Taxonomic Characterization, Evaluation of Toxigenicity, and Saccharification Capability of Aspergillus Section Flavi Isolates from Korean Traditional Wheat-Based Fermentation Starter Nuruk

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Abstract  The most economically important species used in a wide range of fermentation industries throughout Asia belong to Aspergillus section Flavi, which are morphologically and phylogenetically indistinguishable, with a few being toxigenic and therefore a major concern. They are frequently isolated from Korean fermentation starters, such as nuruk and meju. The growing popularity of traditional Korean alcoholic beverages has led to a demand for their quality enhancement, therefore requiring selection of efficient non-toxigenic strains to assist effective fermentation. This study was performed to classify the most efficient strains of Aspergillus section Flavi isolated from various types of traditional wheat nuruk, based on a polyphasic approach involving molecular and biochemical evaluation. A total of 69 strains were isolated based on colony morphology and identified as Aspergillus oryzae/flavus based on internal transcribed spacer and calmodulin gene sequencing. Interestingly, none were toxigenic based on PCR amplification of intergenic regions of the aflatoxin cluster genes norB-cypA and the absence of aflatoxin in the culture supernatants by thin-layer chromatography analysis. Saccharification capability of the isolates, assessed through α-amylase and glucoamylase activities, revealed that two isolates, TNA24 and TNA15, showed the highest levels of activity. Although the degrees of variation in α-amylase and glucoamylase activities among the isolates were higher, there were only slight differences in acid protease activity among the isolates with two, TNA28 and TNA36, showing the highest activities. Furthermore, statistical analyses showed that α-amylase activity was positively correlated with glucoamylase activity (p < 0.001), and therefore screening for either was sufficient to predict the saccharifying capacity of the Aspergillus strain.

Keywords  Aflatoxin, Amylase, Aspergillus, Nuruk, Protease

The traditional Korean fermentation starter, nuruk, composed of a mixed culture of naturally incorporated microbes, is used extensively in the brewing of alcoholic beverages. The diverse natural microflora associated with nuruk facilitates three major processes of saccharification, fermentation, and ripening associated with the traditional preparation of alcoholic beverages. Fungal species are associated with the saccharification process [1], while yeast communities assist in the fermentation process.

Members of the genus Aspergillus are recognized as important contributors of alcoholic fermentation in Asia. Also known as koji molds in Japan and China, they contribute greatly toward the saccharification and ripening processes of alcoholic fermentation [2, 3]. Aspergilli produce amylolytic as well as proteolytic enzymes, such as α-amylase, acid protease, glutaminase, and metallopeptidase, during alcohol fermentation [4]. These enzymes break down complex carbohydrates and proteins to generate sugars and smaller peptides that are utilized by yeasts for fermentation [4-6].
The *Aspergilli* of section *Flavi*, particularly, *A. oryzae*, are well known for their efficiency in solid-state fermentation as they produce high quantities of secretory hydrolases [2]. However, a few members of this group produce secondary metabolites known as aflatoxins, which are potent carcinogens and are barely distinguishable from other strains because of their morphological and phylogenetic similarities. Due to their importance in the fermentation industry, accurate classification along with appropriate selection of non-toxicogenic and highly saccharifying *Aspergilli* strains is essential for the production of safer and efficient starters.

Molecular approaches have long been used to identify the different species of *Aspergillus*, including sequence analyses of the mitochondrial cytochrome b gene [7], the internal transcribed spacer (ITS) region [8, 9], the calmodulin gene (CF) [10], and the aflatoxin gene cluster [11, 12]. Although each method has provided important information about the phylogenetic relationships among species, none used singly has been able to identify all species of *Aspergillus*.

In this study, we isolated and identified as many strains of *Aspergillus* section *Flavi* as possible from several traditional wheat-based *nuruk* samples fermented under different temperatures and moisture levels. Furthermore, we evaluated their toxigenicities and saccharification capabilities. Our results could be used to enhance the quality of traditional fermentation starters.

**MATERIALS AND METHODS**

**Fungal isolates and culture conditions.** *Aspergillus* isolates from four different types of traditional wheat-based *nuruk* were obtained by dilution plating on Dichloran Rose-Bengal Chloramphenicol agar (DRBC agar; Difco Laboratories, Detroit, MI, USA), based on colony morphology. These isolates were maintained on potato dextrose agar (BD Bioscience, Franklin Lakes, NJ, USA) plates and incubated at 25°C for 3 days. Then fungal spores were resuspended in 5 mL sterile 0.85% (w/v) NaCl containing 0.05% (w/v) Tween 20, counted, and equal numbers were inoculated into different media for aflatoxin and biochemical assays. KACC40247 (*A. oryzae*), KACC41403 (*A. flavus*), and KACC45442 (*A. flavus*) were used as reference strains.

**Molecular characterization.** For molecular characterization, DNA was extracted from all of the isolates according to the protocol described by Bal et al. [13]. The ITS region of the rRNA gene and partial CF gene were sequenced from each isolate for molecular identification. The ITS region (spanning part of the 18S rRNA gene, ITS 1, 5.8S rRNA gene, ITS 2, and part of the 28S rRNA gene) was amplified using the primer pair ITS1-5.8S-ITS2 rDNA region using the following primer pair: forward ITS1 (5'-TCC GTA GGT GAA CCT CCG G-3') and reverse ITS4 (5'-TCC TGC TAT TGA TAT GC-3'). CF was amplified with the following primer pair: forward CF1M (5'-AGG CCG AYT CTY TGA CYG A-3') and reverse CF4 (5'-TTT YTG CAT CAT RAG YTG GAC-3') [14]. To confirm the toxigenic nature of the isolates, a portion of the intergenic region and the 5' coding sequences of norB and cypA was amplified by PCR using the primers AP1729 (5'-GTG CCC AGC ATC TTG GTC CAC C-3') and AP3551 (5'-AAG GAC TTG ATG ATT CCT C-3') [12]. After PCR, the products were purified, sequenced, and used in BLASTn searches (http://www.ncbi.nlm.nih.gov) to confirm preliminary identification.

To determine the taxonomic positions of the isolated strains, sequencing data were analyzed using the maximum likelihood method based on the Kimura 2-parameter model [15]. Then these data were used to construct the phylogenetic tree using MEGA7 [16]. To determine the support for each clade, bootstrap analysis was performed with 1,000 replications.

**Quantification of enzyme activity.**

**α-amylase activity:** To measure α-amylase activity, 10³ spores/mL collected from mature culture plates were inoculated into medium containing 0.5% KH₂PO₄, 0.1% NaNO₃, 0.5% soluble starch, 1% peptone, and 0.1% MgSO₄ · 7H₂O, and incubated at 30°C for 48 hr. The culture filtrate, designated as the crude enzyme extract, was harvested by centrifugation at 13,000 rpm. Then α-amylase activity was estimated by mixing with 1% starch solution and a color reagent consisting of sodium potassium tartrate solution and 96 mM 3,5-dinitrosalicylic acid solution, and boiling for 15 min followed by colorimetric observation at 540 nm [17]. A standard curve was plotted using maltose as a standard, and enzyme activity was calculated based on the amount of maltose released.

**Glucoamylase activity:** Glucoamylase activity was measured according to the procedures described by Yang et al. [18]. Briefly, the amount of glucose produced by fungal enzymes was measured in both samples and blanks using the peroxidase/glucose-oxidase enzymatic method, and net glucose production was calculated by subtracting the glucose concentration in the reaction mixture from that in the blank solution. Glucoamylase activity (units/mL) 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/hr under normal assay conditions.

**Acid protease activity:** Acid protease activity was estimated according to a procedure reported previously, with some modifications [19]. Briefly, 5 mL 0.65% casein solution equilibrated at 37°C was mixed with enzyme solution (0.5 mL) and incubated at 37°C for 10 min, and the reaction was stopped by adding 5 mL 110 mM trichloroacetic acid (TCA). The quantity of TCA-soluble peptides in 1 mL filtrate was measured by incubation with 5 mL 0.4 M sodium carbonate solution and 1 mL Folin-Ciocalteu's phenol reagent (Sigma, St. Louis, MO, USA) at 37°C for 30 min. The absorbance at 660 nm was measured relative to distilled water. A calibration curve was constructed using L-tyrosine.
standard solutions (0.05~0.5 μmole). One unit of acid protease activity was defined as the amount of enzyme required to catalyze the release of 1 μmole.

**Aflatoxin assay.** YES medium (sucrose 60 g/L, yeast extract 20 g/L) was inoculated with 10^6 spores/mL and incubated at 180 rpm, 30°C for 5 days. Then 0.1 mL chloroform was added to 1 mL culture suspension and vigorously shaken. The derived aflatoxin was spotted onto thin-layer chromatography (TLC) plates (Silica gel 60 F254; Merck, Whitehouse Station, NJ, USA) and run in diethyl ether-methanol-water (96 : 3 : 1). The developed plate was dried, and the spots were visualized under UV light (365 nm).

**Statistical analysis.** Statistical analyses of the data were performed using SPSS for Windows ver. 18 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was performed to determine the significance of differences and to discriminate between mean enzymatic activities. To examine the relationships among α-amylase, glucoamylase, and acid protease activities, Pearson’s correlation coefficients were calculated and analyzed using GraphPad Prism ver. 6.00 for Mac.

### RESULTS AND DISCUSSION

Members of *Aspergilli* section *Flavi* are of significant economic importance in fermentation industries. However, the toxigenicity of a few species is a major concern due to their indistinguishable molecular and phenotypic characteristics from non-toxic strains. Recent global demands for Korean alcoholic beverages have elicited the need for more efficient and safer starter cultures. *Aspergilli* strains have been frequently isolated from Korean starter cultures nuruk [13, 20-22]. In this study, we isolated *Aspergillus* strains from traditional wheat-based nuruk fermented under different fermentation conditions, including varying temperature and moisture and evaluated their toxigenicity and efficiency. As DRBC is the best medium to support controlled growth of fungi and yeasts without bacterial contamination [13], we applied serially diluted suspensions of traditional wheat-based nuruk from our previous study [13] to DRBC plates and a total of 69 presumed *Aspergillus* species with floccose and grayish yellow to olive colonies were isolated. Further taxonomical identifications were carried out on a molecular basis involving the ITS and CF gene sequences.

**Molecular characterization.** Both ITS and CF sequences of all of the isolates upon alignment and BLASTn search (http://www.ncbi.nlm.nih.gov) revealed 99% sequence similarity to *A. oryzae/flavus*. However, analysis of both ITS and CF sequences could not differentiate clearly between *A. oryzae* and *A. flavus*. Therefore, all of the isolates obtained were assumed to belong to *Aspergillus* section *Flavi*.

To further investigate the genetic relatedness and taxonomic positions of the isolated strains compared to reference

![Fig. 1. Maximum likelihood phylogenetic tree showing taxonomic positions based on internal transcribed spacer sequences of *Aspergillus* section *Flavi* strains isolated from traditional wheat-based *nuruk*. Control strains *A. oryzae* KACC40247, *A. flavus* KACC41403, and *A. flavus* KACC 45441 were included for comparison. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA7. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.](image-url)
strains, maximum likelihood phylogenetic trees were analyzed using MEGA7 (Figs. 1 and 2). The phylogenetic trees inferred from the ITS sequences showed 11 isolates tightly clustered together with the reference strain *A. oryzae* KACC40247 isolated from *meju* and four isolates was indistinguishable from the reference strain *A. flavus* KACC45441. The phylogenetic trees inferred from the CF sequences revealed that only three isolates were tightly clustered with the reference strain *A. flavus* (KACC41403, KACC45441). However, none of them clustered with any of *A. flavus* reference strains based on ITS sequence. These results suggest that, although there were quite degree of genetic diversity among isolated *A. flavus/oryzae* isolates, *A. oryzae* and *A. flavus* are difficult to differentiate based on their ITS and CF gene sequences. In addition, these results were in a good agreement with the previous studies that β-tubulin and CF gene sequences and phenotypic characteristics were not enough to differentiate *A. oryzae* and *A. flavus* [23].

**Characterization through evaluation of toxigenicity.**

As reported previously, due to the close relationship between *A. oryzae* and *A. flavus* both morphologically and phylogenetically, analysis of aflatoxin production capability is the only means differentiating between them [23]. *A. oryzae* does not produce aflatoxin and therefore is considered a domesticated form of *A. flavus* [24]. To evaluate aflatoxin production capability, portions of the intergenic region and the 5’ coding sequences of *norB* and *cypA* were amplified and analyzed [12]. Amplification analysis of the *norB-cypA* region indicated three band patterns: 0.8 kb, 0.3 kb, and no bands (Fig. 3). The reference strain KACC40247 (*A. oryzae*) showed a band of 0.3 kb, whereas KACC41403 (*A. flavus*) and KACC45442 (*A. flavus*) each showed a 0.8 kb band on PCR amplification of the *norB-cypA* region, which resembled the deletion pattern in the *norB-cypA* region responsible for aflatoxin production. Of the 69 isolates, 32 showed the 0.3-kb band, whereas the remaining 37 showed no bands

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**Fig. 2.** Maximum likelihood phylogenetic tree showing taxonomic positions based on calmodulin sequences of *Aspergillus* section *Flavi* strains isolated from traditional wheat-based *nuruk*. Control strains *A. oryzae* KACC40247, *A. flavus* KACC41403, and *A. flavus* KACC 45441 were included for comparison. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA7. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

**Fig. 3.** PCR amplification of the *norB-cypA* region of *Aspergillus* section *Flavi* strains isolated from traditional wheat-based *nuruk*. No PCR amplicons were loaded in blank lanes.
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on PCR amplification of the norB-cypA region. The 0.3-kb band of the 32 isolates resembled that of the reference A. oryzae strain. Therefore, these 32 isolates were considered safe, as they likely cannot produce aflatoxin.

The remaining 37 isolates, in which the norB-cypA region could not be amplified, were further examined for aflatoxin production capability through conventional aflatoxin production tests in culture medium using TLC (Fig. 4). Chloroform-extracted culture filtrate was spotted onto TLC plates and run along with aflatoxin B1 isolated from A. flavus. Aflatoxin was not detected in any of the 37 extracts under UV light, thus confirming the non-toxicogenicity of the Aspargilli isolates in this study. Furthermore, with the absence of the ability to produce aflatoxin, all of the isolates could be classified as A. oryzae. Previously, the non-aflatoxigenic meju strains were designated as A. oryzae, and aflatoxigenic strains were designated as A. flavus [25].

Evaluation of saccharifying capability. The final step in our polyphasic approach to identify and characterize Aspargilli strains was extracellular enzyme activity screening. All 69 isolates along with reference strains were analyzed for α-amylase, glucoamylase, and acid protease activities in culture medium. Ten isolates had relatively high α-amylase activity compared to the control strain, KACC40247; the isolates TNA24 and TNA15 showed highest α-amylase activities of 2.13 ± 0.12 units/mL and 2.08 ± 0.15 units/mL, respectively (Fig. 5). The activity of the control A. oryzae strain was 1.12 ± 0.12 units/mL. In the case of glucoamylase, 45 isolates had higher activity than the control strain; isolates which TNA15 and TNA24 showed the highest activities of 0.43 ± 0.04 units/mL and 0.25 ± 0.01 units/mL (Fig. 6), while that of the control A. oryzae strain was 0.12 ± 0.03 units/mL. Ten isolates showing higher α-amylase and glucoamylase activities were further analyzed statistically to check for the significance of differences compared to the control strains. Among the tested 10 isolates, the enzyme activities of the three isolates TNA24, TNA15, and TNA59 were significantly higher than that of the control A. oryzae strain. These results suggest the possibility of obtaining a better strain for nuruk fermentation. Compared to α-amylase and glucoamylase, the acid protease activities

Fig. 4. Thin-layer chromatography analyses of the culture broth extracts of Aspergillus section Flavi isolates for detection of aflatoxin. A. oryzae KACC40247 was included as a non-aflatoxigenic control, whereas A. flavus KACC41403 and A. flavus KACC45441 were included as aflatoxigenic-positive controls. Aflatoxin B1 isolated from A. flavus (Enzo Life Sciences, Farmingdale, NY, USA) was used as a positive control.

Fig. 5. The α-amylase activities of culture supernatants of Aspergillus section Flavi isolates. The strains TNA24, TNA15, and TNA59 showed significantly higher α-amylase activity than controls. Significant differences in data sets as calculated by ANOVA are represented by **p < 0.01.
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varied less among all of the isolates, with the results being consistent with a previous study of protease activities of Aspergillus isolates from nuruk [20]. About 14 isolates had slightly higher activity than the control strains, with the isolates TNA28 and TNA36 showing activities of 0.76 ± 0.002 units/mL and 0.73 ± 0.09 units/mL, respectively (Fig. 7).

Relationships among different enzymatic activities (α-amylase, glucoamylase, and acid protease) were assessed by Pearson’s correlation analysis (p < 0.001). The correlation coefficients of α-amylase/glucoamylase, α-amylase/acid protease, and glucoamylase/acid protease were 0.663, 0.178, and 0.325, respectively. These results confirm the significant (p < 0.001) moderately positive correlation between the α-amylase and glucoamylase activities in Aspergillus section Flavi isolates from nuruk. α-Amylase/glucoamylase activities showed low correlation with acid protease activity.

In conclusion, we performed molecular characterization and analyzed the toxigenicity and saccharifying ability of Aspergillus section Flavi strains isolated from traditional wheat nuruk. All of the strains were non-toxigenic, with a few showing significantly higher saccharification capabilities than the control strains. These data will facilitate the selection and induction of highly efficient Aspergillus strains for more efficient and safer nuruk preparation.

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Fig. 6. The glucoamylase activities of culture supernatants of Aspergillus section Flavi isolates. The strains TNA15, TNA24, and TNA59 showed significantly higher α-amylase activity than controls. Significant differences in data sets as calculated by ANOVA are represented by **p < 0.01.

Fig. 7. The acid protease activities of culture supernatants of Aspergillus section Flavi isolates.
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