J. Microbiol. Biotechnol. (2017), 27(2), 251–261
https://doi.org/10.4014/jmb.1610.10013

jmb

Characterization of *Aspergillus sojae* Isolated from Meju, Korean Traditional Fermented Soybean Brick

Kyung Min Kim[1], Jaeho Lim[1], Jae Jung Lee[2], Byung-Serk Hurh[2], and Inhyung Lee[1]*

[1]*Department of Bio and Fermentation Convergence Technology, BK21 PLUS Project, Kookmin University, Seoul 02707, Republic of Korea*
[2]*Sempio Fermentation Research Center, Sempio Foods Company, Chungcheongbukdo 28156, Republic of Korea*

Received: October 7, 2016
Revised: November 17, 2016
Accepted: November 21, 2016

First published online
November 23, 2016

**Corresponding author*
Phone: +82-2-910-4771;
Fax: +82-2-910-5739;
E-mail: leei@kookmin.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Initially, we screened 18 *Aspergillus sojae*-like strains from *Aspergillus* spp. isolated from meju (Korean traditional fermented soybean brick) according to their morphological characteristics. Because members of *Aspergillus* section *Flavi* are often incorrectly identified because of their phylogenetic similarity, we re-identified these strains at the morphological and molecular genetic levels. Fourteen strains were finally identified as *A. sojae*. The isolates produced protease and α-amylase with ranges of 2.66–10.64 and 21.53–106.73 unit/g-initial dry substrate (U/g-IDS), respectively, which were equivalent to those of the koji (starter mold) strains employed to produce Japanese soy sauce. Among the isolates and Japanese koji strains, strains SMF 127 and SMF 131 had the highest leucine aminopeptidase (LAP) activities at 6.00 and 6.06 U/g-IDS, respectively. LAP plays an important role in flavor development because of the production of low-molecular-weight peptides that affect the taste and decrease bitterness. SMF 127 and SMF 131 appeared to be non-aflatoxigenic because of a termination point mutation in *aflR* and the lack of the polyketide synthase gene found in other *A. sojae* strains. In addition, SMF 127 and SMF 131 were not cyclopiazonic acid (CPA) producers because of the deletion of *maoA*, *dmaT*, and *pks/nrps*, which are involved in CPA biosynthesis. Therefore, *A. sojae* strains such as SMF 127 and SMF 131, which have high protease and LAP activities and are free of safety issues, can be considered good starters for soybean fermentations, such as in the production of the Korean fermented soybean products meju, doenjang, and ganjang.

Keywords: *Aspergillus sojae*, starter mold, soybean fermentation, protease, leucine aminopeptidase

Introduction

Aspergillus sojae and *Aspergillus oryzae* are used as starter molds (koji) to produce soybean fermentation products in Asia. *A. sojae* has advantages compared with *A. oryzae* as a starter for soybean fermentation because its proteolytic activity is higher than that of *A. oryzae*, which potentially facilitates the more effective breakdown of soybean proteins [1]. In Japan, *A. sojae* is used mainly for soybean fermentation in shoyu (Japanese soy sauce) production because of its high proteolytic activity [2], whereas *A. oryzae* is used widely in sake (Japanese rice wine), miso (Japanese traditional fermented soybean), and soy sauce production because of its high amylolytic activity. *A. sojae* also has advantages in terms of the flavor development in soybean fermentation products because of its high leucine aminopeptidase (LAP; E.C. 3.4.11.1) activity [3]. LAP plays an important role in the production of low-molecular-weight peptides during soybean fermentation, which can improve flavor development [4]. LAP also decreases the bitterness of soybean fermentation products by hydrolyzing hydrophobic amino acid residues, such as leucine, from the amino-terminals of peptides.

Despite of the advantages of *A. sojae* for soybean fermentation, *A. sojae* has seldom been applied in the industrial production of soybean fermentation products such as meju (Korean traditional fermented soybean brick), doenjang (Korean soybean paste), and ganjang (Korean soy

sauce) in Korea; instead, *A. oryzae* has mainly been employed. Because *A. oryzae* is common in meju [5] and it is generally recognized as safe (GRAS), it is a reasonable choice for industrial soybean fermentation. In many cases, a dominant strain from the natural fermentation products is used as a starter for related industrial product fermentation. However, *A. sojae*, which is also GRAS [6–11], has great potential as a starter for Korean soybean fermentation if *A. sojae* strains with high protease and LAP activities can be isolated from traditional soybean fermentation products. There have been a few previous reports of the isolation of *A. sojae* from nature [7, 12–14]. In general, *A. sojae* is considered a domesticated strain of *Aspergillus parasiticus* on the basis of its morphological similarity. It has been suggested that *A. sojae* may have been selected for food fermentation from genetically variable *A. parasiticus* [12]. In this study, we isolated and identified several *A. sojae* strains from our mold culture collections isolated from meju products and evaluated their enzyme production properties.

Materials and Methods

Fungal Strains

All of the *Aspergillus* strains used in this study are listed in Table 1. The reference strains used for the identification of isolated strains comprised *Aspergillus flavus* NRRL 3357 (ATCC 200026), which was obtained from Prof. Nancy P. Keller (University of Wisconsin, USA), and *A. oryzae* NRRL 447, *A. sojae* IFO 30112, and *A. parasiticus* CBS 971.97, IFO 4082, which were obtained from KACC (the Korean Agricultural Culture Collection, Korea). *Aspergillus*-like fungi were isolated from various meju samples collected from different regions over several years according to their morphological characteristics (*i.e.*, a flattened moss shape on the surface of meju with a yellow or green color). They were routinely cultured on potato dextrose agar (PDA) plates, and their spores were collected with 0.1% Tween 80 after culturing the strains on PDA plates for 3 days at 30°C.

Morphological and Physiological Identification

The isolates and reference strains were grown for 5 days as single-point inoculations (1.0×10^5 spores in 10 µl) on PDA plates. The colony morphology and color were noted. The shape and size of the conidia were observed microscopically. To stain the conidia, spores (1.0×10^5 spores in 10 µl) were single-point inoculated onto Czapek Dox media (Cz agar: sucrose 3%, $NaNO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, K_2HPO_4 0.1%, $FeSO_4 \cdot 7H_2O$ 0.001%, agar 1.5%, pH 6.0) and Cz agar containing 0.05% (v/v) *p*-anisaldehyde (Sigma-Aldrich Chemical Co., USA) [15], and they were cultured at 30°C for 10 days. The color of the conidia was compared against that of the reference strains; that is, pink conidia for *A. parasiticus* and green conidia for *A. sojae*. In the bleomycin resistance tests, we used phleomycin (which belongs to the bleomycin family) instead of bleomycin. Spores (1.0×10^5 spores in 10 µl) were single-point inoculated onto modified minimal agar ($MgSO_4$ 0.052%, KCl 0.052%, KH_2PO_4 0.152%, 2 N KOH 0.05% (v/v), glucose 1%, biotin 0.0008%, 1 M proline solution 1% (v/v), trace element solution 0.1% (v/v), agar 1.5%) containing 100 µg/ml phleomycin (InvivoGen, USA), and cultured at 30°C for 4 days [16]. Growth inhibition by phleomycin was compared with that in the reference strains *A. sojae* and *A. parasiticus*.

Analysis of the β-Tubulin (*benA*) and Aflatoxin (AF) Genes

First, 500 µl of spores (5×10^6 spores) were inoculated into 50 ml of potato dextrose broth, and cultured at 30°C for 3 days with shaking at 200 rpm. Next, the mycellia were separated using a Whatman No. 1 filter paper (GE Healthcare, UK) and freeze-dried. Genomic DNA was isolated from the freeze-dried mycelial powder using a Promega Wizard DNA purification kit (Promega, USA) according to the manufacturer's manual.

Fragments containing the β-tubulin region were amplified using the primers Bt2a and Bt2b [17-19]. PCR was performed using a C1000 Thermal Cycler (Bio-Rad, USA). Sequence analysis was performed by Macrogen Inc. (Korea). To identify the isolates, phylogenetic analysis was performed by a neighbor-joining method using the MEGA6 program [20]. The outgroup was set as *A. niger*, which belongs to section *Nigri*, and bootstrapping was conducted with 1,000 replicates. The *benA* sequences from reference *Aspergillus* species were obtained from GenBank (NCBI) and their accession numbers are indicated in Fig. 3. The *benA* sequences of the 14 isolates have been deposited in GenBank under accession numbers KX943510–943523.

The two AF biosynthesis genes, *aflT* and *aflR*, were analyzed by PCR using the primers listed in Table 2. The β-tubulin gene was used as a template control. The amplification mixture comprised 12.5 µl of Go Taq green master mix 2× (Promega), 10 pmol of each primer, and 10 ng of DNA template in a final volume of 25 µl [21]. The afaflT region within the *aflT* gene was analyzed by real-time PCR. The amplification mixture comprised 10 µl of Evagreen 2× (Bio-Rad), 10 pmol of each primer, and 10 ng of DNA template in a final volume of 20 µl. After pre-denaturation at 95°C for 10 min, amplification was performed in 45 cycles as follows: denaturation at 95°C for 10 sec, annealing at 70°C for 2 sec, and elongation at 72°C for 20 sec. Following amplification, melting curve analysis was performed as described by Godet and Munaut [21].

Random amplification of polymorphic DNA (RAPD) analysis was performed to differentiate *A. parasiticus* and *A. sojae* using the primers OPA-04 and OPB-10 [22]. The amplification mixture comprised 12.5 µl of Go Taq green master mix 2× (Promega), 10 pmol of each primer, and 10 ng of DNA template in a final volume of 25 µl.

Enzyme Production and Assay

To evaluate enzyme production by the isolates, 1 ml of spores (1×10^7 spores) from each strain was inoculated into soybean

Table 1. Morphological, physiological, and molecular genetic characteristics of the strains used in this study.

Species	Origin	Morphological characteristics		Physiological characteristics		Molecular genetic characteristics			
		Conidia shape	Conidia size (μm)	Conidia staining	Phleomycin resistance	AfIt	AfaIt	AfIR	RAPD
Reference strains									
A. flavus NRRL 3357	Peanut cotyledons, USA	Smooth	3.5–4.7	-	-	+	+	+	*A. flavus*
A. oryzae NRRL 447^{T}	Tane-koji, Japan	Smooth	3.5–5.2	-	-	+	+	+	*A. oryzae*
A. sojae IFO 30112^{T}	Koji of soy sauce, Japan	Echinulate	4.3–6.4	G[a]	S[c]	+	-	+	*A. sojae*
KACC 45027	Shoyu-koji, Japan	Echinulate	4.2–6.3	G	S	+	-	+	*A. sojae*
KACC 45029	Shoyu-koji, Japan	Echinulate	4.5–5.7	G	S	+	-	+	*A. sojae*
A. parasiticus CBS 971.97	Indian sweets, Akola, India	Echinulate	4.5–6.4	G	R[d]	+	-	+	*A. parasiticus*
IFO 4082^{NT}	Hawaii, USA	Echinulate	4.5–6.1	P[b]	R	+	-	+	*A. parasiticus*
Isolated strains									
SMF 118	Meju, Gwangju, Jeonnam, Korea	Echinulate	4.5–5.7	G	S	+	-	+	*A. sojae*
SMF 120	Meju, Jochiwon, Chungnam, Korea	Echinulate	4.6–5.6	G	S	+	-	+	*A. sojae*
SMF 121	Meju, Gongju, Chungnam, Korea	Echinulate	4.9–7.0	G	S	+	-	+	*A. sojae*
SMF 122	Meju, Okcheon, Chungbuk, Korea	Echinulate	4.2–6.3	G	S	+	-	+	*A. sojae*
SMF 123	Meju, Yeongdong, Chungbuk, Korea	Echinulate	4.5–6.1	G	S	+	-	+	*A. sojae*
SMF 124	Meju, Daegu, Gyeongbuk, Korea	Echinulate	4.9–6.6	G	S	+	-	+	*A. sojae*
SMF 125	Meju, Yeongcheon, Gyeongbuk, Korea	Echinulate	4.4–6.0	G	S	+	-	+	*A. sojae*
SMF 126	Meju, Gwangju, Gyeonggi, Korea	Echinulate	5.1–7.5	G	S	+	-	+	*A. sojae*
SMF 127	Meju, Yangpyeong, Gyeonggi, Korea	Echinulate	5.3–6.5	G	S	+	-	+	*A.sojae*
SMF 129	Meju, Naju, Jeonnam, Korea	Echinulate	4.5–5.5	G	S	+	-	+	*A. sojae*
SMF 131	Meju, Namwon, Jeonbuk, Korea	Echinulate	5.3–6.1	G	S	+	-	+	*A. sojae*
SMF 132	Meju, Sunchang, Jeonbuk, Korea	Echinulate	4.2–6.2	G	S	+	-	+	*A. sojae*
SMF 133	Meju, Yeongdong, Chungbuk, Korea	Echinulate	4.6–6.2	G	S	+	-	+	*A. sojae*
SMF 134	Meju, Okcheon, Chungbuk, Korea	Echinulate	4.0–6.3	G	S	+	-	+	*A. sojae*

^{T}, Type; ^{NT}, Neotype.

[a]Green conidia; [b]Pink conidia by *p*-anisaldehyde; [c]Phleomycin-sensitive; [d]Phleomycin-resistant; (+), amplification product detected; (–), no amplification product detected. NRRL, the Northern Regional Research Laboratory, USDA, Peoria, IL, USA; KACC, the Korean Agricultural Culture Collection, Suwon, Republic of Korea.

media (20 g crushed soybean flakes in 13 ml of 0.22% $ZnSO_4$ solution) and then incubated for 3 days at 30°C, with 50% relative humidity and mixing once after 24 h. Enzymes were extracted for 15 min by shaking at 200 rpm and 25°C after the addition of 190 ml of 2% NaCl solution and followed by homogenization. The extracts were filtered using Whatman No. 2 paper (GE Healthcare) and the

Table 2. Primers used for molecular genetic analyses in this study.

Primer	Sequence (5′-3′)	Target gene or use	Reference
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	β-Tubulin (*benA*)	[17–19]
Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		
OPA-04	AATCGGGCTG	Random amplification for RAPD	[21, 22]
OPB-10	CTGCTGGGAC		
AflT-F	GCACCAAATGGGTCTTTCTCGT	*aflT*	[21, 38]
AflT-R	ATCCACGGTGAAGAGGGTAAGG		
AfaflT-F	CGCGCGAGATACTTCTTATACT	afaflt sequence in the *aflT* gene	[21]
AfaflT-R	GAGCCCACTTCGAAAATACC		
AflR-F	TCGGTACGTAAACAAGGAAC	*aflR*	[21, 39]
AflR-R	TCTGATGGTCGCCGAGTTGA		
As AflR-F	GTATCCCTGCTGCATCGTCTC	Amplification of the *aflR* pretermination region	This study
As AflR-R	GTGTCCAGTGGCTGTCTGAC		
As PksA-F	CAATTCAGGCCCACAACGGC	Amplification of the *pksA* pretermination region	This study
As PksA-R	GAGGAGCTCACCGATGACAG		
MaoA-F	GGCGTTGAAGTCACTGTGCG	*maoA*	This study
MaoA-R	AGGAAGAAGCCATCTGATCG		[28]
DmaT-F	GTCTCTGGATCGTTCCGTCG	*dmaT*	[28]
DmaT-R	GTATAGCACAGCTCCGATGT		
Pks/nrps-F	GAAAGGCCTTGCCAGCGATACT	*pks/nrps* gene	[28]
Pks/nrp-R	GAATGCAAGGAGCCTCTCGT		

filtrates were used as crude enzyme solutions.

The amylase activity was determined on the basis of spectrophotometrical measurement of the reduction of 3,5-dinitrosalicylic acid (DNS) by reducing sugars released from the soluble starch used as substrate [23]. In the α-amylase activity assay, the reaction mixture comprised 180 µl of 0.1 M acetate buffer (pH 5.6) containing 1.0% soluble starch solution and 20 µl of enzyme solution in a total volume of 200 µl [24]. The mixture was incubated at 37°C for 10 min. Next, 200 µl of DNS solution was added and the mixture was incubated for 5 min at 100°C. The absorbance was measured at 535 nm to determine the production of maltose. One α-amylase activity unit was defined as the amount of enzyme required to produce 1 µmol of maltose per minute [23].

The lipase activity was determined as described previously [25]. The reaction mixture comprised 500 µl of 0.1 M Tris-HCl buffer (pH 7.0), 500 µl of substrate solution, and 200 µl of enzyme solution. The substrate solution comprised 0.016 g of *p*-nitrophenyl palmitate (*p*-NPP), 0.017 g of sodium dodecyl sulfate, and 1 ml of Triton X-100 in 100 ml of deionized water. The mixture was incubated at 37°C for 10 min. The absorbance was measured at 405 nm to determine the production of *p*-nitrophenol. One lipase unit was defined as the amount of enzyme required to produce 1 µmol of *p*-nitrophenol per minute.

The protease activity assay was performed according to Sigma's nonspecific protease assay method (Sigma-Aldrich Chemical Co.) which was originally described by Cupp-Enyard [26], with some modifications. Casein solution (0.65%) was prepared as a substrate in 50 mM potassium phosphate buffer (pH 7.5). The reaction mixture comprised 5 ml of 0.65% casein solution and 1 ml of enzyme solution in a total volume of 6 ml. The mixture was incubated at 37°C for 10 min. The enzyme reaction was stopped by adding 5 ml of 110 mM trichloroacetic acid solution and incubating at 37°C for 30 min. The resulting solution was filtered through a 0.45 µm syringe filter (PALL Co., USA). Next, 2 ml of the filtered solution was mixed with 5 ml of 0.5 M sodium carbonate solution and 1 ml of 0.5 M Folin and Ciocalteu's reagent and incubated at 37°C for 30 min. The absorbance was measured at 660 nm using a spectrophotometer. One protease unit was defined as the amount of enzyme required to release 1 µmol of tyrosine equivalents from casein per minute.

The LAP assay was performed as described previously [27]. L-Leucine-*p*-nitroanilide solution (24 mM) was prepared as a substrate in methanol. The reaction mixture comprised 2.8 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.1 ml of substrate solution, and 100 µl of enzyme solution in a 15 ml Falcon tube. The mixture was incubated at 50°C for 10 min, and then stopped by adding 50 µl of acetic acid and placing each Falcon tube on ice. The absorbance was measured at 405 nm. One unit of LAP activity was defined as the amount of enzyme that hydrolyzed 1 µmol of

leucine-*p*-nitroanilide per minute [27].

AF and Cyclopiazonic Acid (CPA) Gene Clusters in *A. sojae* SMF 127 and SMF 131

To evaluate the safety of the candidates for use as starters in fermentation, we analyzed the AF and CPA gene clusters in *A. sojae* SMF 127 and SMF 131, which have high LAP and protease activities. In the AF cluster analysis, the regions of *aflR* and *pksA* genes that are known to contain a pretermination codon were amplified by PCR and sequenced. The amplification program comprised pre-denaturation at 95°C for 5 min, 30 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec, and with a final incubation step at 72°C for 5 min to complete the extension. Sequence analysis was performed by Macrogen Inc. (Korea). The sequences of the *aflR* and *pksA* genes in *Aspergillus* section Flavi were obtained from GenBank and compared with those of *A. sojae* using the MEGA6 program. In the CPA cluster analysis, the *maoA*, *dmaT*, and *pks/nrps* genes were amplified by PCR as described previously [28]. The β-tubulin gene was used as a template control.

Results

Isolation and Identification of *Aspergillus* Strains

Over the recent years, we have isolated *Aspergillus* strains from traditional meju samples collected from different regions of Korea. On the basis of comparisons with the morphological characteristics of reference *Aspergillus* strains, we initially screened 18 *A. sojae*-like strains (SMF 116, SMF 117, SMF 118, SMF 119, SMF 120, SMF 121, SMF 122, SMF 123, SMF 124, SMF 125, SMF 126, SMF 127, SMF 128, SMF 129, SMF 131, SMF 132, SMF 133, and SMF 134). Three strains (SMF 116, SMF 117, and SMF 128) formed yellow-brownish green and floccose colonies similar to the *A. oryzae/flavus* reference strains (data not shown), whereas the remaining strains formed green colonies like the *A. sojae* reference strains (Fig. 1). No strains formed dark green colonies similar to the *A. parasiticus* reference strains. In addition, 15 strains that formed green colonies had shorter conidiophores, which were more similar to those of *A. sojae* than the *A. oryzae/flavus* reference strains. Therefore, these 15 strains were grouped into *A. sojae* and three strains were grouped into *A. oryzae/flavus*. The other distinct characteristic difference between *A. sojae* and *A. oryzae/flavus* is the presence of echinulate-shaped conidia in *A. sojae* and smooth conidia in *A. oryzae/flavus* [29, 30]. As expected, most strains (14 strains) from the *A. sojae* group had echinulate conidia, except one strain (SMF119) (Fig. 1A). Therefore, these 14 strains were tentatively identified as *A. sojae* according to their morphological characteristics (Fig. 1).

A. sojae is considered a domesticated strain of *A. parasiticus*

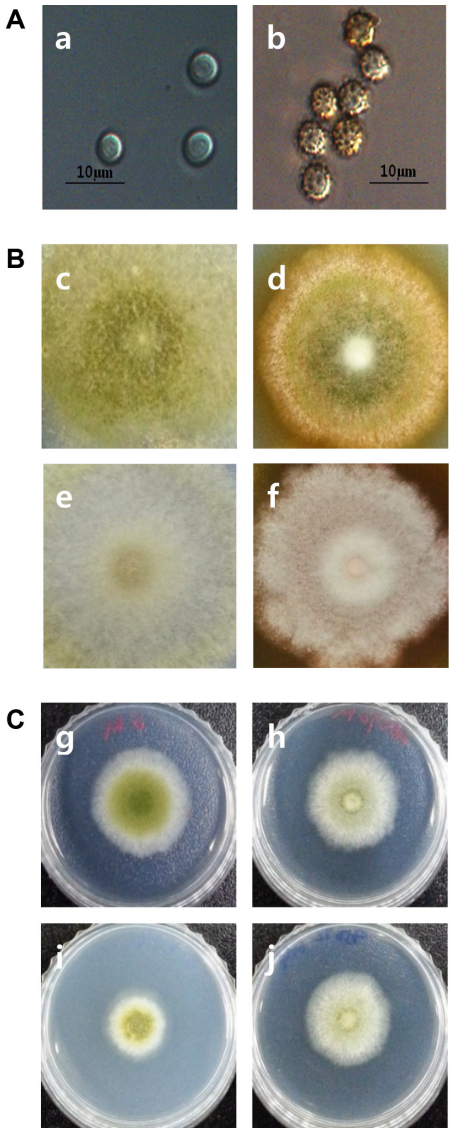

Fig. 1. Morphological and physiological characterization.
(**A**) Each strain was assigned to one of two groups on the basis of its conidial morphology: *A. oryzae/flavus* with smooth conidia (a) and *A. sojae/parasiticus* with echinulate conidia (b). (**B**) *A. sojae* (c, e) were distinguished from *A. parasiticus* (d, f) by staining with *p*-anisaldehyde. Each strain was grown on Cz agar (c, d) and Cz agar containing 0.05% *p*-anisaldehyde (e, f). Conidia produced by *A. sojae* and *A. parasiticus* were green and pink, respectively. (**C**) Each strain was grown on modified minimal agar (MMA; g, h) or MMA containing phleomycin (i, j). The growth of *A. sojae* (g, i) was inhibited in the presence of phleomycin (i) whereas that of *A. parasiticus* (h, j) was not (j).

[13, 22]. Because *A. parasiticus* has echinulate conidia like *A. sojae*, 14 *A. sojae* candidate strains were further characterized according to their physiological characteristics to differentiate them from *A. parasiticus*. *A. parasiticus* produces pink

conidia on Cz agar containing *p*-anisaldehyde, whereas *A. sojae* forms green conidia [15, 30]. All 14 strains with echinulate conidia formed green conidia (Fig. 1B-c, e), whereas the reference strains of *A. parasiticus* formed typical pink (purple) conidia (Fig. 1B-d, f) on *p*-anisaldehyde Cz agar, suggesting that the 14 strains were *A. sojae* and not *A. parasiticus*.

A. parasiticus is resistant to bleomycin, whereas *A. sojae* is sensitive to bleomycin and its growth is inhibited greatly [16, 29]. Bleomycin is a glycopeptide antibiotic that inhibits microbial growth by breaking double-stranded DNA and inhibiting cell-wall biosynthesis. Phleomycin belongs to the bleomycin family and mediates its effects in the same manner as bleomycin, but it is an inexpensive alternative. The growth of the reference *A. parasiticus* strains was minimal, whereas that of the *A. sojae* reference and 14 candidate strains was inhibited greatly by phleomycin (Fig. 1C), suggesting that all 14 strains were probably *A. sojae*.

Analysis of the AF gene cluster, especially the *aflT* and *aflR* genes, is frequently used to identify members of *Aspergillus* section *Flavi* [15, 21]. The *aflT* gene, which encodes a major facilitator superfamily transporter, is present in *Aspergillus* section *Flavi* members such as *A. flavus*, *A. oryzae*, *A. sojae*, and *A. parasiticus*. The afaflT is found within the *aflT* gene, and its amplification by real-time PCR using AfaflT-F and AfaflT-R primers can distinguish *A. flavus/oryzae* group members from *A. parasiticus/sojae* group members. Typically, this sequence is amplified within 30 of 45 cycles (positive) in *A. flavus/oryzae* group members, whereas it is amplified after 30 cycles or amplified at a very low level (negative) in *A. parasiticus/sojae* group members. The *aflR* gene, a regulatory gene in AF biosynthesis, was used to confirm members of the *A. parasiticus*/*sojae* group, from which the *aflR* gene is usually amplified. The *aflT* and *aflR* genes were amplified from all 14 strains that exhibited the morphological and physiological characteristics of *A. sojae*, but afaflT was not amplified (Table 1), suggesting these strains belonged to the *A. parasiticus*/*sojae* group.

RAPD was used to differentiate *A. sojae* from *A. parasiticus* [21]. Using OPA-04 primers, excluding one strain (SMF 118), 13 strains exhibited the characteristic pattern of *A. flavus/oryzae/sojae* (2.2, 1.4, and 0.75 kb bands) but not that of *A. parasiticus* (2.2, 1.4, 0.75, and 0.4 kb bands; or 2.2, 1.4, and 0.6 kb bands). SMF 118 had a similar pattern to *A. parasiticus* (2.2, 1.4, 0.75, and 0.4 kb bands; Fig. 2A). Using OPB-10 primers, all 14 strains were differentiated from *A. flavus/oryzae* (2.6, 1.6, and 1.3 kb bands). All 14 strains produced

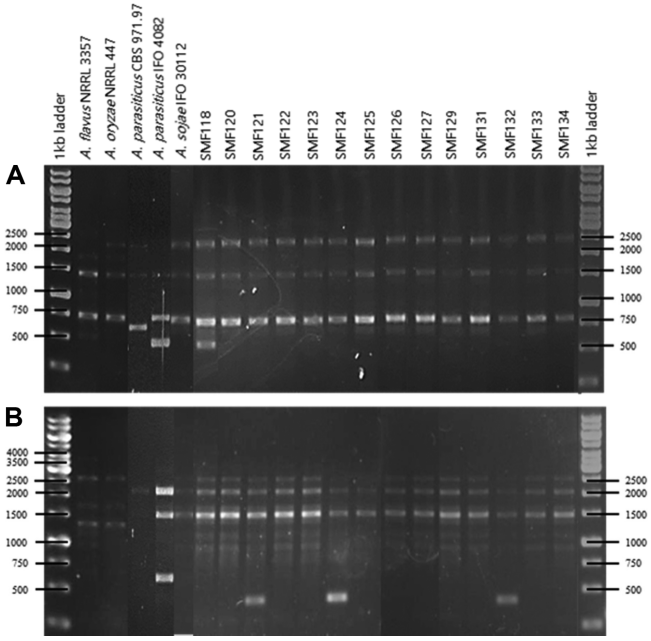

Fig. 2. Random amplification polymorphic DNA patterns obtained for the reference and isolated strains using the primers OPA-04 (**A**) and OPB-10 (**B**).
1 kb ladder; GeneRuler 1 kb DNA ladder.

the characteristic pattern of *A. sojae* (2.6, 2.0, 1.5, and 0.9 kb bands), which differs from that of *A. parasiticus* (2.0, 1.5, and 0.6 kb bands). The 0.6 kb band was not amplified from any of the 14 strains. Among the 14 strains, SMF 121, SMF 124, and SMF 132 produced an additional 0.4 kb band. According to the RAPD patterns obtained using OPA-04 and OPB-10 primers, all 13 strains were probably *A. sojae*, and SMF 118 was considered to be *A. sojae* on the basis of the RAPD pattern obtained with OPB-10 primers.

The internal transcribed spacer (ITS) sequence is generally used for the phylogenetic classification of fungi. However, the similarity of the ITS sequences among *Aspergillus* section Flavi members is too high to differentiate *A. flavus/oryzae/sojae/parasiticus*. By contrast, the β-tubulin sequence is known to be highly variable among species because of the presence of several introns [31]. Therefore, the β-tubulin (*benA*) gene was amplified by PCR, sequenced, and analyzed phylogenetically [17–19]. The *benA* sequences of reference *Aspergillus* species were obtained from GenBank and their accession numbers are indicated in Fig. 3. All 14 isolated strains shared high similarity with *A. sojae* within section Flavi (Fig. 3).

According to their morphological, physiological, and molecular genetic characteristics, 14 of the isolated strains were identified as *A. sojae*.

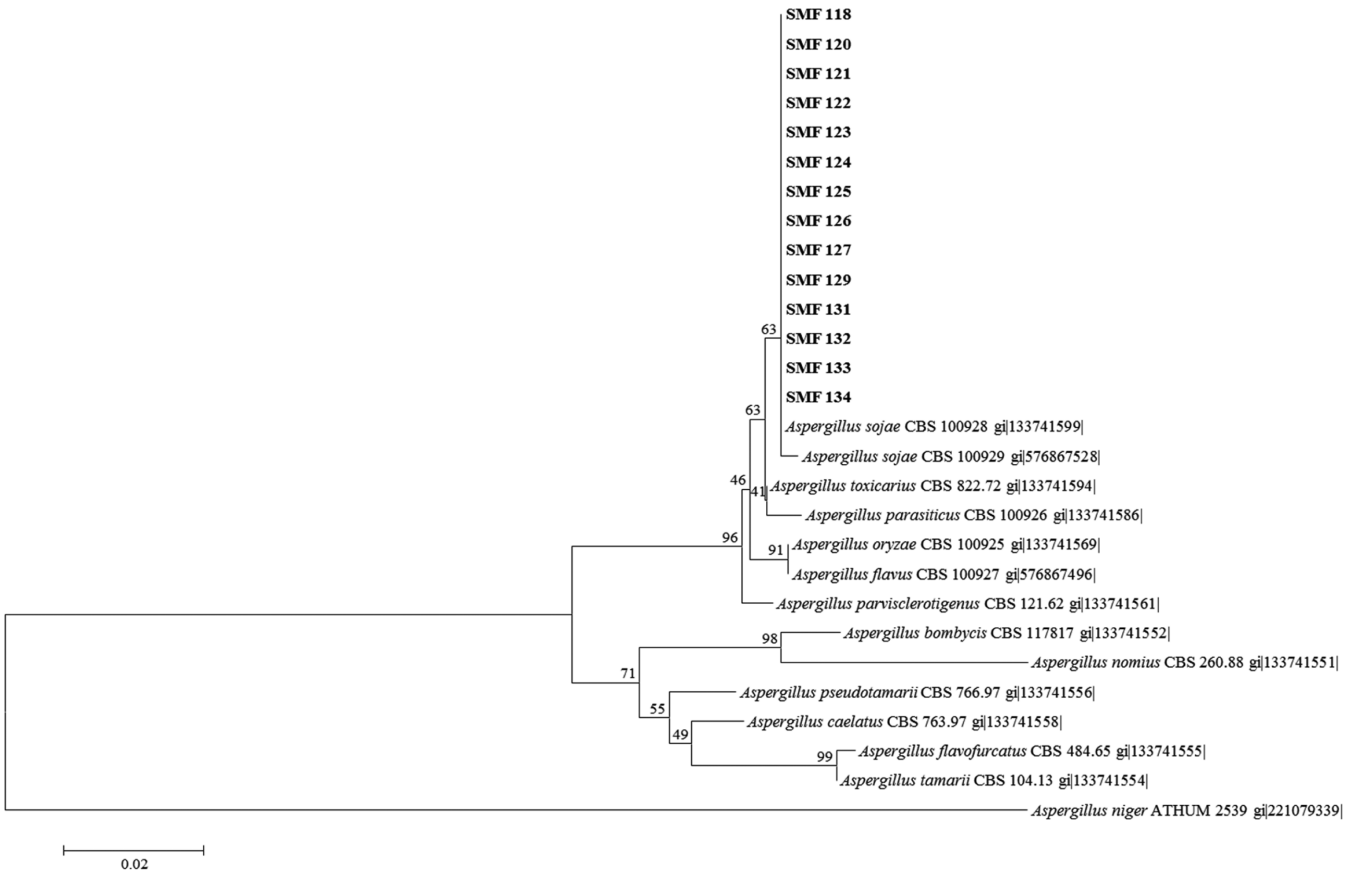

Fig. 3. Neighbor-joining analysis based on sequences of the partial β-tubulin (*benA*) gene in 14 isolates.
Evolutionary history was inferred using the neighbor-joining method [20]. The optimal tree with a summed branch length of 0.39435999 is shown. The percentages of replicate trees where the associated taxa clustered together according to the bootstrap test (1,000 replicates) are shown above the branches [36]. Bootstrap values <40 % are not shown. Evolutionary analyses were conducted with MEGA6 [37].

Enzyme Production by *A. sojae* Isolates

To evaluate the advantages of the isolates as starter molds, we determined the activities of enzymes that are important for soybean fermentation. In particular, protease plays a very important role during soybean fermentation by breaking down soybean proteins into free amino acids or low-molecular-weight peptides. The isolates produced protease in the range of 2.66–10.64 U/g-IDS, where the protease activity of SMF 127 was the highest at 10.64 U/g-IDS (Table 3). The protease levels in the isolates were higher than those in koji strains used to produce Japanese soy sauce, which range from 1.35 to 2.45 U/g-IDS. In addition, amylase activity is required because koji is generally produced using grains that are rich in starch. The α-amylase activity of the isolates was equivalent to that of koji strains used to produce Japanese soy sauce (Table 3). However, the lipase activity of the isolates was lower than that of koji strains used in Japanese soy sauce production (Table 3).

LAP is an exopeptidase that removes the N-terminal L-leucine from peptide substrates. LAP is one of the most important enzymes in the fermentation industry because it plays an important role in flavor development by producing low-molecular-weight peptides that affect the taste, and by decreasing bitterness [4]. SMF 131 had the highest LAP activity at 6.06 U/g-IDS among the isolates and Japanese koji strains. The strains with high protease and LAP activities tended to have low lipase activities. For example, SMF 131 had the highest LAP activity at 6.06 U/g-IDS among the strains tested, but its lipase activity was low at 1.70 U/g-IDS (Table 3). The Japanese koji strains had low LAP activities but much higher lipase activities (6.86–10.33 U/g-IDS) than our isolates.

SMF 127 and SMF 131 had high protease and LAP activities during solid-state fermentation with soybean, and thus they may be considered good starters for use in

Table 3. Production of protease, amylase, lipase, and leucine aminopeptidase (LAP) by isolated *A. sojae* strains in solid-state fermentation (72 h).

Strain		Protease (U/g-IDS)	α-Amylase (U/g-IDS)	Lipase (U/g-IDS)	LAP (U/g-IDS)
Isolated strains from Korean meju	SMF 118	3.35 ± 0.23	37.16 ± 1.68	3.70 ± 0.44	2.41 ± 0.16
	SMF 120	2.66 ± 0.44	33.27 ± 1.25	4.28 ± 0.48	1.92 ± 0.25
	SMF 121	3.15 ± 0.75	31.76 ± 1.03	4.42 ± 0.28	1.58 ± 0.03
	SMF 122	8.76 ± 1.06	87.23 ± 4.75	2.10 ± 0.27	4.91 ± 0.31
	SMF 123	3.39 ± 0.35	21.53 ± 0.16	1.38 ± 0.19	1.12 ± 0.04
	SMF 124	9.22 ± 0.65	76.98 ± 12.07	2.34 ± 0.38	5.06 ± 0.09
	SMF 125	9.99 ± 0.68	106.73 ± 4.67	2.10 ± 0.65	5.55 ± 0.16
	SMF 126	8.09 ± 0.56	68.65 ± 5.70	1.95 ± 0.36	3.64 ± 0.14
	SMF 127	10.64 ± 0.61	96.97 ± 2.07	2.06 ± 0.14	6.00 ± 0.33
	SMF 129	3.70 ± 0.66	27.12 ± 2.78	1.24 ± 0.10	1.32 ± 0.13
	SMF 131	9.44 ± 0.37	89.96 ± 0.58	1.70 ± 0.32	6.06 ± 0.42
	SMF 132	4.74 ± 0.46	56.18 ± 2.37	2.88 ± 0.10	3.03 ± 0.22
	SMF 133	3.20 ± 0.41	42.56 ± 1.11	3.01 ± 0.35	2.57 ± 0.17
	SMF 134	2.98 ± 0.33	37.28 ± 2.36	4.31 ± 0.09	1.79 ± 0.04
Koji strains used in Japanese soy sauce production	IFO 30112^T	2.45 ± 0.11	59.25 ± 6.58	6.86 ± 0.57	1.37 ± 0.11
	KACC 45027	1.53 ± 0.01	60.04 ± 6.01	10.33 ± 0.74	0.93 ± 0.09
	KACC 45029	1.35 ± 0.03	35.36 ± 4.55	8.84 ± 0.68	0.63 ± 0.10

U/g-IDS, unit/g-initial dry substrate.

soybean fermentations such as in the production of meju, doenjang, and ganjang.

AF and CPA Gene Clusters in *A. sojae* SMF 127 and SMF 131

A. sojae is classified as GRAS, but safety assurance is very important before isolated strains can be used in industrial applications because of the close evolutionary relationship between *A. sojae* and pathogenic *Aspergillus* species. In particular, our strains were isolated from nature so they might differ from industrial strains. The *A. sojae* draft genome contains all the orthologs of the AF biosynthesis genes in *A. parasiticus* [31]. Therefore, other isolates are expected to have all the orthologs of the AF biosynthesis genes. However, SMF 127 and SMF 131 appeared to be non-aflatoxigenic like other *A. sojae* strains, because of several mutations in the AF gene cluster, as found in other *A. sojae* strains. The non-production of AF in *A. sojae* is related to a termination point mutation in *aflR* and the absence of the polyketide synthase (PKS) gene [12].

aflR is a regulatory gene that controls transcription of AF biosynthesis gene clusters. As found in other *A. sojae aflR*

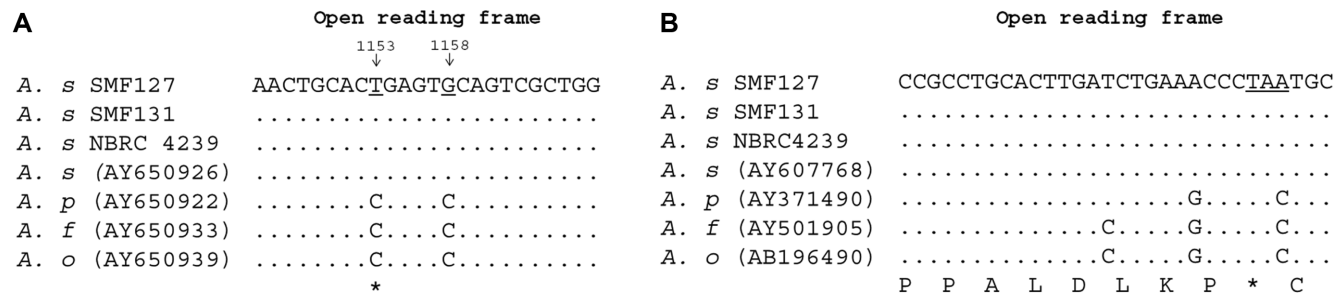

Fig. 4. Comparison of portions of the *aflR* (**A**) and *pksA* (**B**) genes in *A. sojae* SMF 127 and SMF 131 with those in other *A. sojae* and *Apsergillus* section *Flavi* strains.

A. s, A. sojae; *A. p, A. parasiticus*; *A. f, A. flavus*; *A. o, A. oryzae*; (.), sequences identical to *A. sojae* SMF 127; (–), gap. The A in the start codon was designated as +1.

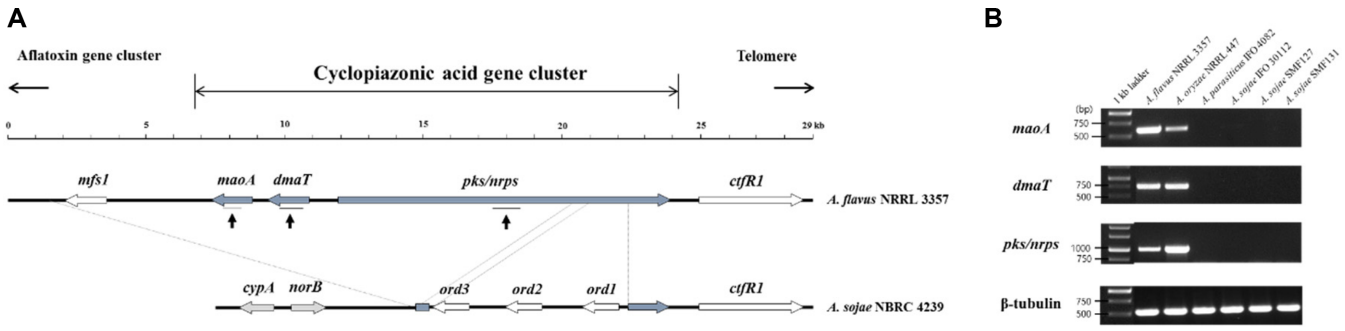

Fig. 5. PCR analysis of the cyclopiazonic acid (CPA) gene cluster in SMF 127 and SMF 131.
(**A**) CPA cluster in *A. flavus* NRRL 3357 and *A. sojae* NBRC 4239. Arrows (↑) indicate the target regions for PCR amplification. (**B**) PCR analysis of the CPA genes. 1 kb ladder, GeneRuler 1 kb DNA ladder.

genes, a premature stop codon (TGA at 1153) was detected in the *aflR* gene of SMF 127 and SMF 131, which results in the loss of 62 C-terminal amino acids and loss of function (Fig. 4A) [12, 32, 33].

The *pksA* gene encode PKS, which comprises acyl carrier protein (ACP), β-ketoacyl-ACP synthase, acyltransferase, and thioesterase (TE) domains [12], where TE hydrolyzes the polyketide chain produced by ACP. In *A. sojae pksA*, a C base is replaced by A to produce a stop codon (TAA) at the 1848th codon, which results in a loss of function because of the absence of the TE domain [12]. The *pksA* gene in *A. sojae* SMF 127 and SMF 131 had the same base change found in other *A. sojae* strains, including NBRC 4239 (Fig. 4B). Therefore, SMF 127 and SMF 131 cannot biosynthesize AF because of their non-functional PksA, which lacks the TE domain. Other base change differences occur between *A. sojae* and *A. parasiticus*, *A. flavus*, and *A. oryzae* (Fig. 4B), but these base changes do not affect the amino acid sequences.

CPA is not as toxic as AF, but is a mycotoxin that can cause nerve disorder when ingested at high levels. The CPA gene cluster comprises the *maoA, dmaT,* and *pks/nrps* genes and it is located in the subtelomeric region close to the AF cluster in *A. flavus* (Fig. 5A) [34]. According to the draft genome sequence of *A. sojae* NBRC 4239, >18 kb has been deleted, including most of the CPA gene cluster, while the *ord3*, *ord2*, and *ord1* genes located in the subtelomeric region downstream of the CPA cluster in *A. flavus* are inserted at the partial *pks/nrps* gene [1]. As expected, the *maoA, dmaT,* and *pks/nrps* genes were not amplified in both SMF 127 and SMF 131 (Fig. 5B), suggesting that SMF 127 and SMF 131 are non-CPA producers because of the loss of all three CPA biosynthetic genes, as found in *A. sojae* NBRC 4239.

Discussion

In this study, several *A. sojae* strains were isolated from meju samples. There have been a few previous reports of the isolation of *A. sojae* from nature, and *A. sojae* is considered a domesticated strain of *A. parasiticus*, which was selected for food fermentation from genetically variable *A. parasiticus* [13]. *A. sojae* is used mainly for soybean fermentation, such as in shoyu (Japanese soy sauce) production in Japan, but it is seldom used for any fermentation in Korea. The close evolutionary relationship between *A. sojae* and pathogenic *A. parasiticus* may have discouraged and limited the applications of *A. sojae* in food fermentation [1, 32]. As expected, our *A. sojae* isolates shared very similar morphological, physiological, and molecular genetic characteristics with *A. parasiticus*. However, our successful isolation of *A. sojae* from various meju samples, which are fermented soybean products obtained by natural inoculation with a mold, argue against that *A. sojae* is a genetic variant of *A. parasiticus* that has been selected in only the industrial environment. *A. oryzae* is also considered as a domesticated strain of AF-producing *A. flavus*, but it can be isolated easily from nature.

The superior protease and LAP activities of some isolates such as SMF 127 and SMF 131 (Table 3) suggest that they may be excellent candidates for use as starters in soybean fermentation, such as meju, doenjang, and ganjang production. At present, these fermentation products are manufactured traditionally by natural microbial inoculation, or industrially by using a starter mold. The traditional process is often hindered by variations in quality and poor product standardization because of the use of non-uniform fermentation environments. In industry, *A. oryzae* is mainly used as a starter because it is the dominant flora in

traditional soybean fermentation. However, *A. sojae* may be a better starter for soybean fermentation because it has higher protease [35] and LAP activities [3], which are important for fermentation and flavor development.

Our analysis of the AF and CPA gene clusters in SMF 127 and SMF 131 indicates that both strains are not producers of AF and CPA (Figs. 4 and 5), and thus they are considered safe for use in industrial applications as starters. Moreover, similar point mutations in the AF gene cluster, as well as gene deletions and insertions in the CPA cluster region, suggest that the natural *A. sojae* flora in meju are also non-aflatoxigenic as well as non-CPA producers, as found with Japanese *A. sojae* koji strains.

In conclusion, *A. sojae* strains such as SMF 127 and SMF 131, which have high protease and LAP activities, and no mycotoxins such as AF and CPA, can be applied in soybean fermentations, such as for meju, doenjang, and ganjang production.

Acknowledgments

This study was supported by the World-Class 300 Project, Small and Medium Business Administration of the Republic of Korea and by the Agriculture, Food and Rural Affairs Research Center Support Program, Ministry of Agriculture, Food and Rural Affairs.

References

1. Sato A, Oshima K, Noguchi H, Ogawa M, Takahashi T, Oguma T, *et al.* 2011. Draft genome sequencing and comparative analysis of *Aspergillus sojae* NBRC4239. *DNA Res.* **18:** 165-176.
2. Kitamoto K. 2002. Molecular biology of the koji molds. *Adv. Appl. Microbiol.* **51:** 129-154.
3. Nampoothiri KM, Nagy V, Kovacs K, Szakacs G, Pandey A. 2005. L-Leucine aminopeptidase production by filamentous *Aspergillus* fungi. *Lett. Appl. Microbiol.* **41:** 498-504.
4. Toldrá F, Aristoy M-C, Flores M. 2000. Contribution of muscle aminopeptidases to flavor development in dry-cured ham. *Food Res. Int.* **33:** 181-185.
5. Kim D-H, Kim S-H, Kwon S-W, Lee J-K, Hong S-B. 2013. Mycoflora of soybeans used for meju fermentation. *Mycobiology* **41:** 100-107.
6. Buyukkileci AO, Tari C, Fernandez-Lahore M. 2011. Enhanced production of exo-polygalacturonase from agro-based products by *Aspergillus sojae*. *Bioresources* **6:** 3452-3468.
7. Chang P-K. 2004. Lack of interaction between AFLR and AFLJ contributes to nonaflatoxigenicity of *Aspergillus sojae*. *J. Biotechnol.* **107:** 245-253.
8. Gurkok S, Cekmecelioglu D, Ogel ZB. 2011. Optimization of culture conditions for *Aspergillus sojae* expressing an *Aspergillus fumigatus* α-galactosidase. *Bioresour. Technol.* **102:** 4925-4929.
9. Heerd D, Yegin S, Tari C, Fernandez-Lahore M. 2012. Pectinase enzyme-complex production by *Aspergillus* spp. in solid-state fermentation: a comparative study. *Food Bioprod. Process.* **90:** 102-110.
10. Lin C-H, Wei Y-T, Chou C-C. 2006. Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi. *Food Microbiol.* **23:** 628-633.
11. Oncu S, Tari C, Unluturk S. 2007. Effect of various process parameters on morphology, rheology, and polygalacturonase production by *Aspergillus sojae* in a batch bioreactor. *Biotechnol. Prog.* **23:** 836-845.
12. Chang P-K, Matsushima K, Takahashi T, Yu J, Abe K, Bhatnagar D, *et al.* 2007. Understanding nonaflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. *Appl. Microbiol. Biotechnol.* **76:** 977-984.
13. Wicklow DT. 1984. Conidium germination rate in wild and domesticated yellow-green aspergilli. *Appl. Environ. Microbiol.* **47:** 299-300.
14. Chang H-Y, Lee Y-B, Bae H-A, Huh J-Y, Nam S-H, Sohn H-S, *et al.* 2011. Purification and characterisation of *Aspergillus sojae* naringinase: the production of prunin exhibiting markedly enhanced solubility with in vitro inhibition of HMG-CoA reductase. *Food Chem.* **124:** 234-241.
15. Murakami H, Hayashi K, Ushijima S. 1982. Useful key characters separating three *Aspergillus* taxa: *A. sojae*, *A. parasiticus*, and *A. toxicarius*. *J. Gen. Appl. Microbiol.* **28:** 55-60.
16. Klich M, Mullaney E. 1989. Use of a bleomycin-containing medium to distinguish *Aspergillus parasiticus* from *A. sojae*. *Mycologia* **81:** 159-160.
17. Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* **61:** 1323-1330.
18. Hubka V, Kolarik M. 2012. β-Tubulin paralogue *tubC* is frequently misidentified as the *benA* gene in *Aspergillus* section *Nigri* taxonomy: primer specificity testing and taxonomic consequences. *Persoonia* **29:** 1.
19. Peterson SW. 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia* **100:** 205-226.
20. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406-425.
21. Godet M, Munaut F. 2010. Molecular strategy for identification in *Aspergillus* section *Flavi*. *FEMS Microbiol. Lett.* **304:** 157-168.
22. Yuan GF, Liu CS, Chen CC. 1995. Differentiation of *Aspergillus parasiticus* from *Aspergillus sojae* by random amplification of polymorphic DNA. *Appl. Environ. Microbiol.* **61:** 2384-2387.
23. Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426-428.
24. Sahnoun M, Kriaa M, Elgharbi F, Ayadi D-Z, Bejar S,

Kammoun R. 2015. *Aspergillus oryzae* S2 alpha-amylase production under solid state fermentation: optimization of culture conditions. *Int. J. Biol. Macromol.* **75:** 73-80.
25. Kum S-J, Yang S-O, Lee SM, Chang P-S, Choi YH, Lee JJ, *et al.* 2015. Effects of *Aspergillus* species inoculation and their enzymatic activities on the formation of volatile components in fermented soybean paste (doenjang). *J. Agric. Food Chem.* **63:** 1401-1418.
26. Cupp-Enyard C. 2008. Sigma's non-specific protease activity assay – casein as a substrate. *J. Vis. Exp.* **19:** e899.
27. Tan PST, Konings WN. 1990. Purification and characterization of an aminopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. *Appl. Environ. Microbiol.* **56:** 526-532.
28. Lee JH, Jo EH, Hong EJ, Kim KM, Lee I. 2014. Safety evaluation of filamentous fungi isolated from industrial doenjang koji. *J. Microbiol. Biotechnol.* **24:** 1397-1404.
29. Jørgensen TR. 2007. Identification and toxigenic potential of the industrially important fungi, *Aspergillus oryzae* and *Aspergillus sojae*. *J. Food Prot.* **70:** 2916-2934.
30. Murakami H. 1971. Classification of the koji mold. *J. Gen. Appl. Microbiol.* **17:** 281-309.
31. Laforgue R, Guérin L, Pernelle JJ, Monnet C, Dupont J, Bouix M. 2009. Evaluation of PCR-DGGE methodology to monitor fungal communities on grapes. *J. Appl. Microbiol.* **107:** 1208-1218.
32. Lee C-Z, Liou G-Y, Yuan G-F. 2006. Comparison of the *aflR* gene sequences of strains in *Aspergillus* section *Flavi*. *Microbiology* **152:** 161-170.
33. Watson AJ, Fuller LJ, Jeenes DJ, Archer DB. 1999. Homologs of aflatoxin biosynthesis genes and sequence of *aflR* in *Aspergillus oryzae* and *Aspergillus sojae*. *Appl. Environ. Microbiol.* **65:** 307-310.
34. Chang P-K, Horn BW, Dorner JW. 2009. Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. *Fungal Genet. Biol.* **46:** 176-182.
35. Sardjono, Zhu Y, Knol W. 1998. Comparison of fermentation profiles between lupine and soybean by *Aspergillus oryzae* and *Aspergillus sojae* in solid-state culture systems. *J. Agric. Food Chem.* **46:** 3376-3380.
36. Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783-791.
37. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30:** 2725-2729.
38. Tominaga M, Lee Y-H, Hayashi R, Suzuki Y, Yamada O, Sakamoto K, *et al.* 2006. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* RIB strains. *Appl. Environ. Microbiol.* **72:** 484-490.
39. Chang P-K, Bhatnagar D, Cleveland TE, Bennett JW. 1995. Sequence variability in homologs of the aflatoxin pathway gene *aflR* distinguishes species in *Aspergillus* section *Flavi*. *Appl. Environ. Microbiol.* **61:** 40-43.