-μm pore size. The filtrate was used for analysis and sensory evaluation.

Rice wine analysis Levels of acetic acid, ethanol, glucose, glycerol, lactic acid, and maltose in rice wine were determined using a refractive index detector after separation at 60°C on a fermentation monitoring column (Bio-Rad Laboratories, Richmond, CA, USA) fitted to a Shimadzu 10A HPLC system (Shimadzu, Kyoto, Japan). The mobile phase was 1 mM H_2SO_4, the flow rate was 0.7 mL/min, and the injection volume was 10 μL. Compounds were identified and quantified by comparing their retention times and peak volumes with standards.

Flavor compounds in rice wine samples (5 mL) were extracted with 2.5 mL dichloromethane and injected (1 μL) into an Agilent 6890 GC column (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and an HP-FFAP capillary column (film thickness 0.5-μm; 50 m length×0.32 mm i.d.). The injector and detector were operated at 250 and 280°C, respectively. The carrier gas was helium at a flow rate of 2 mL/min and the auxiliary gas was nitrogen at a flow rate of 25 mL/min. A split ratio injection of 15:1 with an airflow rate of 400 mL/min was used. The column temperature program was 40 to 55°C for 2.5 min at a rise rate of 2°C/min; 100°C at a rise rate of 3°C/min; 200°C at a rise rate of 10°C/min; and then 10 min at 200°C. The compounds were identified and quantified by comparing retention times and concentrations with standards. Hunter L, a, and b values of rice wine samples were measured using a Minolta chroma meter CT-310 (Minolta Camera Co., Osaka, Japan).

Sensory evaluation Five male panelists (aged 31-46 years) who had 2-20 years of experience of this type of sensory evaluation were recruited. Assessments were conducted orthonasally (by sniffing) without tasting. Samples were provided as 100 mL aliquots in 200-mL plastic bottles with lids. The panelists were requested to select coded sample bottles randomly, swirl the contents well, and sniff the headspace vapor. Sensory attributes (fruitiness, cereal/*koji* odor, off-odor, and overall harmony) of rice wine were evaluated on a 9-point graduated scale with a maximum score of 9 for extremely strong or extremely high and a minimum of 1 for none or extremely low.

Aflatoxin analysis Aflatoxin B1, B2, G1, and G2 levels in rice *koji* samples were analyzed using an AFLASCAN assay kit (Rhône Diagnostics Technologies, Glasgow, Scotland). Fluorescence intensity was compared to a fluorescent test card to obtain semi-quantitative results.

Statistical analysis Data were analyzed using the SPSS for Windows version 18 software (SPSS Inc., Chicago, IL, USA). Pearson correlation coefficients were calculated to examine relationships among α-amylase, glucoamylase, acid carboxypeptidase, and acid protease activities, and between enzyme activities and quality characteristics such as ethanol production and sensory attributes (overall harmony). Analysis of variance (ANOVA) and Duncan's multiple range test ($p<0.05$) were performed to determine statistical differences and to discriminate between mean sensory evaluation scores.

Results and Discussion

Enzymatic activities α-Amylase and glucoamylase activities were determined for 47 *A. oryzae* strains isolated from *nuruk* in a previous study (15). The means and standard deviations of α-amylase and glucoamylase activities for 47 *A. oryzae* strains were 208.5±280.3 and 114.5± 46.6 unit/g, respectively. The activities of α-amylase were more variable than those of glucoamylase for *A. oryzae* strains. Sixteen *A. oryzae* strains with relatively high amylolytic activities were selected for analysis of their proteolytic activities (Table 1). Of the *A. oryzae* strains, DG-C3, YI-A6, and YI-A7 showed a very high α-amylase activity. Furthermore, YI-A7 showed the highest gluco-amylase activity of all strains tested. DG-B3 and JC-A4 had high acid carboxypeptidase activity. There were only slight differences in acid protease activity among the

Table 1. Enzymatic activities of selected *A. oryzae* isolates

Strain	Enzyme activity (unit/g *koji*)			
	α-Amylase	Gluco-amylase	Acid carboxy-peptidase	Acid protease
AD-B2	294.0	129.6	3,898.6	1,285.7
AD-B4	251.8	120.9	4,179.1	1,371.4
DG-B3	362.8	166.9	5,790.4	1,289.0
DG-B4	285.5	129.6	5,248.3	1,302.2
DG-C3	1,110.3	152.5	4,380.8	1,190.1
GS-A2	235.3	118.9	504.9	1,170.3
GS-A3	278.0	141.9	4,543.7	1,453.8
HP-A1	223.8	73.9	4,792.8	1,209.9
HS-A6	282.5	120.0	5,637.6	1,526.4
IC-A3	304.0	157.4	2,075.5	1,104.4
JC-A4	264.0	162.1	6,058.3	1,453.8
SC-A3	163.8	169.8	5,301.0	1,483.5
SEJ-A5	214.8	128.5	420.7	1,111.0
YI-A5	304.0	205.3	1,795.1	1,364.8
YI-A6	1,297.3	132.2	1,178.0	1,256.0
YI-A7	1,227.3	231.3	3,506.0	1,262.6

strains. Nunokawa (17) previously described the enzymatic activities of rice *koji* used for brewing sake, the Japanese rice wine (α-amylase, 870-1,710; glucoamylase, 125-305; acid protease, 2,686-4,316; and acid carboxy-peptidase, 3,265-7,550 in unit/g). Several *A. oryzae* strains used in this study, including DG-C3, YI-A6, and YI-A7, had enzyme activities similar to industrial strains used for Japanese *sake*, except that their acid protease activities were lower.

Relationships among different enzymatic activities (α-amylase/glucoamylase, acid carboxypeptidase/acid protease, and amylolytic enzymes/proteolytic enzymes) were assessed using a Pearson correlation analysis ($p<0.05$). The correlation coefficients of α-amylase/glucoamylase, acid carboxypeptidase/acid protease, and amylolytic enzymes/proteolytic enzymes were 0.367, 0.643, and −0.186, respectively. Therefore, the 2 proteolytic enzymatic activities of *A. oryzae* strains showed a significant correlation at the 95% confidence level.

Rice wine fermentation Small-scale rice wine fermentation was performed with each of the 16 selected *A. oryzae* strains. The fermentation rates of JC-A4 and YI-A7 were higher than those of other strains, while HP-A1 exhibited the slowest fermentation. However, the fermentation rate of all *nuruk* strains tested was lower than that of the industrial *koji* strain used as a control (Fig. 1).

Characteristics of rice wine brewed with selected *A. oryzae* strains After fermentation for 12 days, the ethanol concentration of rice wines was analyzed by HPLC (Table 2). The ethanol concentration ranges of *A. oryzae* wines were 10.2-14.3%. YI-A7 produced more ethanol than the industrial *koji* control did, and GS-A3 showed good ethanol production. In addition, saccharifying ability, indicated by the amounts of residual sugars (including maltose and glucose), was highest for YI-A6 and YI-A7. Furthermore, glycerol concentration was slightly higher following YI-A6 and YI-A7 fermentation than following fermentation by other strains. Glycerol has been shown to play a role in protecting yeast against osmotic stresses and can boost mellowness, thus generating fermented alcoholic beverages with good qualities (18,19). Therefore, our results show that strains used in this study can be used to produce rice wines with different properties.

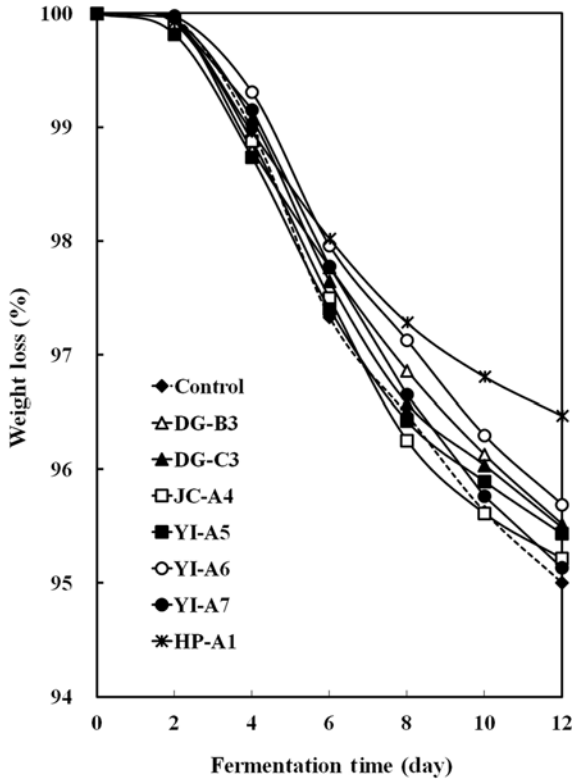

Fig. 1. Rice wine fermentation profiles for representative *A. oryzae* strains.

The color of rice wine products influences shelf life during distribution. The particular *Aspergillus* strain used for making *koji* can affect rice wine color. For example, by disrupting the ferrichrysin biosynthesis gene, Watanabe *et al*. (20) developed *A. oryzae* strains that did not produce ferrichrysin, an iron-chelating siderophore that causes an undesirable brown color in fermented rice wine products. The Hunter parameter 'a' values, on a scale from green (–a) to red (+a), did not differ markedly in wines produced using different strains. However, 'L' values (indicating lightness) were higher in wines produced using JC-A4, GS-A3, and DG-B3 strains than those in wines produced

Table 2. HPLC analyses of rice wines fermented using selected *A. oryzae* strains

Strain	Concentration (%)[1)]					
	Maltose	Glucose	Lactate	Glycerol	Acetate	Ethanol
Control[2)]	1.5	1.5	0.4	0.3	0.5	14.2
AD-B2	1.0	1.2	0.4	0.2	0.4	12.1
AD-B4	0.8	1.1	0.4	0.2	0.5	12.6
DG-B3	1.3	1.3	0.4	0.2	0.4	12.7
DG-B4	1.2	1.2	0.4	0.2	0.4	12.4
DG-C3	1.3	1.3	0.4	0.2	0.5	12.8
GS-A2	0.9	1.1	0.4	0.2	0.5	11.6
GS-A3	0.8	1.1	0.4	0.3	0.4	13.6
HP-A1	1.2	1.1	0.4	0.2	0.6	10.2
HS-A6	0.9	1.3	0.4	0.2	0.5	12.8
IC-A3	1.2	1.5	0.4	0.3	0.4	12.4
JC-A4	1.1	1.3	0.4	0.3	0.5	12.7
SC-A3	1.0	1.3	0.4	0.2	0.4	12.6
SEJ-A5	1.0	1.2	0.4	0.2	0.4	11.9
YI-A5	0.9	1.3	0.4	0.2	0.5	12.9
YI-A6	2.6	2.7	0.4	0.4	0.3	12.4
YI-A7	2.0	2.0	0.4	0.4	0.3	14.3

[1)]Concentration of ethanol is v/v, and that of all other components is w/v.
[2)]Fermented using an industrial *koji* molded with *A. oryzae* strains

using other strains. The 'b' values, on a scale from blue (−b) to yellow (+b), were lowest in rice wine produced using DG-B3, JC-A4, and SEJ-A5 strains (Table 3). These *A. oryzae* strains thus produce wine with good color characteristics because, in general, high 'L' values and low 'b' values are required for a prolonged shelf life.

Table 3. Color of rice wine fermented with selected *A. oryzae* strains

Strain	Hunter color value[1)]		
	L	a	b
Control[2)]	87.49	-0.33	3.57
AD-B2	85.75	-0.30	3.70
AD-B4	89.04	-0.35	3.42
DG-B3	89.42	-0.30	2.89
DG-B4	88.86	-0.40	4.16
DG-C3	88.27	-0.30	3.90
GS-A2	83.37	-0.24	3.38
GS-A3	89.46	-0.37	3.60
HP-A1	86.68	-0.31	3.53
HS-A6	89.02	-0.38	4.16
IC-A3	88.55	-0.36	4.15
JC-A4	91.16	-0.33	2.80
SC-A3	89.31	-0.40	4.48
SEJ-A5	91.12	-0.34	2.68
YI-A5	88.55	-0.33	3.04
YI-A6	87.24	-0.20	3.65
YI-A7	83.68	-0.24	4.49

[1)]Hunter L, a, and b values of each rice wine were measured with a Minolta chroma meter CT-310.
[2)]Fermented using an industrial *koji* molded with *A. oryzae* strains

Several flavor compounds responsible for rice wine quality have been identified by investigating flavor characteristics of *Aspergillus* strains from *nuruk* (21,22). Therefore, the flavor profiles for representative rice wine samples with high amylolytic or proteolytic enzymatic activities were made (Table 4). All rice wine samples contained lower levels of off-flavor compound (acetaldehyde and furfural) than the control did. It was found that levels of higher alcohol and ester compounds varied among samples. In particular, DG-B3 exhibited high phenethyl alcohol, ethyl caproate, and ethyl lactate concentrations, and YI-A7 produced high concentrations of isoamyl alcohol and ethyl acetate. The levels of fruity flavor compounds, such as ethyl acetate (fruity), isoamyl acetate/isoamyl alcohol (banana-like aroma), ethyl caproate (apple or peach-like aroma), ethyl caprylate (apple-like aroma), ethyl lactate (buttery and creamy), and phenylethyl acetate/phenethyl alcohol (fruity and flowery), are closely linked to the odor and taste of fermented alcoholic beverages (23). Because yeast plays the main role in the formation of flavor compounds in rice wine fermentation, many studies have focused on the development of yeast strains that produce large amounts of various flavor compounds (24,25). However, little attention has been paid to the influence of *Aspergillus* fungi on the formation of flavor compounds during rice wine fermentation (22,26,27). Our data suggest that the flavor profile of rice wine depends on the particular *A. oryzae* strain used. Interestingly, strains used in this study produced only small quantities of both off-flavor and flavor

Table 4. Flavor profiles of rice wines fermented with representative *A. oryzae* strains

Flavor compound	Concentration (ppm)					
	Control[1)]	DG-B3	DG-C3	JC-A4	YI-A6	YI-A7
Acetaldehyde	27.8	22.3	21.9	23.5	18.3	20.3
Furfural	0.2	0.2	0.1	0.1	0.1	0.1
Isoamyl alcohol	193.1	163.3	156.9	150.7	166.1	182.3
Phenethyl alcohol	152.9	144.8	138.8	138.0	131.3	136.3
Ethyl acetate	51.4	36.4	35.5	36.2	30.5	36.6
Ethyl caproate	1.2	1.6	1.5	1.4	1.4	1.2
Ethyl caprylate	0.6	0.7	0.7	0.6	0.4	0.5
Ethyl lactate	78.4	107.5	99.6	94.1	80.8	85.8
Isoamyl acetate	3.1	1.8	1.6	1.7	1.7	1.7
Phenethyl acetate	2.2	1.3	1.1	1.3	1.2	1.0

[1)]Fermented using an industrial *koji* molded with *A. oryzae* strains

compounds than the control did, indicating that these strains have clean odor and/or taste characteristics. However, further studies are necessary to determine how *Aspergillus* fungi influence the formation of flavor compounds during rice wine fermentation.

Sensory odor evaluation The various sensory attributes of Korean rice wines have been investigated (28,29). To simultaneously evaluate the sensory characteristics of a large number of rice wine samples (n=17), only the most noticeable sensory attributes were evaluated. In addition, rice wine samples were assessed orthonasally without tasting to avoid bias due to alcohol consumption. An ANOVA test conducted on the sensory evaluation scores showed that mean sensory attribute values (cereal/*koji* odor, fruitiness, off-odor, and overall harmony) differed significantly among rice wine samples (p<0.05; Table 5). Wine made using YI-A5 had significantly higher 'fruitiness' than all other samples did, followed by wine made using DG-C3 and JC-A4. The AD-B2 wine sample had a significantly higher 'cereal/*koji* odor' than all others did. HP-A1 wine exhibited a significantly higher 'off-odor' than all other samples did. In addition, wine made using YI-A5 showed significantly higher 'overall harmony' than all other samples did, followed by wine made using DG-B3, JC-A4, and YI-A7. The mean scores for representative rice wine samples with high levels of amylolytic or proteolytic enzymatic activities are plotted in Fig. 2. Several strains from *nuruk* showed higher intensities than the control did. It was found that sensory data differed slightly from the results of flavor compound analysis and assume that the balance or interactions among flavor compounds, including off-flavors, determine the overall sensory profile.

Table 5. Sensory characteristics of rice wines fermented with selected *A. oryzae* strains

Strain	Sensory attributes			Overall Harmony
	Fruitiness	Cereal/ *koji* odor	Off-odor	
Control[1)]	$4.6^{abcd2)}$	2.8^{ab}	2.0^{c}	5.0^{bcd}
AD-B2	3.2^{cd}	4.6^{a}	2.8^{bc}	4.8^{bcd}
AD-B4	3.8^{bcd}	3.4^{ab}	2.4^{bc}	5.0^{bcd}
DG-B3	5.0^{abc}	3.2^{ab}	2.0^{c}	6.0^{ab}
DG-B4	4.4^{abcd}	3.6^{ab}	2.6^{bc}	5.0^{bcd}
DG-C3	5.6^{ab}	2.8^{ab}	2.6^{bc}	5.4^{abc}
GS-A2	2.8^{d}	3.4^{ab}	3.2^{abc}	4.4^{cd}
GS-A3	4.0^{bcd}	3.4^{ab}	2.8^{bc}	5.2^{abcd}
HP-A1	3.2^{cd}	3.8^{ab}	4.2^{a}	4.0^{d}
HS-A6	4.0^{bcd}	3.6^{ab}	2.6^{bc}	5.0^{bcd}
IC-A3	3.6^{bcd}	3.8^{ab}	2.6^{bc}	4.4^{cd}
JC-A4	5.6^{ab}	2.6^{ab}	2.0^{c}	6.0^{ab}
SC-A3	3.8^{bcd}	3.0^{ab}	3.4^{ab}	4.4^{cd}
SEJ-A5	3.6^{bcd}	3.2^{ab}	2.6^{bc}	4.8^{bcd}
YI-A5	6.0^{a}	2.4^{b}	2.2^{bc}	6.4^{a}
YI-A6	4.6^{abcd}	2.8^{ab}	2.2^{bc}	5.4^{abc}
YI-A7	4.8^{abcd}	3.0^{ab}	2.2^{bc}	5.6^{abc}

[1)]Fermented using an industrial *koji* molded with *A. oryzae* strains
[2)]Values within a column not sharing a letter are significantly different according to Duncan's multiple range tests (p<0.05).

Pearson correlation coefficients were significant between glucoamylase activity and both ethanol production (concentration) and overall harmony attributes in the sensory evaluation (p<0.01; Table 6). No correlation was found between α-amylase and acid protease activities and any other characteristic. Therefore, our data suggests that screening *Aspergillus* strains for suitable rice wine fermentation properties can be simplified by measuring only their glucoamylase activity.

Aflatoxin production Aflatoxin production was investigated in rice *koji* samples with high amylolytic or proteolytic enzymatic activities. No aflatoxins were detected in any of the 6 *A. oryzae* strains (DG-B3, DG-C3, JC-A4, YI-A5, YI-A6, and YI-A7) tested. Aflatoxins have previously been detected in Korean rice (30). However, to our

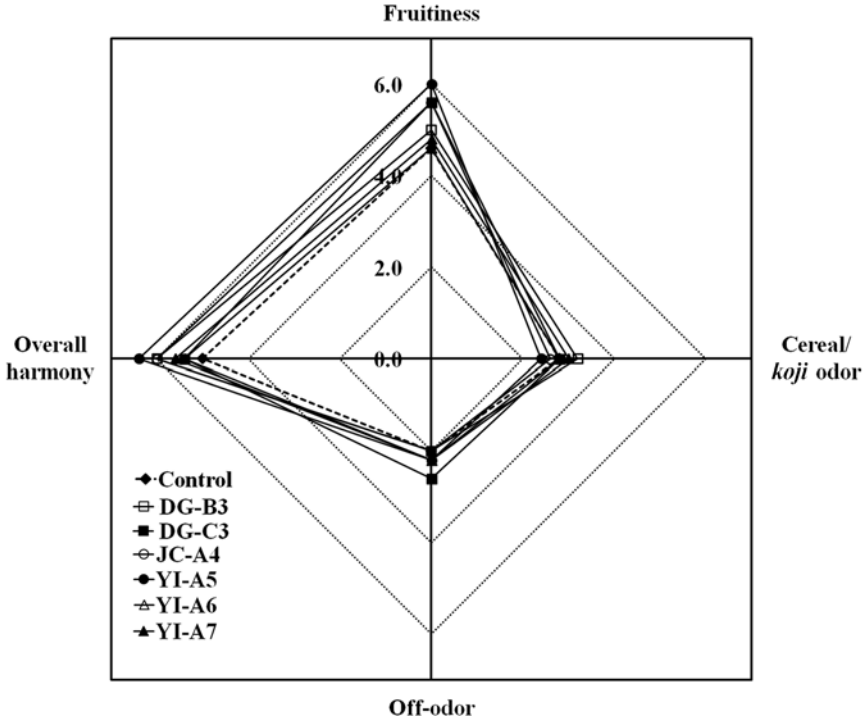

Fig. 2. Sensory analysis of representative rice wines by using a 9-point scale.

Table 6. Pearson correlation analysis between each enzymatic activity and the rice wine quality characteristics

Enzyme	Ethanol production	Overall harmony score
α-Amylase	0.397	0.328
Glucoamylase	0.782*[1)]	0.653*
Acid carboxypeptidase	0.166	0.133
Acid protease	0.410	0.301

[1)]*Correlation is significant at the $p<0.01$ level (2-tailed).

knowledge, there have been no reports of aflatoxin in Korean *nuruk*, rice wine, or in fungi isolated from *nuruk*.

In conclusion, we investigated the characteristics of *A. oryzae* strains suitable for industrial rice wine fermentation, including enzyme activities, ethanol production, color patterns, flavor profiles, and aflatoxin production. These data will facilitate the generation of an *Aspergillus* strain pool for industrialization. In addition, glucoamylase and ethanol production both correlated with a sensory attribute (overall harmony), indicating that glucoamylase activity is the most important factor for screening *Aspergillus* strains for use in industrial rice wine fermentation.

Acknowledgments We would like to thank Dr. Yasuo Konishi for valuable advice during the study. Lee Y-W was supported by the National Research Foundation of Korea (NRF) grant by the Korean government (MEST) (2012-0000575).

References

1. Aidoo KE, Rob Nout MJ, Sarkar PK. Occurrence and function of yeasts in Asian indigenous fermented foods. FEMS Yeast Res. 6: 30-39 (2006)
2. Teramoto Y, Okamoto K, Ueda S, Kayashima S. Rice wine brewing with sprouting rice, sprouting rice infected with *Aspergillus oryzae* and rice *koji*. J. I. Brew. 99: 467-471 (1993)
3. Hesseltine CW. Microbiology of oriental fermented foods. Annu. Rev. Microbiol. 37: 575-601 (1983)
4. Wood BJB. Oriental food uses of *Aspergillus*. pp. 481-498. In: The British Mycological Symposium Series No 1. Genetics and Physiology of *Aspergillus*. Smith JE, Pateman JA (eds). Academic Press, London, UK (1977)
5. Kim HS, Hyun JS, Kim J, Ha, HP, Yu JS. Characteristics of useful fungi isolated from traditional Korean *nuruk*. J. Korean Soc. Food Sci. Nutr. 26: 767-774 (1997)
6. Park JW, Lee KH, Lee CY. Identification of filamentous molds isolated from Korean traditional *nuruk* and their amylolytic activities. Korean J. Appl. Microbiol. Biotechnol. 23: 737-746 (1995)
7. Yu TS, Kim J, Kim HS, Hyun JS, Ha HP, Park MG. Bibliographical study on microorganisms of traditional Korean *nuruk* (since 1945). J. Korean Soc. Food Sci. Nutr. 27: 789-799 (1998)
8. Kim HS, Hyun JS, Kim J, Ha HP, Yu TS. Enzymological characteristics and identification of useful fungi isolated from traditional Korean *nuruk*. Korean J. Appl. Microbiol. Biotechnol. 26: 456-464 (1998)
9. Narahara H. *Koji*-mold and its product *koji*. J. Brew. Soc. Japan 89: 873-881 (1994)
10. Iemura Y, Takahashi T, Yamada T, Furukawa K, Hara S. Properties of TCA-insoluble peptides in *kimoto* (traditional seed mash for sake brewing) and conditions for liberation of the peptides from rice protein. J. Biosci. Bioeng. 88: 531-535 (1999)
11. Li P, Zhang Q, Zhang W. Immunoassays for aflatoxins. Trends Anal. Chem. 28: 1115-1126 (2009)
12. Watson AJ, Fuller LJ, Jeenes DJ, Archer DB. Homologs of aflatoxin biosynthesis genes and sequence of *aflR* in *Aspergillus oryzae* and

Aspergillus sojae. Agr. Biol. Chem. Tokyo 65: 307-310 (1999)
13. So MH. Characteristics of a modified *nuruk* made by inoculation of traditional *nuruk* microorganisms. Korean J. Food Sci. Technol. 12: 219-225 (1999)
14. Kim JY, Koh JS. Screening of brewing yeasts and saccharifying molds for foxtail millet-wine making. J. Korean Soc. Appl. Bi. 47: 78-84 (2004)
15. Yang S, Lee J, Kwak J, Kim K, Seo M, Lee YW. Fungi associated with the traditional starter cultures used for rice wine in Korea. J. Korean Soc. Appl. Bi. 54: 933-943 (2011)
16. Shinoki S, Mikami S, Ueki, T, Shimada T. Studies on the assay method of rice-*koji* α-amylase. J. Brew. Soc. Japan 80: 879-882 (1985)
17. Nunokawa Y. Enzyme production of *koji*. pp. 315-322. In: *Kojigaku*. Murakami H (ed). The Brewing Society of Japan, Tokyo, Japan (1986)
18. Omori T, Umemoto Y, Ogawa K, Kajiwara Y, Shimoda M, Wada H. A novel method for screening high glycerol-and ester-producing brewing yeasts (*Saccharomyces cerevisiae*) by heat shock treatment. J. Ferment. Bioeng. 83: 64-69 (1997)
19. Lubbers S, Verret C, Voilley A. The effect of glycerol on the perceived aroma of a model wine and a white wine. Lebensm. - Wiss. Technol. 34: 262-265 (2001)
20. Watanabe H, Hatakeyama M, Sakurai H, Uchimiya H, Sato T. Isolation of industrial strains of *Aspergillus oryzae* lacking ferrichrysin by disruption of the *dffA* gene. J. Biosci. Bioeng. 106: 488-492 (2008)
21. Han EH, Lee TS, Noh BS, Lee DS. Volatile flavor components in mash of *takju* prepared by using different *nuruks*. Korean J. Food Sci. Technol. 29: 563-570 (1997)
22. Lee TS, Han EH. Volatile flavor components in mash of *takju* prepared by using *Aspergillus oryzae nuruk*s. Korean J. Food Sci. Technol. 33: 366-372 (2001)
23. Lilly M, Bauer F, Lambrechts M, Swiegers J, Cozzolino D, Pretorius I. The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. Yeast 23: 641-659 (2006)
24. Akita O, Ida T, Obata T, Hara S. Mutants of *Saccharomyces cerevisiae* producing a large quantity of β-phenethyl alcohol and β-phenethyl acetate. J. Ferment. Bioeng. 69: 125-128 (1990)
25. Ichikawa E, Hosokawa N, Hata Y, Abe Y, Suginami K, Imayasu S. Breeding of a *sake* yeast with improved ethyl caproate productivity. Agr. Biol. Chem. Tokyo 55: 2153-2154 (1991)
26. Kuriyama K, Ashida S, Saito Y, Hata Y, Suginami K, Imayasu S. Ethyl caproate synthesis and hydrolysis activity of *koji*. Hakkokogaku 64: 247-251 (1986)
27. Lee JH, Sato T, Kawai Y, Enei H. Purification and properties of extracellular esterases of *Aspergillus oryzae* which synthesize ethyl caproate. J. Microbiol. Biotechn. 5: 274-279 (1995)
28. Lee SJ, Ahn BH. Sensory profiling of rice wines made with *nuruk*s using different ingredients. Korean J. Food Sci. Technol. 42: 119-123 (2010)
29. Kim JY, Sung KW, Bae HW, Yi YH. pH, acidity, color, reducing sugar, total sugar, alcohol, and organoleptic characteristics of puffed rice powder added *takju* during fermentation. Korean J. Food Sci. Technol. 39: 266-271 (2007)
30. Park JW, Choi SY, Hwang HJ, Kim YB. Fungal mycoflora and mycotoxins in Korean polished rice destined for humans. Int. J. Food Microbiol. 103: 305-314 (2005)