Making Soy Sauce Using Direct Fermentation of Defatted Soybean Meal without the meju (Soybean Koji) Preparation Process

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Received September 29, 2015
Revised January 15, 2016
Accepted January 15, 2016
Published online June 30, 2016

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pISSN 1226-7708
eISSN 2092-6456

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Abstract Soy sauce prepared via direct fermentation of defatted soybean meal (DFSM) using halophiles without addition of dried, fermented soybeans or meju was evaluated. DFSM was fermented using single and mixed cultures of *Oceanobacillus kimchii* and *Bacillus pumilus* under 18% salinity conditions. Amounts of total organic nitrogen, free amino acids, and organic acids in soy sauce prepared with the mixed culture were slightly higher than sauces prepared with single culture. The ingredient content was higher in soy sauce prepared via direct fermentation of DFSM than soy sauce prepared with meju. Microorganisms detected in DFSM fermentation were not detected in the meju culture, except for the 2 halophiles, based on metagenomic analysis. Direct fermentation of DFSM is better than using meju for preparation of soy sauce.

Keywords: meju, soy sauce-fermenting culture, *Oceanobacillus kimchii*, *Bacillus pumilus*, metagenomic analysis

Introduction

Meju is a traditional Korean soybean fermentation starter prepared via natural fermentation without specific microbial seeding or control of microbial contamination. Most microorganisms for meju fermentation originate from dried rice straw, human hands, tools, the fermentation room, and surrounding air (1). Remarkably, dried rice straw is generally and traditionally used as a flooring material for support or as a binding material for hanging meju dough in the meju preparation process (2).

Some of the bacteria and fungi that are naturally inoculated into meju dough may be responsible for production of amino acids and peptides, but some may produce unwanted metabolites (3). The dominant microorganisms observed during traditional meju fermentation are bacterial species belonging to genus *Bacillus*, and fungal species belonging to genus *Aspergillus* (4). Meju quality is determined based on microbial metabolites, free amino acids, and peptides generated from soybean protein via extracellular enzyme catalysts (5). Microbial metabolites generated in meju fermentation may be changed opportunistically via variations in dominant microorganisms and microbial diversity. These changes may be caused by environmental conditions, time, location, and differences in preparation (6). Korean soy sauce is traditionally prepared via simple steeping of meju in brine followed by ripening for >6 months

(7). Microbial metabolites, amino acids, and peptides in fermented meju are naturally extracted during ripening after microorganism inactivation under high salt brine conditions (8).

The quality of traditional Korean soy sauce is determined based on the quality of the meju, which is characterized by meju contents, metabolites, amino acids, and peptides in fermented meju (9). All amino acids, organic acids, and peptides are necessary factors for soy sauce, but some microbial metabolites are unnecessary or unwanted. The diversity of the unnecessary and unwanted metabolites may be proportional to the diversity of microorganisms in meju during fermentation. The diversity of microbial species is opportunistically amended each time meju dough is prepared because dough is randomly inoculated with microorganisms from different sources (10). Generally, meju dough is prepared in the dry and cold season ranging from late autumn to winter. This can reduce contamination of meju dough with microorganisms from the natural environment. Microbial communities inhabiting natural environments tend to be dormant in the fall and winter seasons (11). The seasonal limitation of the meju preparation process may be a passive action for microorganism control; however, a temperature change in the meju fermentation process can actively select thermophilic microorganisms. The temperature for natural meju fermentation facilitates growth of mesophilic microorganisms that cannot grow under thermophilic conditions (45–60°C) (1). Salinity control in the meju-making or soy

sauce preparation process may also act to actively select halophilic microorganisms (12).

In this study, defatted soybean meal with the 2 halophiles *Oceanobacillus kimchii* and *Bacillus pumilus* that are capable of degrading soybean protein under >16% saline conditions was fermented for evaluation of a simplified soy sauce preparation process.

Materials and Methods

Isolation of halophilic bacteria capable of degrading soybean protein Soybean pastes (doenjangs) obtained from 24 different makers of traditional home-made soybean paste were used for isolation of halophilic bacteria capable of producing extracellular proteolytic enzymes. Soybean paste was 100 times diluted with sterilized 16% solar salt solution and spread on soybean powder plate medium composed of 5 g/L defatted soybean powder, 0.1 g/L yeast extract (Duksan, Seoul, Korea), 160 g/L solar salt, and 20 g/L agar (Duksan), then incubated using an incubator (MIR-153; Sanyo, Tokyo, Japan) at 35°C. Clear zone-forming bacterial colonies were picked up and transferred to a soybean powder broth medium composed of 20 g/L defatted soybean powder, 1 g/L yeast extract, and 180 g/L solar salt, then incubated using an incubator at 35°C. Bacterial culture grown in the soybean powder broth medium were streaked on the soybean powder plate medium using a sterilized loop for verification of physiological functionality for extracellular proteolytic enzyme production. Finally, a clear zone-forming bacterial colony was picked up and transferred to fresh soybean powder broth medium and cultivated using an incubator at 35°C. The 16S-rRNA that was amplified from the genomic DNA of the isolates via direct polymerase chain reaction was submitted to Basic Local Alignment Search Tool (BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) in order to identify based on the similarity between the submitted sequence and the database sequences released in the BLAST system.

Qualitative extracellular proteolytic enzyme assays of cell-free culture fluids Isolated bacteria were grown in soybean powder broth medium using an incubator for 48 h at 35°C and centrifuged using a high speed centrifuge (Supra 21K; Hanil Science, Seoul, Korea) at 10,000×g and 4°C for 40 min. The centrifugal supernatant was filtered using 0.22 µm pore membrane filter (Millipore, Billerica, MA, USA) to obtain sterilized cell-free culture fluids. Cell-free culture fluids were absorbed onto sterilized 8 mm paper discs of 1.5 mm thickness (Advantec, Tokyo, Japan) and put in sterilized petri dish (diameter 35 mm, Corning, Corning, NY, USA) in clean bench. The petri dish with paper discs was put in vacuum desiccator (Nalgen, Rochester, NY, USA) and gas was sucked out from the desiccator using a vacuum pump (ABM Greiffenberger Atriebsterchnik, GmbH, Markterdewitz, Germany) under −0.05 MP for 60 min at room

temperature. This procedure was 5 times repeated for concentration of culture fluids in the paper disc. Dried paper discs were placed on soybean protein plate composed of 5 g/L soybean protein concentrate (SPC; ESFood, Gunpo, Korea), 160 g/L solar salt, 20 g/L agarose (HydraGene, Piscataway, NJ, USA) then incubated using an incubator at 35°C for 6 h. The agar plate thickness was adjusted to 3 mm by pouring of 15 mL melting soybean protein agarose gel on sterilized petri dish (diameter 80 mm) using a micropipette (Pipetman; Gilson, Middleton, WI, USA) and sterilized tip. Clear zones around paper discs are qualitative indicators of proteolytic enzyme activity.

Quantitative extracellular proteolytic enzyme assays of cell-free culture fluids Isolated *O. kimchii* and *B. pumilus* were cultivated in soy broth medium for 72 h at 35°C and centrifuged using a high speed centrifuge at 10,000×g at 4°C for 40 min. Sterilized cell-free culture fluids were obtained using 0.22 µm pore membrane filtration of centrifuged supernatants. Approximately 5 g of SPC was mixed with 1 L of cell-free culture fluid and incubated using an incubator at 35°C for 6 h, then the insoluble portion was separated from a supernatant by centrifugation using a high speed centrifuge at 10,000×g and 4°C for 60 min. Sterilized distilled water was used instead of cell-free culture fluids as a control. Residual soybean proteins that were not hydrolyzed by extracellular proteolytic enzymes were completely dried at 110°C using a dry oven (MOV-212; Sanyo) for 6 h and weighed using a precision balance (E02130; Ohaus, Parsippany, NJ, USA) to a constant weight. Extracellular proteolytic activities of cell-free culture fluids of *O. kimchii* and *B. pumilus* were determined based on testing in triplicate.

Direct fermentation of soy sauce using isolates A soybean meal slurry composed of 100 g/L defatted soybean meal, 180 g/L solar salts, and 5 g/L yeast extract was pasteurized via heat treatment using an autoclave at 90°C for 90 min. *O. kimchii* and *B. pumilus* previously cultivated in soy broth medium for 3 days were used as seeds separately or mixed into the pasteurized soybean meal slurry. A soy sauce-fermenting culture was prepared using seed culture inoculation at 3%, v/v of the pasteurized soybean meal slurry. Soy sauce-fermenting cultures were aerobically incubated with shaking incubator (SI-600R; Jeiotech, Daejeon, Korea) at 120 rpm at 35°C for 20 days, then statically cultivated in an incubator without shaking at 35°C for 130 days.

Preparation of soy sauce A 150 day incubated soy sauce-fermenting culture was pasteurized using heat treatment at 70°C using water bath (Jeiotech SWB-20; Daejeon, Korea) for 60 min, then allowed to settle statically for natural precipitation of fine particles. Soy sauce was obtained from the clear supernatant by filtration of floating particles through cheesecloth. Finished soy sauce was chemically analyzed on the basis of necessary ingredients for soy sauce.

Analysis of organic nitrogen compounds and ammonium Soy sauce organic nitrogen compounds were analyzed using the micro-Kjeldahl method (13) and found to be soluble in a basic solution. The total organic nitrogen content as amino acids (AAs) and peptides was determined using a nitrogen-to-protein conversion factor of 6.25 (14). Ammonium was analyzed using a Dionex DX-500 ion chromatography (Dionex, Herisau, Switzerland) equipped with an IonPacDionex CS12A cation column. Methanesulfonic acid (20 mM 100%) was used as a mobile phase. The column temperature and flow rate were set at 35°C and 0.8 mL/min.

Analysis of free AAs Free AAs of soy sauce was analyzed based on a general technique used for food analysis. Soy sauce was diluted 20x with a sodium citrate buffer and filtered through a Sartorius 0.45- μ m filter (GmbH, Gottingen, Germany). Free AAs were determined using an LCA k04/Na ammonia filtration column (4.6 $\times$ 100 mm) (Sykam, GmbH, Eresing, Germany) equipped with a Sykam S433 automatic AA analyzer. All buffers used for hydrolysate program AA analysis were purchased from Sykam.

Analysis of minerals Soy sauce minerals were analyzed using an inductively coupled plasma optic emission (ICPOE) spectrometer (Spectro Analytical Instrument, Kleve, Germany). Soy sauce was filtered through a Sartorius 0.45- μ m membrane filter and diluted 20x with double-distilled water. Diluted filtrates were then directly injected into the ICPOE under the specific wavelengths of 279.553 nm for Mg, 589.592 nm for Na, 766.491 nm for K, 396.847 nm for Ca, 257.61 nm for Mn, 213.856 nm for Zn, and 324.745 nm for Cu. Mineral concentrations were calculated based on absorbance values by ICPOE spectrometer obtained using standard materials (AccuTrace™ Reference Standard, AccuStandard, New Haven, CT, USA) and dilution rates for determination of suitable analytical concentration.

Analysis of organic acids Soy sauce organic acid contents were analyzed using an HPLC apparatus (Beckman, Coulter System Gold, Brea, CA, USA) with a Shodex RSpak KC-811 ion-exclusion column (Showa Denko, Tokyo, Japan) and a Shodex RI-101 refractive index detector (Munich, Germany). The column and detector were set at 40°C. The mobile phase was 6 mM HClO₄ and the flow rate was adjusted to 0.8 mL/min. Analytical sample of soy sauce was prepared by centrifugation using a high speed centrifuge for 30 min at 12,000 $\times$ g and 4°C and filtration through a 0.45- μ m membrane filter and subsequent desalting using an Amberlite IR120 ion-exchange column (Rohm and Haas, Philadelphia, PA, USA). Thirty μ L of a filtered and desalted soy sauce sample was injected. Organic acid concentrations were calculated based on peak areas in chromatograms generated using standard materials.

Metagenomic analysis of soy sauce-fermentation cultures Cultures of defatted soybean meal for soy sauce fermentation were statically incubated using an incubator for 120 days were subjected to

metagenomic analysis. Slurry-type cultures were centrifuged using a high speed centrifuge at 8,000 $\times$ g and 4°C for 20 min to separate precipitates, including bacterial cells. Precipitates were frozen at -85°C using a deep freezer (MDF-U53V; Sanyo) and transported on dry ice to MacroGen Ltd. (Seoul, Korea) where metagenomic analysis was performed. DNA was extracted from bacterial cells using a stool Power water® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), and a genetic library was prepared using PCR products following the GS FLX plus library prep guide (Roche Diagnostics GmbH, 454 Sequencing, Cat. No. 05 608 228 001, 68298 Mannheim, Germany: www.roche-applied-science.com). Emulsion (em) PCR, for clonal amplification of a purified library, was carried out using a GS-FLX plus emPCR Kit (454; Life Sciences, Branford, CT, USA). The 16S universal primers 27F (5' GAGTTTGATCMTGGCTCAG 3') and 518R (5' WTTACCGCG-GCTGCTGG 3') were used for amplification of 16s rRNA genes using the FastStart High Fidelity PCR system (Roche, Basel, Switzerland). After the PCR reaction was complete, products were purified using AMPure beads (Beckman Coulter). Sequencing was performed at MacroGen Ltd. using a 454 GS-FLX plus system based on the Next Generation Sequencing technique (Illumina, Sandiego, CA, USA: www.illumina.com). All sequences were compared against the Silva rRNA database (<http://www.arb-silva.de>) using BLAST, and taxonomical hierarchies were assigned on the basis of more than 97% similarity for species, 94% for genus, 90% for family, 85% for order, 80% for class, and 75% for phylum. CD-HIT-OTU software (Research Company of Weizhong Li, San Diego, CA, USA) was used for clustering by using computational technique and analysis of operational taxonomic units (OTU) as indicators of community richness (15,16). Microbial communities, Shannon-Weaver diversity index values, and Simpson index values were analyzed using Mothur software.

Results and Discussion

Phylogenetic analysis of halophilic isolates Fermented foods are useful sources of isolate bacteria that can be used as fermentation starters and probiotics for humans and livestock (17). *O. kimchii* KFCC11607P isolated from soybean paste was phylogenetically close to *O. kimchii* X50 that originated from traditional Korean fermented foods. *B. pumilus* KFCC11608P was phylogenetically close to several *Bacillus* genera related to fermented soybean foods (Fig. 1). Use of *O. kimchii* and *B. pumilus* for food applications may not require experimental verification because these taxa originate from Korean traditional soybean paste that has been ripened under 16-20% salinity for >1 year and used for cooking and seasoning (18,19). However, some *B. pumilus* strains that originate from soil and feces have been reported to cause plant and human diseases (20). Pathogenic bacteria may be isolated generally from clinical samples handled in hospitals but fermenting bacteria are isolated generally from fermented foods (21). Accordingly, the halophiles used in this

Fig. 1. Phylogenetic tree constructed based on 16S-rDNA sequences obtained from *Oceanobacillus kimchii* (KFCC11607P) and *Bacillus pumilus* (KFCC11608P).

study were safe bacteria for soy sauce fermentation and cannot cause foodborne illness because of bacterial isolation from fermented foods.

Extracellular proteolytic activities of cell-free cultures The function of *O. kimchii* and *B. pumilus* was to degrade protein of defatted soybean meal using extracellular proteases under high salt conditions for production of free amino acids and peptides. Cell-free *O. kimchii* and *B. pumilus* cultures degraded soybean proteins that were dispersed in an agar gel plate (Fig. 2). Specific activities of hydrolytic enzymes in cell-free *O. kimchii* and *B. pumilus* cultures on insoluble soybean protein were 24.82 and 24.29 mg/L/mg protein/h, respectively, evaluated on the basis of a weight difference in soybean proteins before and after reactions in incubation time units of mg/protein/h (Table 1). The specific activity of the cell-free *O. kimchii* culture was slightly higher than the activity of the cell-free *B. pumilus* culture. Thus, soluble organic nitrogen consisting of free amino acids and peptides accumulated in soy sauce-fermenting cultures in proportion to the specific activity of extracellular enzymes and incubation time (22,23). The main nutrient for bacterial cell growth is soybean protein, which induces bacterial cells to excrete proteolytic enzymes and to produce the major free amino acid and peptide ingredients of soy sauce. Qualitative and quantitative assay results for proteolytic activities showed that *O. kimchii* and *B. pumilus* exerted the physiological actions required to produce soy sauce via direct fermentation of defatted soybean meal.

Total nitrogen and free amino acids in soy sauce During the

Fig. 2. Proteolytic activities of cell-free *Oceanobacillus kimchii* (A) and *Bacillus pumilus* (B) culture fluids for soy protein concentrates.

bacterial growth that is responsible for soy sauce fermentation, extracellular enzymes that are excreted can degrade proteins after bacterial growth has stopped. Products generated from soybean proteins via enzymatic degradation are mostly peptides and amino acids that are detected as total organic nitrogen compounds in soy sauce (24). Some of the peptides and free amino acids may be consumed by growing bacterial cells; however, some may accumulate in soy sauce-fermenting cultures (25). The accumulation-to-consumption ratio of peptides and free amino acids may be higher under oxygen-limited conditions due to a bacterial growth limitation. Soy sauce-fermenting cultures were incubated under oxygenated conditions for 20 days to activate bacterial growth, then incubated under static conditions for induction of free amino acid and peptide accumulation for 130 days. Amounts of 28,880, 29,130, and 30,360 mg/L of total organic nitrogen were accumulated in soy sauce prepared via

Table 1. Quantitative analysis of soy protein hydrolysis by cell-free culture fluids of *O. kimchii* and *B. pumilus* culture

Factors for assay	Hydrolysis of soy protein (mg/L) by hydrolysis of cell free culture fluid		
	Control	<i>O. kimchii</i> culture	<i>B. pumilus</i> culture
Initial weight (mg/L)	5,160±4	5,085±6	5,105±3
Final weight (mg/L)	5,196±21	5,006±18	5,008±23
Variation (mg/L)	+32±2	-79±4	-97±3
Protein conc. of a cell-free culture fluid (mg/mL)	-	0.53	0.66
Soy protein degradation (mg/L/mg protein/h)	-	24.82	24.49

Table 2. Contents of organic nitrogen, ammonium, and free amino acids in soy sources prepared via fermentation of defatted soybean meal using *Oceanobacillus kimchii* (A), *Bacillus pumilus* (B), a mixture of *O. kimchii* and *B. pumilus* (C), and prepared with meju

Classification	Contents	Soy sauces			
		A	B	C	Meju
Total Nitrogen (mg/L)	Soluble organic nitrogen	28,880±347	29,130±312	30,360±384	19,386±163
	Ammonium	12.0±0.3	11.0±0.3	13.0±0.4	62.8±1.7
Free amino acids (%)	Aspartic acid	0.12±0.00	0.11±0.00	0.12±0.00	0.07±0.00
	Threonine	0.11±0.00	0.12±0.00	0.12±0.00	0.05±0.00
	Serine	0.08±0.00	0.08±0.00	0.09±0.00	0.06±0.00
	Glutamic acid	0.25±0.01	0.27±0.01	0.28±0.01	0.17±0.01
	Proline	0.08±0.00	0.08±0.00	0.08±0.00	0.04±0.00
	Glycine	0.06±0.00	0.07±0.00	0.07±0.00	0.05±0.00
	Alanine	0.16±0.00	0.17±0.00	0.17±0.00	0.12±0.00
	Cysteine	0.00	0.00	0.00	0.00
	Valine	0.11±0.00	0.12±0.00	0.12±0.00	0.08±0.00
	Methionine	0.00	0.00	0.00	0.00
	Isoleucine	0.11±0.00	0.11±0.00	0.12±0.00	0.08±0.00
	Leucine	0.19±0.00	0.18±0.00	0.19±0.00	0.12±0.00
	Tyrosine	0.09±0.00	0.08±0.00	0.08±0.00	0.05±0.00
	Phenylalanine	0.13±0.00	0.14±0.00	0.15±0.00	0.09±0.00
	Histidine	0.18±0.00	0.19±0.00	0.19±0.00	0.11±0.00
	Lysine	0.07±0.00	0.08±0.00	0.08±0.00	0.05±0.00
	Arginine	0.06±0.00	0.06±0.00	0.06±0.00	0.00
	Sum (mg/L)	1.78±0.04 (17,800±390)	1.86±0.03 (18,600±280)	1.92±0.03 (19,200±330)	1.22±0.03 (12,200±250)

fermentation using *O. kimchii*, *B. pumilus*, and a mixed culture of the 2 strains, respectively (Table 2). Conversely, approximately 19,300 mg/L total organic nitrogen was accumulated in soy sauce prepared using meju, which was related to the specific activity of the extracellular enzyme. The significant difference in total organic nitrogen between soy sauce prepared via direct fermentation and prepared with meju may have been related to differences between accumulation and consumption of soluble organic nitrogen by microorganisms growing in soy sauce-fermenting and meju-fermenting cultures. Limited oxygen dissolution into the soy sauce-fermenting culture under static conditions can cause an increase in the ratio of accumulation-to-consumption of soluble organic nitrogen in proportion to a reduction in the rate of bacterial growth. However, soluble organic nitrogen may be actively consumed by microorganisms in meju because meju is fermented in air while hanging together with rice straw throughout the fermentation period. Free amino acid amounts in soy sauces prepared via direct fermentation and fermentation with meju were approximately 1.78-1.92 and 1.22 mg/

L, respectively, which were proportional to amounts of soluble organic nitrogen (Table 2).

Tastes of Thr, Ser, Gly, Ala, and Lys amino acids are sweet, Asp, Glu, and Cys are savory, and Met, Ile, and Leu are bitter. Others have no taste. The taste of soy sauce is determined based on mixing sweet, savory, and bitter tastes and not by single amino acids. Tastes of peptides are sweet, bitter, umami, sour, and salty (26). Accordingly, the taste of soy sauce may be improved in proportion to the soluble organic nitrogen content as peptides and total amino acids. Meanwhile, ammonium contents in soy sauce prepared via direct fermentation and fermentation with meju were approximately 11-13 and 162 mg/L, respectively. The ammonium content in soy sauce prepared via direct fermentation may have been too low to influence taste, but the ammonium content in soy sauce prepared with meju fermentation may have influenced the taste. Accordingly, direct fermentation of defatted soybean meal using a single culture of either *O. kimchii* or *B. pumilus* or a mixed culture of *O. kimchii* and *B. pumilus* may be a useful technique for production of high quality soy

Table 3. Contents of minerals and heavy metals in soy sources prepared via fermentation of defatted soybean meal using *Oceanobacillus kimchii* (A), *Bacillus pumilus* (B), a mixture of *O. kimchii* and *B. pumilus* (C), and prepared with meju

Inorganic compounds (mg/Kg)	Soy sauces			
	A	B	C	Meju
Mg	3,987±101	4,123±112	3,992±98	4,152±88
Ca	689±32	707±28	693±16	681±13
Na	16,186±132	16,333±162	16,093±148	16,210±126
K	7,066±184	6,889±202	6,915±189	6,926±178
Zn	>0.02	>0.02	>0.02	>0.02
Cu	>0.01	>0.01	>0.01	>0.01
Mn	>0.01	>0.01	>0.01	>0.01
Pb, As	>0.005	>0.005	>0.005	>0.005

Table 4. Contents of organic acids in soy sauces prepared via fermentation of defatted soybean meal using *Oceanobacillus kimchii* (A), *Bacillus pumilus* (B), a mixture of *O. kimchii* and *B. pumilus* (C), and prepared with meju

Organic acids (mg/kg)	Soy sauces			
	A	B	C	Meju
Citric acid	7,469±218	7,237±202	7,339±196	2,235±89
Malic acid	0	0	0	3,103±77.2
Succinic acid	0	0	0	104.2±3.9
Tartaric acid	0	0	0	0
Levulinic acid	0	0	0	0
Lactic acid	1,508±31	1,532±57	1,621±43	656±21
Acetic acid	1,088±27	1,152±19	1,177±23	1,862±46
Pyroglutamic acid	1,088±43	1,086±34	1,046±43	294±13

sauce based on the total organic nitrogen compound content, including total free amino acids.

Minerals in soy sauce Most minerals in soy sauce probably originate from solar salt. Others may originate from defatted soybean meal considering that 100 g/L of defatted soybean meal or 200 g/L of meju were submerged in 180 g/L of solar salt for fermentation or ripening. Sodium, potassium, magnesium, and calcium contents in soy sauce prepared via direct fermentation and with meju fermentation were not significantly ($p>0.05$) different (Table 3). The mineral content may not be a factor that influences the taste and flavor of soy sauce, but must be considered in regard to nutritional quality (27).

Organic acids in soy sauce Citric, lactic, malic, acetic, and pyroglutamic acids were detected in soy sauce prepared with meju, and citric, lactic, acetic, and pyroglutamic acids were detected in soy sauce prepared via direct fermentation. Organic acid contents in soy sauce prepared via direct fermentation using only a single or both bacterial species were not significantly ($p>0.05$) different, but were significantly ($p<0.05$) higher than contents in soy sauce prepared with meju (Table 4). Acetic acid is volatile and the level was approximately 1.5 times higher in soy sauce prepared with meju than in sauce prepared via direct fermentation. Non-volatile organic acids affect soy sauce taste. Specifically, citric and lactic acids that improve soy sauce taste were present at levels approximately 3.5 and 2.5

times higher in soy sauce prepared via direct fermentation than in sauce prepared with meju (28). Malic acid was not detected in soy sauce prepared via direct fermentation, but was detected at 3,103 mg/kg in soy sauce prepared with meju. Malic acid is a source of undesirable flavors in wines; however, the effect of malic acid on soy sauce has yet to be determined (29).

Diversity of bacteria detected in a soy sauce-fermenting culture

The solar salt concentration for soy sauce-fermenting cultures was maintained at 18%, which inhibits growth of general heterotrophic bacteria. Theoretically, some halo-tolerant and heterotrophic bacteria can opportunistically contaminate soy sauce-fermenting cultures. If other halo-tolerant and heterotrophic bacteria grow in a soy culture, then soy sauce quality can change on the basis of contained ingredients. Bacterial species detected in soy sauces prepared via direct fermentation of defatted soybean meal using either a single or both bacterial species were not different from bacterial species used as seeds for inoculation (Table 5). The bacterial diversity indices of OTU and Chao 1 were commonly 5 in soy sauce-fermenting cultures of *O. kimchii*, *B. pumilus*, and mixture of *O. kimchii* and *B. pumilus* but Shannon and Simpson were low or close to zero (30,31). A 180 g/L solar salt content in soy sauce-fermenting cultures may prevent microorganism contamination but is a suitable condition for the inoculated bacteria *O. kimchii* and *B. pumilus*.

In this study, 2 halophiles isolated from deonjang were used for direct fermentation of defatted soybean meal under 18% salinity

Table 5. Bacterial community detected in soy sauce-fermenting cultures that were initially inoculated with *Oceanobacillus kimchii* (A), *Bacillus pumilus* (B), and a mixture of *O. kimchii* and *B. pumilus* (C)

Community diversity	Soy sauce-fermenting cultures		
	A	B	C
	<i>Oceanobacillus</i> sp 99.5%	<i>Bacillus</i> sp 99.6%	<i>Oceanobacillus</i> sp 47.7%
	Others 0.4%	Others 0.3%	<i>Bacillus</i> sp 51.2%
	Chloroplast 0.1%	Chloroplast 0.1%	Chloroplast 0.1%
Detected bacteria			
Operational taxonomic unit (OTU)	5	5	5
Within-community diversity (Chao1)	5	5	5
Popular diversity index (Shannon)	0.21	0.85	0.55
Biodiversity index of habitat (Simpson)	0.05	0.37	0.26

conditions without using the *Meju* preparation process. Microorganisms were not detected in soy sauce-fermenting cultures, except for *O. kimchii* and *B. pumilus*, which were used in the initial inoculum. The diversity of metabolites accumulated in soy sauce was proportional to the diversity of microorganisms in a meju-fermenting culture or inoculated into a soy sauce-fermenting culture. The proportion of unsuitable metabolites comprising soy sauce ingredients should be proportional to the diversity of metabolites accumulated in soy sauce. Metabolites produced by a specific microorganism can be biochemically identified on the basis of metabolic information, but metabolites produced by unidentified microorganisms are impossible to identify. The quality of soy sauce prepared via direct fermentation of defatted soybean meal can be effectively and repeatedly controlled, similar to brewery sauces, but sauce prepared using meju cannot be controlled because of natural fermentation without microbial inoculation and contamination control. In conclusion, direct fermentation of defatted soybean meal using specific microorganisms grown under saline conditions can be a useful technique for the production of good-quality soy sauce.

Disclosure The authors declare no conflict of interest.

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