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Safety Evaluation of Filamentous Fungi Isolated from Industrial *Doenjang Koji*

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A few starters have been developed and used for *doenjang* fermentation but often without safety evaluation. Filamentous fungi were isolated from industrial *doenjang koji*, and their potential for mycotoxin production was evaluated. Two fungi were isolated; one was more dominantly present (90%). Both greenish (SNU-G) and whitish (SNU-W) fungi showed 97% and 95% internal transcribed spacer sequence identities to *Aspergillus oryzae/flavus*, respectively. However, the *Sma*I digestion pattern of their genomic DNA suggested that both belong to *A. oryzae*. Moreover, both fungi had morphological characteristics similar to that of *A. oryzae*. SNU-G and SNU-W did not form sclerotia, which is a typical characteristic of *A. oryzae*. Therefore, both fungi were identified to be *A. oryzae*. In aflatoxin gene cluster analysis, both fungi had *norB-cypA* genes similar to that of *A. oryzae*. Consistent with this, aflatoxins were not detected in SNU-G and SNU-W using ammonia vapor, TLC, and HPLC analyses. Both fungi seemed to have a whole cyclopiazonic acid (CPA) gene cluster based on PCR of the *maoA*, *dmaT*, and *pks-nrps* genes, which are key genes for CPA biosynthesis. However, CPA was not detected in TLC and HPLC analyses. Therefore, both fungi seem to be safe to use as *doenjang koji* starters and may be suitable fungal candidates for further development of starters for traditional *doenjang* fermentation.

Keywords: *Aspergillus oryzae*, mycotoxin, aflatoxin, cyclopiazonic acid, *doenjang koji*

Introduction

Doenjang, a Korean traditional fermented soybean paste, is made using *meju*, in which fermentation is mainly dependent on natural inoculation of various microorganisms such as bacteria, yeasts, and molds [17, 21, 24]. In *meju*, *Aspergillus* spp. have been the primary molds used for soybean fermentation. Supporting this, *A. oryzae* was dominantly found in various types of *meju*, regardless of regional origins and fermentation time [14].

Koji using a starter has been developing for industrial production of *doenjang* because it is easier for mass production with uniform quality [27]. *A. oryzae* has been included in most starters, and other molds are also additionally used to take advantage of their beneficial characteristics. For example, *Aspergillus sojae* has been used because of its high activities of protease and amylase [16], and *Aspergillus usami* and *Rhizopus* sp. are included to speed up the maturation process [1]. Most molds used as starters for *doenjang* fermentation are based on isolates that originate from *meju*.

Because of the long history of *doenjang* consumption, molds involved in fermentation get little attention with regard to safety issues. However, the World Health Organization (WHO) and United Nations Food and Agriculture Organization (UN FAO) recommend continuous monitoring of mycotoxins because they could cause various diseases [13, 25, 28]. According to some reports, the aflatoxigenic *Aspergillus flavus* was isolated from Korean traditional fermentation products [20]. The aflatoxigenic *A. flavus* comprised 2.2% of the molds isolated from home-made *meju* [12].

In many cases, *A. oryzae*-like molds are used without thorough evaluation of safety after simple identification,

and sometimes even without identification. However, because fungi belonging to *Aspergillus* section *Flavi* are so phylogenetically close that they may sometimes be misidentified, it is possible that molds producing mycotoxins may accidentally be used.

A. oryzae is generally regarded as safe (GRAS); however, *A. flavus*, which is very closely related to *A. oryzae*, is a producer of aflatoxins (AFs). AFs are known to be the strongest naturally found carcinogens. There are four main types of AFs-AFB_1: AFB_2, AFG_1, and AFG_2. AFB_1 is more prevalent than other AFs in contaminated food grains and has been considered to be the most mutagenic and carcinogenic [6]. Even the identification of a strain as *A. oryzae* does not guarantee its inability to produce AFs or other toxic metabolites. Some *A. oryzae* produce cyclopiazonic acid (CPA), which is an indole-tetramic acid mycotoxin that could be a health threat to humans [26]. Although CPA has not been considered to be a serious mycotoxin and its contamination has not been regulated for food safety, there have been reports on the toxicity of CPA. CPA co-contamination with AF is considered to be a cause of "X-disease," which has killed ducks [32]. CPA also causes "kodua poisoning," which is accompanied by symptoms of dizziness and vomiting [29]. Therefore, the toxigenic potential must be specifically determined for individual strains of *A. oryzae*.

Although *A. oryzae* has mainly been used for a starter in *doenjang koji*, thorough evaluation of its mycotoxin production is required, because it may produce CPA and the strain used may not be *A. oryzae* because of its very close relationship to aflatoxigenic *A. flavus*. In this study, we isolated and identified molds from industrial *doenjang koji* and evaluated their AF and CPA production.

Materials and Methods

Chemicals and Media

AF mix (B_1, B_2, G_1 and G_2), CPA, and Ehrlich solution were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). All HPLC-grade solvents were from Samchun Pure Chemical Co. (Pyeongtaek, Korea). Potato dextrose agar (PDA), malt extract agar (MEA), dichloran rose bengal chloroamphenicol agar (DRBC), and dichloran 18% glycerol agar (DG18) were from Acumedia (Baltimore, MD, USA). *Doenjang koji* used for manufacturing SNU *doenjang* was provided by Prof. S. H. Choi (Seoul National University, Seoul, Korea).

Fungal Strains and Culture Conditions

A. flavus NRRL 3357 (AF+) and *A. oryzae* SRRC 266 (AF-) obtained from Prof. Nancy P. Keller (University of Wisconsin, Madison, WI, USA) were used as reference strains to identify the unknown strains and for AF production analysis. As for reference strains for CPA production analysis, *A. oryzae* NBRC 4177 (CPA+) and *A. oryzae* RIB 40 (CPA-) obtained from KACC (Korean Agricultural Culture Collection, Suwon, Korea) were used.

Spores of strains were harvested using a 0.1% Tween 80 buffer after culturing the strains on PDA plates for 3 days at 30°C.

Isolation of Filamentous Fungi from *Doenjang Koji*

To isolate residing filamentous fungi from *doenjang koji*, a sample was diluted from 10^{-1} to 10^{-7} using 0.1% peptone in water. Diluted samples were inoculated onto PDA, MEA, DRBC, and DG18 and incubated at 30°C for 5 days. Colonies with different morphological characteristics were pure-cultured and further analyzed.

Morphological Examination

The isolates and two reference strains, *A. flavus* NRRL 3357 and *A. oryzae* SRRC 266, were grown for 5 days as 1-point inoculations (1.0×10^4 spores in 10 µl) on PDA. The morphology and color of colonies were noted. For conidial head and mycelia observation, spores were inoculated onto a PDA agar block (1 cm^2) on a glass slide, cultured for 5 days, and then observed using a stereoscope (Olympus, Tokyo, Japan). The size of the conidia and the shape of the mycelia were observed microscopically. For sclerotia formation, spores (1.0×10^4 spores in 10 µl) were 1-point inoculated onto Czapek Dox medium (sucrose 3%, $NaNO_3$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, K_3HPO_4 0.1%, agar 1.5%, pH 6.2) and cultured at 30°C for 15 days.

Analysis of the ITS Region, and of the AF and CPA Gene Clusters

Fungal strains were cultured in PDB at 30°C and genomic DNA was isolated as previously described [31]. Fragments containing the internal transcribed spacer (ITS) region were amplified using the primers ITS1 and ITS4 [35]. The amplification program consisted of pre-denaturation at 94°C for 2 min; 30 cycles at 94°C for 30 sec, 48°C for 30 sec, 72°C for 30 sec; and a final incubation at 72°C for 5 min to complete the final extension. PCR was performed using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Sequence analysis was performed at Macrogen Inc. (Seoul, Korea). To identify the isolates, the sequences were analyzed using the NCBI BLAST program (National Center for Biotechnology Information, Bethesda, MD, USA).

The restriction enzyme fragment polymorphism analysis of genomic DNA was performed to differentiate *A. oryzae* and *A. flavus* [19]. About 5 µg of genomic DNA was digested using *Sma*I and analyzed by agarose gel electrophoresis, according to standard protocols [30].

The five AF biosynthetic genes (*norB-cypA*, *aflR*, *omtA*, *nor-1*, and *ver-1*) and three CPA biosynthetic genes (*moaA*, *dmaT*, and *pks-nrps*) were analyzed by PCR using the primers listed in Table 1. The β-tubulin gene was used as a template control. The same PCR conditions applied for the ITS region were used, except

Table 1. PCR primers used in this study.

Primer	Sequence (5′ → 3′)	Amplicon (bp)	Target gene	Reference
norB-cypA F	GTG CCC AGC ATC TTG GTC CA	400(I)/800(II)	*norB-cypA*	[3]
norB-cypA R	AGG ACT TGA TGA TTC CTC GTC			
aflR F	TAT CTC CCC CCG GGC ATC TCC CGG	1,032	*aflR*	[15]
aflR R	CCG TCA GAC AGC CAC TGG ACA CGG			
omtA F	GTG GAC GGA CCT AGT CCG ACA TCA C	797	*omtA*	[15]
omtA R	GTC GGC GCC ACG CAC TGG GTT GGG G			
nor-1 F	ACC GCT ACG CCG GCA CTC TCG GCA C	400	*nor-1*	[5]
nor-1 R	GTT GGC CGC CAG CTT CGA CAC TCC G			
ver-1 F	GCC GCA GGC CGC GGA GAA AGT GGT	537	*ver-1*	[5]
ver-1 R	GGG GAT ATA CTC CCG CGA CAC AGC C			
dmaT F	GTC TCT GGA TCG TTC CGT CG	765	*dmaT*	[4]
dmaT R	GTA TAG CAC AGC TCC GAT GT			
pks-nrps F	GAA AGG CCT TGC CAG CGA TAC T	986	*pks-nrps*	This study
pks-nrps R	GAA TGC AAG GAG CCT CTC GT			
maoA F	GAC CGT CGC TGT CGT TGA AC	1,205	*maoA*	This study
maoA R	AGG AAG AAG CCA TCT GAT CG			
β-tubulin F	CCA AGA ACA TGA TGG CTG CT	277	β-tubulin	[34]
β-tubulin R	CTT GAA GAG CTC CTG GAT GG			

for the time for extension (30 sec for AF genes and 1 min 30 sec for CPA genes) and the annealing temperature (55°C for *norB-cypA*, 68°C for *aflR*, 69°C for *omtA*, 69°C for *nor-1*, 68°C for *ver-1*, 55°C for *moaA*, 56°C for *dmaT*, 56°C for *pks-nrps*, and 55°C for β-tubulin).

Analysis of AF Production

For the rapid evaluation for AFB_1 production of the isolates, the ammonium vapor method (AVM) was carried out as previously described [15]. Ten microliters of spores (1×10^4 spores) was inoculated at the center of the PDA and yeast extract sucrose agar medium (YES; 2% yeast extract, 15% sucrose, 1.5% agar) at 30°C for 3 days. The cultured strains were exposed to a drop of 25% ammonium hydroxide in the lid of an inverted petri dish. In each colony, color change to red was noted.

For the evaluation of AF production by TLC and HPLC, 0.5 ml of spores (1.0×10^7/ml) was inoculated in 50 ml of PDB and incubated at 30°C with shaking at 200 rpm for 5 days. The cultures were filtered with Whatman No. 1 filter paper (Whatman PLC, Buckinghamshire, UK). The extraction and TLC analysis of AF were performed as described previously [15]. The filtrate was mixed with an equal volume of chloroform and vortexed for 20 min. The chloroform phase was separated from the aqua phase by centrifugation at 1,750 ×*g* for 3 min. Chloroform in the AF extract was removed using a rotary evaporator (EYELA, Tokyo, Japan) in a 40°C water bath, and the samples were redissolved in 1 ml of chloroform. Five microliters of each sample and AF standard (B_1, B_2, G_1, and G_2; Sigma, St. Louis, MO, USA) was spotted onto a Silica 60 F254 plate (Merck, Darmstadt, Germany) and the plate was developed using acetone:chloroform (15:85). The developed plate was dried, and the spots were visualized under UV light (365 nm).

The AF extracts were filtered using a 0.22 PTFE membrane filter (Target, National Scientific Co., Rockwood, TN, USA), and 20 µl was injected into the HPLC system (Agilent Technologies, Wilmington, DE, USA) equipped with a Hypersil Gold column (C18, 4.6 mm × 150 mm, 5 µm; Thermo, San Jose, CA, USA). The mobile phase was acetonitrile:methanol:water (10:40:50), pumped at a constant flow rate of 0.5 ml/min for 30 min.

Analysis of CPA Production

For the TLC and HPLC analyses of CPA production, 0.5 ml of spores (1.0×10^7/ml) was inoculated in 50 ml of CAM media (15% sucrose, 2% yeast extract, 1% peptone, pH 6.0 [26]) and incubated at 30°C with shaking at 200 rpm for 5 days. The culture supernatant prepared by the same method for AF extraction was mixed with an equal amount of chloroform. The CPA extraction and sample preparation for TLC and HPLC were the same as that for AF analysis. A sample was reconstituted in 1 ml of methanol and 10 µl was spotted onto a Silica 60 F254 plate (Merck, Germany) and developed using ethyl acetate/methanol/ammonium hydroxide (85:15:10). The developed plate was dried, and a CPA spot was visualized under UV light (254 nm) after staining with Ehrlich's solution [4].

The extraction samples were filtered using a 0.22 PTFE membrane filter (Target, National Scientific Co., Rockwood, TN, USA) and 20 µl was injected into the HPLC system equipped with

a Hypersil Gold column (C18, 4.6 mm × 150 mm, 5 µm; Thermo, USA). The mobile phase was acetonitrile: 0.1% trifluoroacetic acid water (50:50 (v/v)), pumped at a constant flow rate of 1 ml/min. CPA was detected using a UV detector (254 nm).

Results and Discussion

The safety of a starter for industrial *doenjang* fermentation is a very important issue. Many starters have been developed based on molds isolated from soybean fermentation products without safety evaluation. Although *A. oryzae* is the mold most commonly used for a starter and regarded as safe, care should be taken as it is hard to distinguish from *A. flavus*, a well-known carcinogenic AF producer. In addition, some *A. oryzae* are known to produce an indole-tetramic acid mycotoxin, CPA.

Isolation of Fungi from Industrial *Doenjang Koji*

SNU *doenjang* has a good reputation because of its high quality. To evaluate fungal flora involved in SNU *doenjang* fermentation, fungi were isolated from SNU *doenjang koji* by a plating method using PDA, MEA, DRBC, and DG18 media. DRBC and DG18 media were used to ensure that no slow-growing and/or xerophilic fungi were missed. The dilution of a *doenjang koji* sample at 10^{-6} yielded about 100 colonies after a 3-day culture in all the media used. Only two types of fungi appeared: one formed greenish-colored colonies (SNU-G) whereas the other formed white colonies (SNU-W) (Fig. 1A). About 90% of the colonies were SNU-G and about 10% of them were SNU-W.

Identification of SNU-G and SNU-W

To identify the two isolates, their morphological characteristics were examined and compared with known *A. oryzae* SRRC 266 and *A. flavus* NRRL 3357. SNU-G formed floccose colonies (similar to *A. oryzae* SRRC), which is the typical colony characteristic of *A. oryzae* [7, 11]. Supporting the floccose colonies were obvious aerial hyphae in SNU-G (Fig. 1B). The colony color of SNU-G was yellow-green, while that of SNU-W was white during a 2–3-day incubation, changing gradually to an olive color (Fig. 1A). The spore size of SNU-G and SNU-W was 1.5 times bigger (3–5 µm) than that of *A. flavus* (2–3 µm) (Fig. 1D), and the spore size is known to be bigger in *A. oryzae* than in *A. flavus* [7]. SNU-G and SNU-W did not form sclerotia, which are very common in *A. flavus* but rare in *A. oryzae* (Fig. 1E). Altogether, SNU-G and SNU-W had more similar morphological characteristics to *A. oryzae* than to *A. flavus*.

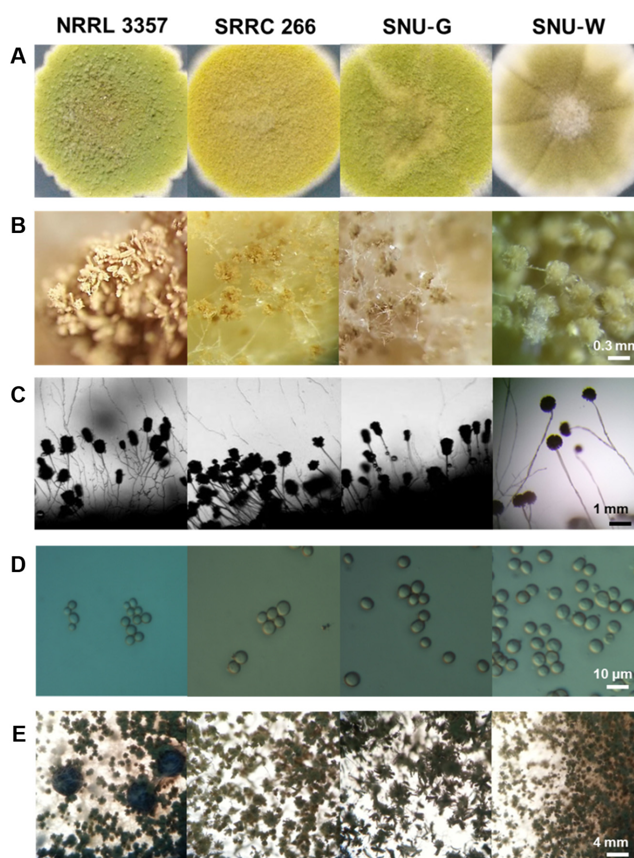

Fig. 1. Morphological characteristics of *A. flavus* NRRL 3357, *A. oryzae* SRRC 266, SNU-G, and SNU-W.
(**A**) Colony morphology in PDA medium. (**B**) Aerial hyphae and conidia head examined using a stereoscopic microscope. (**C**) Conidia head on slide culture. (**D**) Conidia size: 2–3 µm diameter for NRRL 3357; 3–5 µm for SRRC 266; 3–5 µm for SNU-G; and 3–5 µm for SNU-W. (**E**) Sclerotia formation. NRRL 3357 only formed sclerotia in PDA medium.

The identification of *Aspergillus* at the genus level is carried out using the ITS regions [9]. The ITS regions of SNU-G and SNU-W were amplified by PCR using ITS1F and ITS4R primers, both of which yielded 578 bp fragments. Sequence analysis revealed that SNU-G and SNU-W showed 97% and 95% sequence similarity to *A. oryzae/flavus*, respectively. However, analysis of the ITS sequence did not differentiate whether SNU-G and SNU-W were *A. flavus* or *A. oryzae*. The method to distinguish *A. oryzae* from *A. flavus* has been developed depending on the *Sma*I digestion pattern of genomic DNA [8, 19]. The 3.8 kbp fragments were only observed in *A. flavus* NRRL 3357, whereas both 2.7 kbp and 1.0 kbp fragments were in SNU-G, SNU-W, and *A. oryzae* SRRC 266, as expected for *A. oryzae* (Fig. 2). Therefore, both SNU-W and SNU-W were identified as

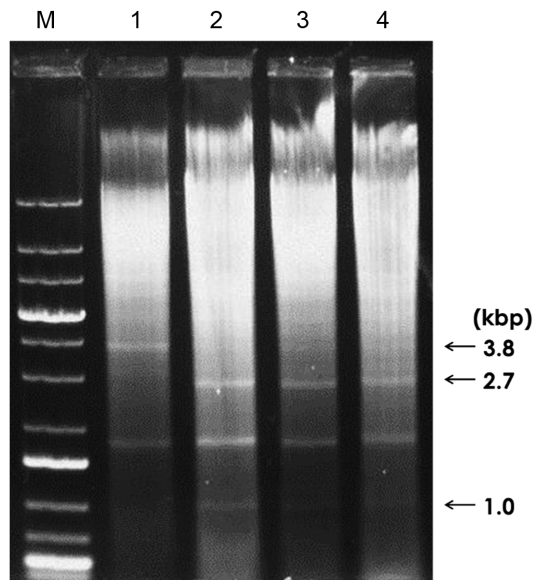

Fig. 2. *Sma*I digestion pattern of genomic DNA.
Lanes: M, molecular size markers (1 kbp ladder); 1, NRRL 3357 (3.8 kbp band pattern); 2, SRRC 266 (2.7 kbp and 1 kbp band patterns); 3, SNU-G; 4, SNU-W.

A. oryzae based on the morphological characteristics and the *Sma*I digestion pattern of genomic DNA. Supporting this identification, SNU-G and SNU-W had the similar AF gene cluster to *A. oryzae* and did not produce AF (see the following section). Therefore, SNU-G and SNU-W were designated as *A. oryzae* SNU-G and *A. oryzae* SNU-W, respectively.

Evaluation of AF Production in *A. oryzae* SNU-G and *A. oryzae* SNU-W

Although both SNU-G and SNU-W were identified to be *A. oryzae*, their aflatoxigenicity was evaluated to ensure that they are non-AF producers. Presently, various methods are used to distinguish aflatoxingenic from non-aflatoxigenic strains. The most common methods are PCR of the AF biosynthetic genes and AF detection by TLC and HPLC [10, 15, 22].

A. oryzae is very closely related to *A. flavus*, a notorious AF producer in various contaminated foods. Most *A. oryzae* strains possess the AF biosynthesis gene cluster as in *A. flavus*, but do not produce AF because of deletions or mutations in the gene cluster [10, 18]. The AF gene cluster is composed of 25 genes, among which the deletion or mutation has been reported in the *norB-cypA*, *aflR*, *omtA*, *nor-1*, and *ver-1* genes. When evaluated by PCR, the expected sizes of fragments from the *aflR*, *omtA*, *nor-1*, and *ver-1* genes were amplified in *A. flavus* NRRL 3557, *A. oryzae* SRRC 266, SNU-G, and SNU-W. However, for the *norB-cypA* gene, the type I-deleted form of the *norB-cypA* gene (400 bp) was detected in both SNU-G and SNU-W as well as in *A. oryzae* SRRC 266; however, it was not detected in *A. flavus* NRRL 3557, in which the type II-deleted form (800 bp) was amplified [2] (Fig. 3B). Supporting this analysis, AF was not detected by AVM, TLC, and HPLC (Fig. 4). AVM is a quick test for AFB_1 production that is based on versicolorin, a precursor of AFB_1, which can turn to red under alkaline conditions [17].

Evaluation of CPA Production in *A. oryzae* SNU-G and *A. oryzae* SNU-W

Some *A. oryzae* strains are known to produce CPA. Although CPA has not been considered as serious as AF, its toxicity is clearly documented. For example, "kodua poisoning," which is accompanied by symptoms of dizziness and vomiting, is caused by CPA [29]. To evaluate CPA production of *A. oryzae* SNU-G and SNU-W, the CPA biosynthetic gene cluster was analyzed. The CPA biosynthetic gene cluster is located next to the AF gene cluster [4]. At present, three genes (*maoA*, *dmaT*, and *pks-nrps*) are known to be important for CPA biosynthesis. Some *A. oryzae*, such as *A. oryzae* RIB 40, do not produce CPA because of deletion in *pks-nrps* [4, 23, 32, 33]. In the PCR analysis of the *maoA*, *dmaT*, and *pks-nrps* genes, *A. oryzae* SNU-G and SNU-W showed the amplification pattern of *A. oryzae* NBRC 4177, which is a CPA producer (Fig. 3C). However, CPA was not detected in the culture extracts of *A. oryzae* SNU-G and SNU-W as in *A. oryzae* RIB 40, which is a non-CPA producer (Fig. 5). The reasons why *A. oryzae* SNU-G and SNU-W do not produce CPA despite the presence of all three CPA biosynthetic genes remain unclear. One possibility is that we may not detect CPA because production levels are too low to detect at our conditions. Another possibility involves some deletions or mutations in the regulatory elements for CPA biosynthesis. Currently, there is no report on the regulatory genes in CPA production.

Because of a long history of consumption, *doenjang* production often excludes the issue of food safety. However, mycotoxins have been detected, suggesting that its safety should not be taken for granted. In particular, starters that are increasingly used in the industrial production of fermented foods should be evaluated for safety, because the misuse of starters could cause serious problems for consumer health. In this study, we evaluated the mycotoxin production potential of starters isolated from industrial *doenjang koji* that have previously been used without proper species identification. Such evaluation would guarantee

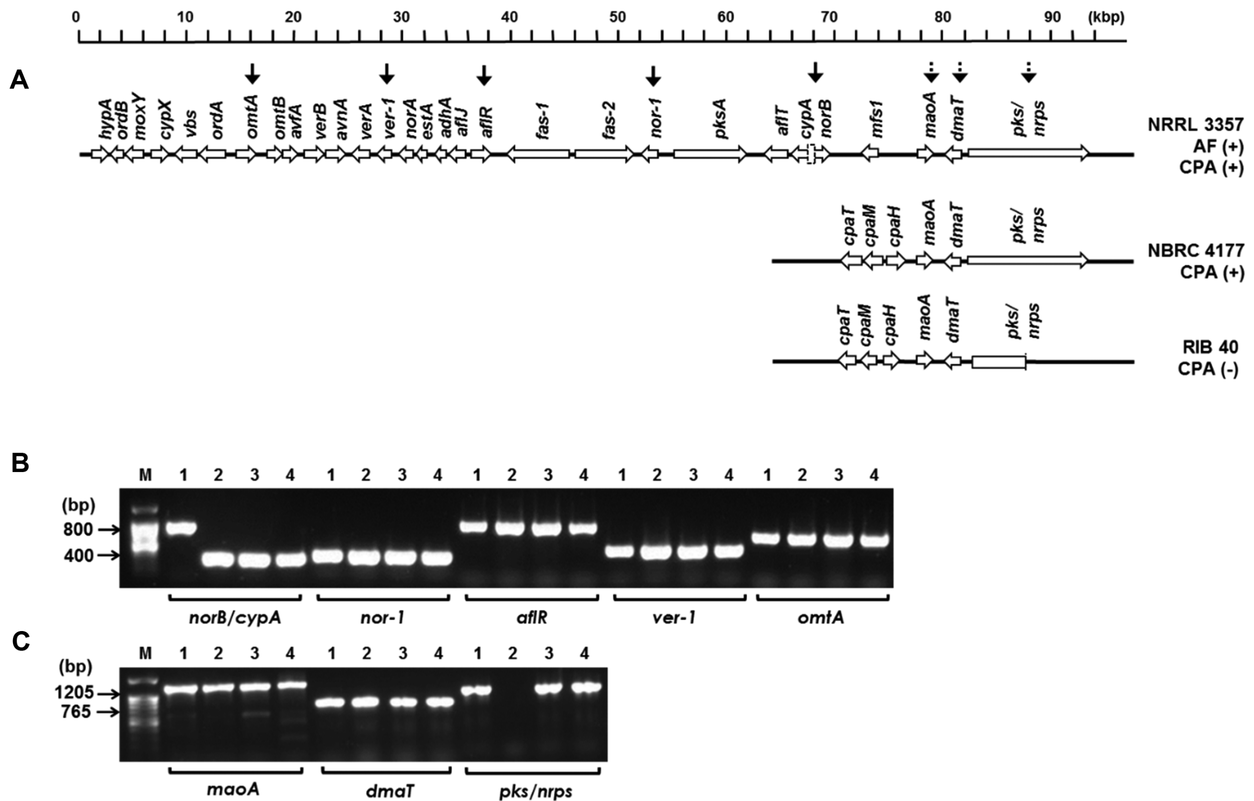

Fig. 3. PCR analysis of the AF and CPA gene clusters.

(**A**) AF and CPA gene clusters in *A. flavus* NRRL 3357, *A. oryzae* NBRC 4177, and *A. oryzae* RIB 40. Solid and dotted arrows indicate the target AF and CPA genes for PCR amplification, respectively. (**B**) PCR amplification of AF genes. 1, NRRL 3357; 2, SRRC 266; 3, SNU-G; 4, SNU-W. Amplicon sizes were 400 bp (I) and 800 bp (II) for *norB-cypA*, 400 bp for *nor-1*, 1,032 bp for *aflR*, 537 bp for *ver-1*, and 797 bp for *omtA*. (**C**) PCR amplification of the CPA genes. 1, NBRC 4177; 2, RIB 40; 3, SNU-G; 4, SNU-W. Amplicon sizes were 1,205 bp for *maoA*, 765 bp for *dmaT*, and 986 bp for *pks-nrps*.

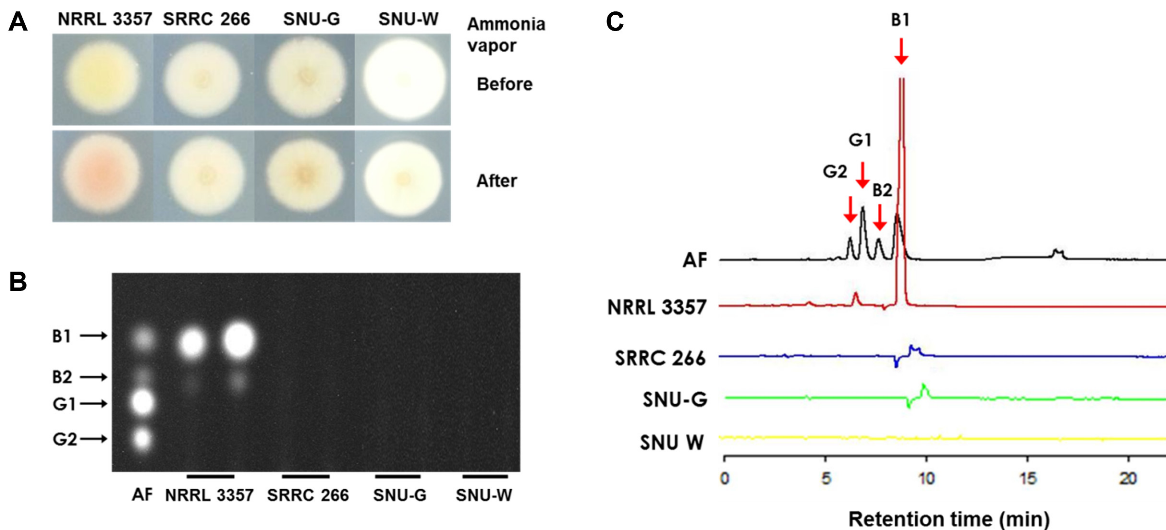

Fig. 4. Evaluation of AF production.

(**A**) AVM. (**B**) TLC. (**C**) HPLC. AF: AF standard; B1: AFB_1; B2: AFB_2; G1: AFG_1; G2: AFG_2; NRRL 3357: *A. flavus* NRRL 3357; SRRC 266: *A. oryzae* SRRC 266; SNU-G; and SNU-W. AFB_1 was only detected in *A. flavus*, with a retention time of 8.3 min.

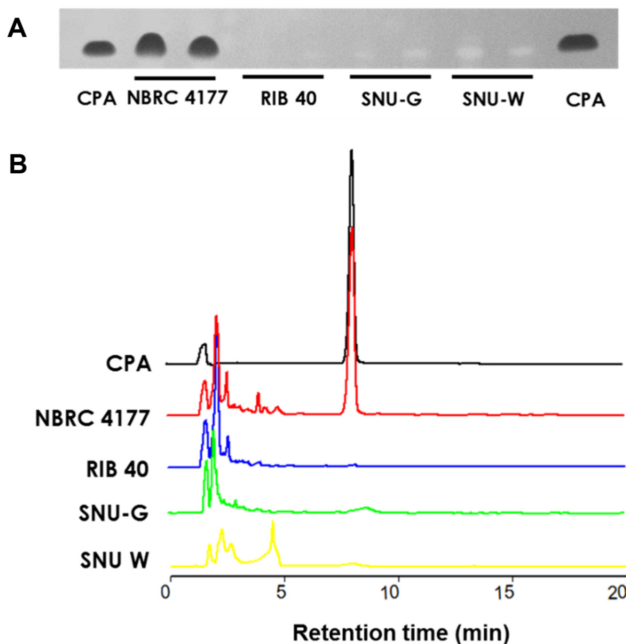

Fig. 5. Evaluation of CPA production.
(**A**) TLC. (**B**) HPLC. CPA: CPA standard; NBRC 4177: *A. oryzae* NBRC 4177; RIB 40: *A. oryzae* RIB 40; SNU-G; and SNU-W.

the safety of products manufactured using these starters. In addition, the two strains identified in this study can safely be used as fungal candidates for the development of other starters for soybean fermentation.

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