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Research Note

Mycoflora and Enzymatic Characterization of Fungal Isolates in Commercial Meju, Starter for a Korean Traditional Fermented Soybean Product

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Abstract Mycoflora was assessed in the commercial meju from four well-separated geographic origins. A total of 112 fungal isolates were identified by phenotypic characteristics and molecular taxonomy using sequencing the internal transcribed spacer of the rDNA and revealed 19 species from 13 genera. Enzymatic characteristics of protease and amylase, and mycotoxin production were analyzed.

Keywords Amylase, Meju, Mycoflora, Mycotoxin, Protease

Korean traditional meju, a brick of dried fermented soybeans, serves as an important starting material of several Korean condiments such as soybean paste (doenjang), soy sauce (ganjang), and hot pepper paste (gochujang) [1]. Meju is made by soaking, steaming, and mashing soybeans, and then fermenting them with various microorganisms such as bacteria, yeast, and fungi for a prolonged term, usually 2~3 mon, until it used for further process. Fungi particularly perform a crucial role in decomposition organic matter on meju [1, 2].

Although several factors determine differences in the taste and aroma of traditional fermented soybean products such as the raw materials and process techniques, the most significant factor is the extracellular enzymatic activities of the various microorganisms involved in the fermentation process with starter material, meju [3]. The fungi of meju or soybean products are commonly evaluated as being safe. However, it is possible to be contaminated with mycotoxin-producing fungi under the natural fermentation [4].

It has been known that fungi perform an important role in fermentation and decomposition of organic matters of the soybeans into small nutrient functional molecules as amino acids, sugars, organic acids, alcohol, and esters, which underline the typical flavor of meju [1, 3]. Therefore, the mycoflora study of fermented soybean products have been reported that *Aspergillus* spp., *Cladosporium* spp., *Eurotium* spp., *Fusarium* spp., *Lichtheimia* spp., *Mucor* spp., *Penicillium* spp., *Rhizopus* spp., and *Scopulariopsis* spp. participated in general meju fermentation [5-9].

In order to better comprehend the complex progresses that appear during the slow fermentation of commercial meju, analysis of the fungal diversity in each product will have taken a great interest. Therefore, the aims of this study were to assess the mycoflora of meju fermentation on traditionally manufactured commercially available meju samples and to compare these fungal species with those analyzed in meju. In addition, extracellular enzymatic characteristics of the fungal isolates related to proteins and carbohydrates degradation were carried out. Finally, mycotoxin producing capacity was evaluated using thin layer chromatography (TLC).

Fungal strains were isolated from commercial manufactured meju samples from four different regions in Korea (Table 1). Ten loaves of each molded meju sample, including the surface and the inside of the sample, were collected. Each loaf was smashed to paste and 2 g from smashed each loaf was mixed for fungal isolation. Twenty grams of the mixed preparation for each sample was added to 180 mL of sterile

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Table 1. Representative fungal strains isolated from meju used in this study

Sample designation	Geographic origin	Morphological group	No. of isolates	Scientific nomenclature	ITS identification				Enzyme activity[a]	
					GenBank accession No.	Sequence length (bp)	Coverage (%)	Identity (%)	Protease activity	Amylase activity
A	Gyeonggi, Paju	A - a	4	*Rhizopus stolonifer*	FN401529.1	882	100	100	−	−
		A - b	2	*Mucor circinelloides*	FN598919.1	639	92	99	+	−
		A - c	14	*Aspergillus flavus*	JQ269826.1	597	84	99	++	+++
		A - d	3	*Mucor racemosus*	AY213659.1	638	98	100	+	+
		A - e	12	*Mucor racemosus*	HM641690.1	638	99	99	+	+
		A - f	1	*Penicillium chrysogenum*	HM371375.1	540	79	100	+	+
		A - g	1	*Mucor racemosus*	HM641690.1	638	98	99	+	+
		A - h	1	*Penicillium polonicum*	AF033475.1	531	91	100	+	+
		A - i	1	*Penicillium polonicum*	JQ082508.1	531	100	99	+	+
		A - j	2	*Penicillium crustosum*	JN252103.1	532	100	100	++	++
B	Gyeongbuk, Yecheon	B - a	7	*Mucor circinelloides*	AY213658.1	639	95	99	+	−
		B - b	1	*Rhizomucor variabilis*	DQ119007.1	640	95	99	+	−
		B - c	4	*Lichtheimia* sp.	FJ719383.1	743	90	100	+	−
		B - d	7	*Aspergillus flavus*	JQ269826.1	596	82	100	++	+++
		B - e	3	*Aspergillus oryzae*	GU385811.1	638	83	99	++	+++
		B - f	4	*Aspergillus oryzae*	GU385811.1	597	88	99	++	+++
		B - g	1	*Trichosporon asahii*	AB369919.1	543	69	100	+	−
		B - h	1	*Eurotium amstelodami*	HM145963.1	556	75	100	−	+
C	Jeonnam, Gangjin	C - a	3	*Lichtheimia corymbifera*	HQ285657.1	766	94	99	+	−
		C - b	2	*Gibberella intermedia*	JQ69083.1	560	71	99	−	+
		C - c	1	*Pichia burtonii*	AM420292.1	452	59	98	−	++
		C - d	4	*Pichia burtonii*	EU714323.1	449	69	99	−	++
		C - e	2	*Aspergillus flavus*	JQ269826.1	596	82	100	++	+++
		C - f	1	*Aspergillus flavus*	JQ269826.1	597	87	99	++	+++
		C - g	3	*Aspergillus oryzae*	JN561266.1	597	82	100	++	+++
		C - h	2	*Aspergillus flavus*	JQ269826.1	597	82	100	++	+++
		C - i	2	*Aspergillus flavus*	HQ340109.1	596	74	100	++	+++
		C - j	2	*Aspergillus flavus*	HQ010119.1	596	86	100	++	+++
D	Gangwon, Yeongwol	D - a	1	*Absidia corymbifera*	DQ658188.1	771	94	99	−	−
		D - b	11	*Aspergillus flavus*	JQ269826.1	597	82	100	++	+++
		D - c	1	*Rhizomucor pusillus*	AB369914.1	629	86	99	+	+++
		D - d	4	*Scopulariopsis brevicaulis*	KC311514.1	631	99	98	++	+
		D - e	2	*Scopulariopsis brevicaulis*	JN942890.1	630	92	98	++	+
		D - f	2	*Eupenicillium* sp.	JQ828843.1	616	84	86	++	++

ITS, internal transribed spacer.

[a]Enzymatic activity was assigned based on distance of the clearance zone from the colony margin (+++, > 10 mm; ++, 5~10 mm; +, < 5 mm; −, absent).

water, vigorously vortexed for 30 sec, and maintained at room temperature for 30 min. Serial dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were prepared and 0.3 mL of each of these dilutions was spread onto the surface of potato dextrose agar medium (PDA) containing 17 mg/L chloramphenicol.

The plates were incubated for 2~5 days at 30°C, the isolated fungal colonies were transferred to a new PDA plate and incubated for 3~7 days at 30°C to obtain a pure culture. Agar blocks containing actively growing mycelia were stored as previously described for further study [10].

The whole of the four meju samples yielded a total of 112 distinctive fungal colonies. Based on characteristics of colony morphology, growth rate, and pigmentation, 41, 28, 22, and 21 isolates from samples A~D could be assigned to 10, 8, 10, and 6 groups, respectively. Each isolates were identified in genus standard methods [11]. At least two representative isolates for each type (except for those with a single isolate) were classified by sequence comparison of the internal transcribed spacer (ITS) region of the nuclear ribosomal gene (rDNA). The ITS region was amplified using with primers ITS1 (5′-CTAGCGGAGACGGGCTGC-3′) and ITS4 (5′-GGGTCTCTTGGTATGGTC-3′). The resulting PCR amplicon was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and four clones per one isolate were sequenced from both directions using the dideoxynucleotide method with universal and synthetic oligonucleotide primers. A total of 112 fungal isolates were identified as belonging to 13 genera, 19 species, and four *Lichtheimia* and two *Eupenicillium* isolates of unclassified species. Seven, seven, five, and five species were identified for samples A~D, respectively (Table 1). Even though some representative isolates showed slightly different cultural characteristics of colony morphology, growth rate, and pigmentation, they were identified as the same species. Among classified isolates, the genera *Aspergillus* and *Mucor* were the predominant fungi; 41 and 10 isolates of *A. flavus* and *A. oryzae*, respectively, and 16 and 9 isolates of *M. racemosus* and *M. circinelloides*, respectively, were obtained (Fig. 1). The mycoflora of meju fermentation showed a

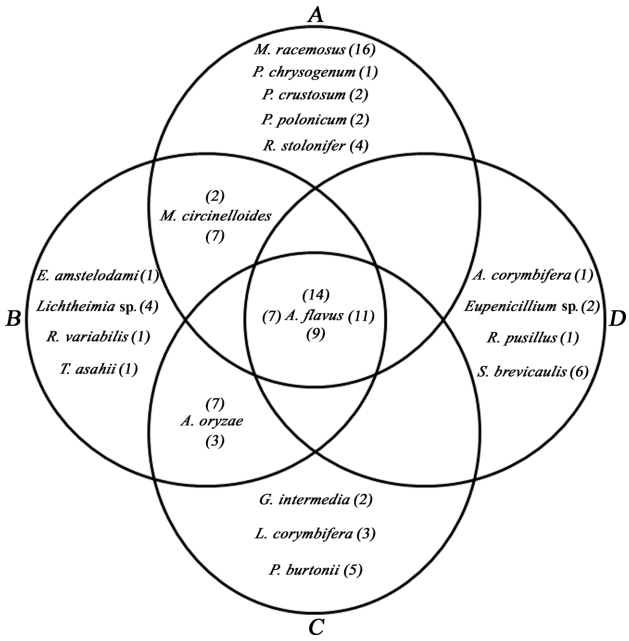

Fig. 1. Venn diagram summarizing the number of unique and shared fungal species in the four meju samples from four well-separated geographic origins. A, B, C, and D, as indicated in Table 1. Numbers in parentheses represent the number of identified fungal isolates from each sample. Parentheses of shared species are shown according to the relative sample locations. Note that only one species was found in all four meju samples and six species were present in two different samples.

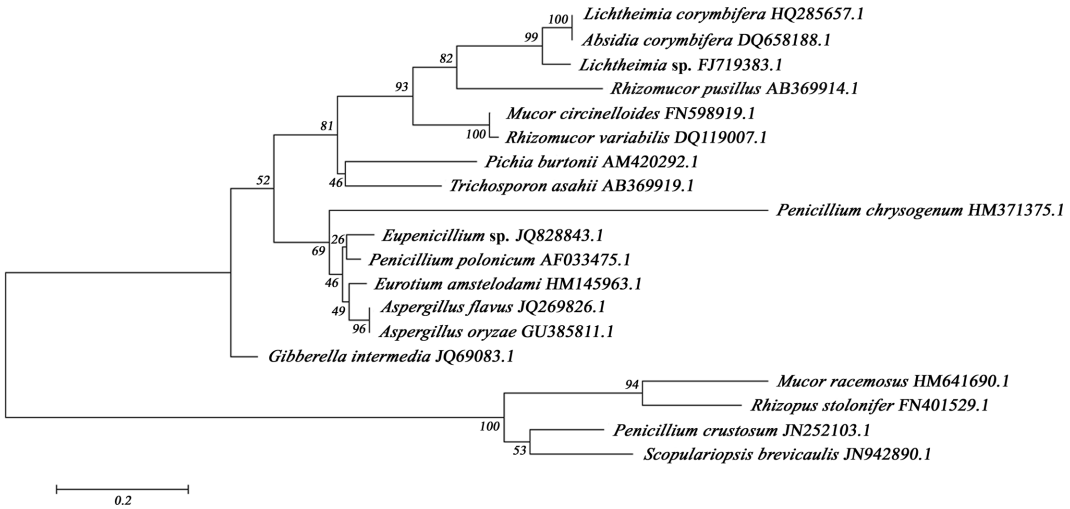

Fig. 2. Neighbor-joining tree resulting from the gene sequences of the internal transcribed spacer region of rDNA in 19 identified fungal strains derived from meju, starter material for fermented soybean products. Bootstrap values supporting branch points are expressed as the percentages and confidence values for individual branches were determined by 1,000 replication.

different pattern in that common species were isolated from different samples. For example, *A. flavus* was isolated from all four meju samples, *A. oryzae* was isolated from samples B and C, and *M. circinelloides* was found in samples A and B (Fig. 1). All identified species had been observed in previous studies [4, 6, 8, 12, 13] except for *Trichosporon asahii* and *Scopulariopsis brevicaulis*. Although *S. chartarum* and *S. trigonospora* have been reported in a previous study [8], *S. brevicaulis* has not previously been isolated. In addition, this is the first report of the genus *Trichosporon* in the mycoflora of a fermented soybean product.

The phylogenetic relationships of all identified isolates were presented (Fig. 2). A clade with significant support by bootstrap analysis included species from different meju samples. These results suggested that common origin of fungal flora in soybean products irrespective of the separated geographic origin and fermentation method.

Extracellular enzymatic characteristics that are believed to be important for traditional fermentation, such as protease and amylase activities, were analyzed for all isolated fungi (Table 1). To measure protease and amylase activities, agar blocks containing actively growing mycelia from 10-day-old cultures were transferred to the center of petri dishes containing casein [14] and starch media [15], respectively. The dishes in triplicate were maintained at 30°C for 7 days. To confirm the presence of true proteolytic and amylolytic activities, an acid solution of mercury chloride and Gram's iodine solution, respectively, were added to each plate. Extracellular enzymatic activity was certificated by the formation of a transparent halo zone. These procedures revealed differences hydrolysis region around the colony which indicated the protease and amylase producing ability of the fungal strains [14, 15]. All *Aspergillus* isolates showed high levels of both enzymes. In addition, most of the isolates from the meju showed at least one of the enzymatic activities except for *A. corymbifera* and *R. stolonifer*. *A. corymbifera*, which is known as a common causal agent of mucormycosis requiring high enzymatic activities necessary for food processing and pathogenesis and also found in another meju study [13], did show no enzymatic activity under the current culture conditions. Therefore, although it has not been detected in this study, it will be of great interest to analyze the enzyme activities of *A. corymbifera* on sterile culture and different culture conditions. Although several studies have shown a broad spectrum of enzymatic activity from *R. stolonifer*, including starch utilization [16], our assay did not successfully detect amylase activity, possibly because of differences in the assay conditions or culture conditions.

Mycotoxin contamination of fermented food has attracted worldwide attention because of its adverse effects on human health and livestock [17, 18]. It is possible for meju to be contaminated with aflatoxins (AFs; Sigma, St. Louis, MO, USA) produced by *A. flavus* or *A. parasiticus*, which have similar morphological and biochemical properties to *A. oryzae* or *A. sojae* [19-21]. In addition, 3 strains among 51 isolates of the *A. flavus* and *A. oryzae* group present in meju were previously shown to be aflatoxinogenic [4]. Since we found 51 isolates of the *A. flavus* and *A. oryzae* group, we analyzed for the production of mycotoxin from all isolates. To determine production of sterigmatocystin (ST; Sigma) or AF, 1×10^6 conidia/mL for each isolates were cultured in the fungal complete media 3 mL under constant dark condition at 30°C for 7 days without agitation. The whole mycelia and culture filtrate were then extracted by 1 mL chloroform thoroughly. The ST/AF in 50 µL of chloroform-extract was separated by silica gel TLC plate (Merck, Darmstadt, Germany) in toluene : ethyl acetate : acetic acid (80 : 10 : 10 [v/v/v]) solvent system, followed by treatment of 10% aluminum chloride in 95% ethanol to enhance ST/AF fluorescence upon exposure to a longwave UV light [22]. As shown in Fig. 3, we did not detect the presence of AF or ST from any of the isolates in our culture conditions.

In conclusion, the current study examined the mycoflora from the starting material for fermented soybean products, meju in four well-separated geographic origins. From four meju samples, a total of 112 fungal isolates were obtained and identified as belonging to 13 genera and 19 species. There were four isolates of *Lichtheimia* and two isolates of

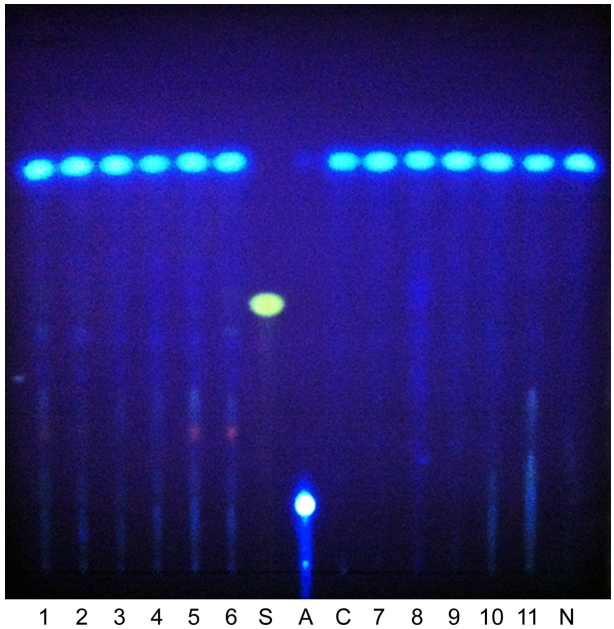

Fig. 3. Thin layer chromatography (TLC) analysis of sterigmatocystin and aflatoxin from culture extracts of *Aspergillus flavus* and *A. oryzae* strains in this study. A representative TLC plate of the organic extracts from culture filtrate of 12 *A. flavus* and *A. oryzae* strains is shown. Strains are indicated under the panel by the lane number. Lanes 1~6 contain extracts from *A. flavus* strains and lanes 7~11 contain extracts from *A. oryzae* strains. Lanes labeled S, A, C, and N represent sterigmatocystin standard, aflatoxin standard, culture medium alone, and a culture extract of no aflatoxigenic *A. oryzae* strain (KACC 44823) [23] as a negative control, respectively.

Eupenicillium of unclassified species. Biochemical analysis of protease and amylase activities, which are closely related to fermentation, indicated that extracellular enzymatic activity was observed in most isolated strains and was particularly high in general fungal species of *Aspergillus*. No aflatoxigenic fungal isolates were detected in this study.

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