Making soy sauce from defatted soybean meal without the meju process by submerged cultivation using thermophilic bacteria

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Abstract The diversity of thermophilic bacteria was not significantly altered while growing in a defatted soybean meal (DFSM) slurry at 60 °C for 10, 20, and 30 days. Five species of thermophilic bacteria, which belong to the genera *Aeribacillus* (temperature gradient gel electrophoresis [TGGE] band no. 1), *Saccharococcus* (TGGE band no. 2), *Geobacillus* (TGGE band no. 3), *Bacillus* (TGGE band no. 4), and *Anoxybacillus* (TGGE band no. 5), were detected in the fermenting DFSM slurry. The cell-free culture fluid obtained from the fermenting DFSM slurry on day 14 hydrolyzed starch and soy protein at 60 °C but not at 30 °C. Soy sauce (test soy sauce) was prepared from the fermented DFSM slurry after a 30 day cultivation at 60 °C and a 60 day ripening at 45 °C. Free amino acid (AA) and organic acid contents in the soy sauce increased in proportion to the fermentation period, whereas ammonium decreased proportionally. Mg and Ca contained in the soy sauce decreased proportionally during fermentation and were lower than those in the non-fermented DFSM extract (control). Spectral absorbance of soy sauce prepared from the fermented DFSM slurry was maximal at 430 nm and increased slightly in proportion to the fermentation period. The aroma and flavor of the test soy sauce were significantly different from those of traditional Korean soy sauce. Conclusively, soy sauce may be prepared directly from the fermented DFSM slurry without meju-preparing process and fermentation period may be a factor for control of soy sauce quality.

Keywords Soy sauce · Thermophile · Defatted soybean meal · Submerged culture · Phylogenetic analysis

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Introduction

Korean soy sauce (Ganjang) is traditionally prepared by extracting meju with brine made of solar salt. Meju is prepared by naturally fermenting cooked soybeans using microorganisms originating generally from rice straw (Heu et al. 1999). The microorganisms that originate from rice straw are primarily *Bacillus* and *Aspergillus*, which are dominant in meju-fermenting cultures (Kim et al. 2013a, b; Lee et al. 2008). Some microorganisms from rice straw may be responsible for meju fermentation, whereas others may be microbial contaminants that produce unwanted or unnecessary compounds. The contaminants may cause the soy sauce to deteriorate by producing unnecessary or unwanted metabolites (Jung et al. 2012); however, a pure culture technique is difficult to employ during the traditional Korean meju-fermenting process. The meju-fermentation technique has been empirically and domestically developed without a system for sterilization and aseptic fermentation using natural microorganisms.

Meju fermentation conditions can be influenced by several factors, including the time, place, and maker because dominant species and the diversity of microorganisms that are responsible for meju fermentation or contamination may be opportunistically altered according to these factors (Kim et al. 2011a, b). Soy sauce quality is determined by meju quality, considering that soy sauce is prepared by simple extraction of soluble compounds contained in meju using brine (Kim et al. 2011a, b; Ham et al. 2004). Extracting soluble compounds containing amino acids (AAs), peptides, organic acids, and minerals may not influence soy sauce quality; however, the long-term ripening achieved during the soluble compound-extracting process from meju may chemically stabilize the soy sauce ingredients. The period for Korean meju fermentation may be >5 weeks but <10 weeks, whereas ripening of Korean soy sauce may take >12 months. This long period of ripening may chemically neutralize unwanted soy sauce ingredients

that originate from the meju naturally fermented by various microbial communities.

Moderate thermophiles, which thrive in temperatures of 45–65 °C, can grow in the temperature range for mesophiles and may be inoculated opportunistically into meju dough with mesophilic bacteria. Accordingly, most bacteria growing in meju-fermenting culture incubated under temperature conditions for human living must be mesophiles (Lee et al. 1977; Reysenbach et al. 2001; Brock 1978). In contrast, thermophilic bacteria can be dominant or selectively grow in a meju-fermenting culture incubated at 60 °C. Hot springs and saunas are common thermal environments for normal growth of thermophilic bacteria (Ward et al. 1998; Hugenholtz et al. 1998). Some thermophilic bacteria have been isolated from a sauna and grew at 60 °C (Kim et al. 2013a, b; Lee and Park 2012). In this study, we evaluated the possibility of directly preparing soy sauce by submerged fermentation of a defatted soybean meal (DFSM) slurry using thermophilic bacteria but without a process to prepare the meju.

Materials and Methods

Experimental data

Mean and standard deviation values of results were obtained from triplicate tests. All analytical data were mean values of triplicate experiments except the DNA used for temperature gradient gel electrophoresis (TGGE) and the cell-free fluid of the fermented DFSM slurry for the extracellular enzyme assay was obtained from a mixture of three experiments.

Fermentation of the DFSM slurry

A total of 1,000 g of DFSM was steeped in 3,000 g of drinking water. The DFSM slurry was cooked (sterilized) for 30 min in an autoclave and then the temperature was adjusted to 65 °C to protect growth of mesophilic bacteria. Ten g of sliced rice straw (5–10 mm length) was added to the 65 °C-heated DFSM slurry for inoculation and then cultivated at 60 °C for 10, 20, and 30 days in a 50 rpm-rotating condition. The boiled DFSM slurry prepared for the control experiment was not inoculated but was incubated at 60 °C for 30 days in a 50 rpm-rotating condition.

Extracellular enzyme assay

The activity of the extracellular enzymes produced by thermophilic bacteria grown in the fermenting DFSM slurry was qualitatively evaluated based on the clear zone generated around a paper disk containing cell-free culture fluid from the fermenting DFSM slurry. Ten mL of culture was isolated from the fermenting DFSM slurry cultivated for 30 days and

centrifuged at 5,000×g for 30 min. The supernatant was filtered with a 0.22 µm membrane filter (Millipore, Milford, MA, USA) for sterilization and absorbed on a paper disk (thickness 1.5 mm, diameter 8 mm; Advantec, Tokyo, Japan) that was sterilized previously. Agar plates containing soybean protein or starch were prepared previously without pH control. Five g/L of soybean protein or corn starch slurry and 40 g/L of agar slurry was separately autoclaved and then mixed together equivalently after autoclaving. Ten mL of the mixture was put in a Petri dish (diameter, 90 mm; height, 15 mm) and coagulated at room temperature. The paper disks containing 100 µL cell-free culture fluid were aseptically dried in a refrigerator for 120 min, placed on an agar plate, incubated at 30 °C and 60 °C for 6 h, and the zones of hydrolysis were observed. After the incubation, 1 mL of potassium iodide solution (2.5 %) was spread on the agar plate to clearly separate hydrolyzed protein or starch from non-hydrolyzed protein or starch.

Soy sauce preparation

The fermented DFSM slurry incubated for 10, 20, and 30 days was salted by adding 200 g/L Solar salt and then ripened at 45 °C for 60 days. The serially fermented, salted, and ripened DFSM slurry was filtered to separate solid particles from the liquid fraction, which was the test soy sauce. The non-fermented (non-inoculated) DFSM slurry was directly salted with 200 g/L Solar salt and ripened at 45 °C for 60 days as a control. The liquid fraction separated from the non-fermented but salted and ripened DFSM slurry by filtration was the DFSM extract but not soy sauce. The appearance (color) of the prepared soy sauce and the DFSM extract was externally and spectrally compared to analyze the effect of fermentation and ripening on the general features of the soy sauce.

TGGE

Bacterial DNA was directly extracted from the fermenting DFSM slurry incubated for 10, 20, and 30 days to analyze variations in the bacterial community according to incubation time. DNA was directly extracted from the mixture of the fermenting DFSM slurry using a Power Soil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) and a FastPrep-24 bead beater (MoBio). 16S-rDNA was amplified using DNA extracted from the DFSM culture. A variable region of the 16S-rDNA was amplified with the forward primer (eubacteria V3 region, 341f 5'-CCTACGGGAGGCAGCA G-3') and the reverse primer (universal V3 region, 518r 5'-ATTACCGCGGCTGCTGG-3'). A GC clamp (5'-CGCCCG CCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG G-3') was attached to the 5'-end of the GC341f primer (Cheung and Kinkle 2001). The TGGE system (Dcode™, Bio-Rad Hercules, CA, USA) was operated in accordance

with the manufacturer's specifications. Aliquots (45 μ L) of the polymerase chain reaction products were electrophoresed on gels containing 8 % acrylamide, 8 M urea, and 20 % formamide in a 1.5 $\times$ concentrated TAE (Tris, acetate, and EDTA) buffer system at a constant voltage of 100 V for 12.5 h, followed by 40 V for 0.5 h, with a temperature gradient of 39–52 °C. The gel was equilibrated to the temperature gradient for 30–45 min prior to electrophoresis (Jeon and Park 2011).

Amplification and identification of DNA extracted from the TGGE bands

DNA was extracted from the TGGE bands and purified using a DNA gel purification kit (Accuprep; Bioneer, Daejeon, Korea). The purified DNA was subsequently amplified using the same primers and procedures employed for TGGE sample preparation, but the GC clamp was not attached to the forward primer. The species-specific identities of the amplified variable 16S-rDNAs were determined based on sequence homology, according to the information released in the GenBank database system.

Phylogenetic analysis

A phylogenetic tree for the five DNAs extracted from the TGGE gel was constructed using the neighbor-joining method in the Molecular Genetic Analysis computational program (Tamura et al. 2007). Bootstrap proportions were used to assess the robustness of the tree with 1,000 bootstrap replicates. The phylogenetic analysis was performed by correlating a comparison of the sequences of the five DNAs extracted from the TGGE gel without reference DNA.

Analysis of free AAs and ammonium

Free AAs in the soy sauce were analyzed based on a general technique employed for food analysis (Jeon et al. 2002). Soy sauce and the DFSM extract were diluted 20-fold with sodium citrate buffer and filtered through a 0.45 μ m filter (Satorius, GmbH, Gottingen, Germany) and were used for AA analysis. Free AAs were determined with an ammonia filtration column (LCA, k04/Na, 4.6 $\times$ 100 mm, Sykam, GmbH, Eresing, Germany) equipped with an S433 automatic AA analyzer (Sykam). All buffers used for AA analysis with the hydrolysate program were purchased from Sykam: Buffer A-1, buffer B-1, regeneration solution, and dilution buffer. Ammonium was analyzed using an ion chromatograph (Dionex DX-500, Sunnyvale, CA, USA) equipped with a cation column (IonPac, Dionex CS12A). A 100 % 20 mM methanesulfonic acid solution was used as the mobile phase. Column temperature and flow rate were adjusted to 35 °C and 0.8 ml/min, respectively.

Analysis of organic acids

Organic acid content of the soy sauce and the DFSM extract were analyzed by high performance liquid chromatography (HPLC) (Coulter System Gold; Beckman, Brea, CA, USA) with an ion-exclusion column (Shodex, Rspak KC-811, Showa Denko, Tokyo, Japan) and a refractive index detector (Shodex, RI-101). The column and detector were adjusted to 40 °C. The mobile phase was 6 mM HClO₄, and flow rate was adjusted to 0.8 ml/min. The samples were prepared via 30 min of centrifugation at 12,000 $\times$ g and 4 °C, filtered through a 0.45 μ m membrane filter (Sartorius), and subsequently desalted by passing them through an ion-exchange column (Amberlite IR120, Rohm and Haas, Philadelphia, PA, USA). Thirty μ l of filtered and desalted sample was injected into the HPLC. Organic acid concentration was calculated based on the peak area in the chromatogram generated using standards.

Analysis of minerals

Minerals were analyzed using an inductively-coupled plasma optic emission (ICPOE) spectrometer (SPECTRO Analytical Instrument, Kleve, Germany). The soy sauce and the DFSM extract were centrifuged at 12,000 $\times$ g and 4 °C for 30 min, filtered through a 0.45 μ m membrane filter (Sartorius), and diluted 20-fold with double-distilled water. The diluted filtrate was then directly injected into the ICPOE under specific wavelengths for Mg (279.553 nm), Na (589.592 nm), K (766.491 nm), Ca (396.847 nm), Mn (257.61 nm), Zn (213.856 nm), and Cu (324.745 nm). Mineral concentrations were calculated based on the absorbance obtained using standards (AccuTraceTM Reference Standard, AccuStandard, New Haven, CT, USA) and dilution rates.

Organoleptic test

After 30 days of fermentation and 60 days of ripening, the aroma and flavor of the soy sauce were organoleptically compared by 12 panelists. Aroma was compared by inhaling air around the mouth of test tubes (soy sauce volume, 10 ml; tube volume, 15 ml) containing the undiluted soy sauces. The distance between the nose and test tube mouth was adjusted horizontally to 10 cm. Flavor was compared using the tongue without swallowing by dropping undiluted soy sauce (100 μ l) on the center of the upper part of the tongue. For the double comparison, the soy sauce was used twice as a test group and two traditional Korean soy sauces were used as references. The similarity between the references and the test group was determined based on the following five criteria: completely the same (5 points), almost the same (4 points), slightly different (3 points), different (2 points), and completely different (1 point). Preference was determined based on the following five criteria: very good (5 points), good (4 points),

same (3 points), bad (2 points), and very bad (1 point). Aroma and flavor were comprised of a comparative value, and preference was an absolute value. The result of sensory tests was statistically analyzed using one-sample *t*-test, in which the statistical significance was determined based on the probability value ($p < 0.05$).

Results and Discussion

Variations in the bacteria grown in the fermenting DFSM slurry

The temperature of the DFSM slurry was maintained at 60 °C during the 30 days after inoculation with dried rice straw. Thermophilic or thermotolerant bacteria originating from the rice straw may have selectively grown and survived due to the plentiful nutrients. As shown in Fig. 1, the bacterial community that grew in the DFSM-submerged culture was not significantly influenced by the incubation period of 10, 20, and 30 days. Five bacterial species were survived or maintained in fermenting DFSM slurry during incubation at 60 °C

Fig. 1 Temperature gradient gel electrophoresis profiles of 16S-rDNA amplified with DNA extracted from bacteria grown in the fermenting defatted soybean meal (DFSM) slurry on days 10, 20, and 30, respectively

according to the TGGE band patterns. This result indicates that some of the thermophiles originating from the rice straw might adapt physiologically to 60 °C and grow stably in the nutritional conditions of the DFSM slurry for at least 30 days (Rannekleiv and Bååth 2001).

Identification of bacteria grown in the fermenting DFSM slurry

The variable region of 16S-rDNA separated on a TGGE gel (Fig. 1) was identified based on the sequence homology shown in Table 1. Bacteria grown in the DFSM slurry were 97–99 % homologous with thermophiles, including *Aeribacillus pallidus* (TGGE band no. 1), *Saccharococcus thermophilus* (TGGE band no. 2), *Geobacillus thermoglucosidasius* (TGGE band no. 3), *Bacillus smithii* (TGGE band no. 4), and *Anoxybacillus gonensis* (TGGE band no. 5) and functioned as a biocatalyst to make the soy sauce from soybean meal (Fig. 4). However, all thermophilic bacteria were not employed as biocatalysts for soy sauce preparation before and were not presently employed. This may be a natural consequence because the fermentation process for meju and the ripening process for soy sauce are performed at room or ambient air temperature. This was a clue that some thermophilic bacteria are distributed in temperature conditions for mesophilic bacteria and can selectively grow under the thermal conditions of DFSM slurry incubated at 60 °C (Knaysi 1964; Ratkowsky et al. 1982). Thus, both mesophilic and thermophilic bacteria can be inoculated into the DFSM slurry but thermophilic bacteria selectively grow at 60 °C (Knaysi 1964).

Table 1 GenBank accession codes and general character of bacteria identified based on the sequence homology of 16S-rDNA extracted from a temperature gradient gel electrophoresis (TGGE) gel (Fig. 1)

TGGE band No	Bacterial genus or species (homology, GenBank accession code): General characters
1	<i>Aeribacillus pallidus</i> (98 %, NR026515): A Gram-positive, thermophilic, neutrophilic, facultative anaerobic, and spore-forming bacterium
2	<i>Saccharococcus thermophilus</i> (98 %, NR036770): A Gram-positive, thermophilic, aerobic, endospore-forming bacterium
3	<i>Geobacillus thermoglucosidasius</i> (99 %, NR036987): A Gram-positive, thermophilic, starch-hydrolyzing glucosidase-producing bacterium
4	<i>Bacillus smithii</i> (97 %, NR036987): A Gram-positive, facultative thermophilic, starch-hydrolyzing bacterium
5	<i>Anoxybacillus gonensis</i> (99 %, NR025667): A Gram-positive, thermophilic, spore-forming bacterium

Fig. 2 Neighbor-joining phylogenetic tree was constructed based on variable region (bases 341–518) of 16S-rDNA extracted from the DNA band on a temperature gradient gel electrophoresis gel. Bootstrap values > 50 % (1,000 replicates) are given at nodes. Branch length in the tree is proportional to the numbers of nucleotide substitutions as measured by the scale bar (1.0 % sequence divergence)

Phylogenetic analysis of bacteria grown in the fermenting DFSM slurry

The five DNAs extracted from the TGGE gel were classified into TGGE band nos. 1–5 for the phylogenetic analysis (Fig. 1). TGGE band nos. 1 and 4 were supported by a 57 % bootstrap value and TGGE band nos. 2 and 3 were supported by 84 % bootstrap value, as shown in Fig. 2. This result may not support that the phylogenetic similarity between TGGE band nos. 1 and 4 was lower than that between TGGE band nos. 2 and 3 because the phylogenetic analysis was performed without an out-group as a reference and the five different bacterial genera (Table 1) belonged to same family of *Bacillaceae* (Schmidt et al. 2011). The five bacterial species grown in the fermenting DFSM slurry were supposed to

belong to a monophyletic clade based on the dendrogram. It is supposed that the physiological identity of the five thermophilic bacteria for survival or growth at 60 °C may be related with phylogenetic similarity on the basis of the phylogenetic tree.

Hydrolysis of soy protein and starch by the fermenting DFSM slurry

Enzymatic hydrolysis of proteins and carbohydrates in the DFSM slurry by extracellular enzymes may be essential to induce production of organic soy sauce ingredients. The cell-free fluid of the fermented DFSM slurry must contain thermophilic enzymes for hydrolysis of protein and starch, considering that bacteria grew with the DFSM as the sole nutrition source at 60 °C (Connelly et al. 2004). Some of the soluble compounds produced by the extracellular enzymes may have been substrates for bacterial growth but most of them were probably soy sauce ingredients. As shown in Fig. 3, the cell-free fluid of the fermented DFSM slurry catalyzed hydrolysis of both soybean protein and starch at 60 °C but not at 30 °C. The pH of the cell-free fluid from the fermented DFSM slurry was 4.51 ± 0.13 , and the pH of the agar plate for the extracellular enzyme assay was 6.5. Accordingly, the extracellular enzymes excreted by the thermophilic bacteria grown in the DFSM slurry may be active in a pH range between 4.5 and

Fig. 3 Activity of cell-free fluid obtained from fermenting the defatted soybean meal (DFSM) slurry for starch degradation at 60 °C (a) and 30 °C (b), and soybean protein degradation at 60 °C (c) and 30 °C (d)

Table 2 Contents of free amino acids and ammonium in soy sources prepared from serially fermented, salted, and ripened defatted soybean meal (DFSM) slurry

Amino Acids (%)	DFSM extract	Fermentation periods, days		
		10	20	30
Aspartic acid	0.01±0.0003	0.08±0.007	0.09±0.005	0.10±0.004
Threonine	0.01±0.0002	0.07±0.005	0.08±0.005	0.09±0.006
Serine	0.02±0.0005	0.11±0.009	0.12±0.006	0.14±0.008
Glutamic acid	0.05±0.001	0.26±0.01	0.27±0.008	0.33±0.01
Proline	0.00±0.0	0.00±0	0.03±0.0001	0.03±0.001
Glycine	0.01±0.0004	0.05±0.0005	0.06±0.0007	0.06±0.0007
Alanine	0.02±0.0002	0.13±0.006	0.14±0.005	0.17±0.06
Cysteine	0.00±0.0	0.00±0	0.00±0	0.00±0
Valine	0.02±0.0004	0.08±0.002	0.09±0.002	0.10±0.001
Methionine	0.00±0.0	0.02±0.0003	0.03±0.0002	0.03±0.0002
Isoleucine	0.01±0.0003	0.07±0.001	0.08±0.002	0.09±0.001
Leucine	0.02±0.001	0.12±0.004	0.14±0.002	0.16±0.003
Tyrosine	0.01±0.002	0.04±0.001	0.05±0.002	0.05±0.001
Phenylalanine	0.01±0.001	0.07±0.001	0.07±0.0009	0.07±0.001
Histidine	0.03±0.001	0.15±0.009	0.16±0.008	0.19±0.01
Lysine	0.01±0.0003	0.04±0.001	0.07±0.002	0.08±0.002
Arginine	0.01±0.0004	0.01±0.0002	0.07±0.0006	0.05±0.0006
Total	0.24±0.0086	1.30±0.047	1.55±0.038	1.74±0.084
Ammonium (mg/L)	1.6±0.11	3.2±0.15	2.0±0.11	0.6±0.04

6.5. This is useful for soy protein and carbohydrate hydrolysis at <60 °C because the pH of the DFSM slurry must be gradually changed from neutral to acidic during fermentation.

Effect of fermentation period on free AA content in the soy sauce

Free AAs contained in the soy sauce originate from soybean protein, which is produced by catalysis of extracellular proteolytic enzymes produced by the bacteria grown in the DFSM-submerged culture (Bae et al. 1983; Seo and Lee 1992). Some free AAs may be consumed by growing bacteria, whereas others may be soy sauce ingredients (Bumison and Morita 1974). As shown in Table 2, the

free AAs contained in the soy sauce increased in proportion to the fermentation period of the DFSM slurry. The free AAs (0.24 %) in the DFSM extract may be chemically produced from soy protein by ripening under a salted condition or intrinsically contained in the DFSM (Park et al. 1994). The ammonium content in the soy sauce decreased in proportion to fermentation period, whereas the ammonium in the DFSM extract was relatively higher than that in the soy sauce prepared with the 20 day-fermented DFSM slurry. Free AAs are a positive factor but ammonium is a negative factor when determining soy sauce quality. Accordingly, fermentation of the DFSM slurry may be a major factor and ripening may be a minor factor influencing soy sauce quality.

Table 3 Contents of organic acids in soy sources that were prepared from serially fermented, salted, and ripened defatted soybean meal (DFSM) slurry

Organic acids (mg/kg)	DFSM extract	Fermentation periods, days		
		10	20	30
Citric acid	123±4.9	1,959±54.7	3,233±93.4	4,034±106.2
Malic aci7d	86.6±3.3	1,045±37.3	2,358±57.1	3,466±89.6
Succinic Racid	52.6±1.9	785±29.3	1,283±51.3	1,524±66.2
Lactic acid	2.4±0.1	193±7.93	294±16.6	350±13.9
Acetic acid	9.2±0.42	283±11.7	463±20.8	588±24.7
Pyroglutamic acid	4.3±0.13	102±4.3	178±7.7	239±12.4

Table 4 Contents of minerals and heavy metals in soy sources that were prepared from serially fermented, salted, and ripened defatted soybean meal (DFSM) slurry

Inorganic compounds (mg/Kg)	DFSM extract	Fermentation periods, days		
		10	20	30
Mg	2,832±69	2,408±54	2,292±58	2,016±51
Ca	1,056±59	808±28	768±31	711±36
Na	16,560±621	16,450±526	16,994±593	16,220±638
K	3,992±121	4,036±126	4,008±119	4,076±128
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	0.05>	0.05>	0.05>	0.05>

Effect of fermentation period on organic acid content in the soy sauce

Organic acids are produced by fermentative metabolism of carbohydrates and proteins of the bacterial community growing in the DFSM slurry. Organic acids such as citric acid, malic acid, succinic acid, lactic acid, acetic acid, and pyroglutamic acid contained in the soy sauce are mostly metabolites produced by bacteria growing in the DFSM slurry, considering they are contained in the DFSM extract, as shown in Table 3. All of the organic acids tended to increase in proportion to the DFSM fermentation period and showed the same tendency as the free AAs in the soy sauce (Table 2). Acetic acid is a volatile fatty acid that may be a negative factor in soy sauce quality but none of the other organic acids was volatile. Non-volatile organic acids affect the taste of soy sauce. In particular, citric acid and lactic acid effectively improve the taste of soy sauce (Ham et al. 2004). Malic acid is known as a source of undesirable flavor during wine-making; however, its effect on soy sauce has yet to be elucidated (Nielsen and Richelieu 1999). Lactic acid is responsible for flavor in dairy products; however, its effect on soy sauce has yet to be assessed (Urbach 1995).

Effect of fermentation period on mineral content in the soy sauce

Most minerals contained in soy sauce originate from the brine prepared with sun-dried salt and some may have originated from the DFSM. Organic acids produced by bacterial metabolism in the fermenting DFSM slurry may function as a chelator for divalent cations (Ca and Mg). This may cause Mg and Ca contents in soy sauce to vary or be altered by the organic acid content. As shown in Table 4, Mg and Ca contents in the soy sauce were slightly lower than those in the DFSM extract, as expected, and decreased gradually in proportion to the fermentation period and organic acid content (Table 3). The kinds and contents of minerals in soy sauce

except Na are important factor influencing soy sauce quality and may be a concern for healthy cooking.

Fig. 4 Appearance of defatted soybean meal (DFSM) extract and soy sauces prepared with the fermented DFSM slurry for 10, 20, and 30 days, respectively (upper). Absorption spectra of 10 fold-diluted DFSP extract and soy sauce (lower)

Table 5 Organoleptic evaluation of soy sauce made of fermented defatted soybean meal (DFSM) slurry and traditional Korean soy sauce (reference) by comparison of the aroma and flavor difference, and preference level

Test items	Comparison of test soy sauce with traditional Korean soy sauce (reference)			
	Test soy sauce	Reference 1	Test soy sauce	Reference 2
Aroma similarity	2.67±0.65	2.75±0.75		
Flavor similarity	2.67±0.49	2.50±0.52		
Preference level	4.08±0.67	2.42±0.52	4.25±0.62	2.58±0.67

Appearance of the soy sauce

The color of soy sauce may indicate a universal feature of soy sauce because color is determined by soluble organic compounds generated from the DFSM during fermentation. The apparent color between the DFSM extract and soy sauce was significantly different. The DFSM extract obtained by salting and ripening of the DFSM slurry without fermentation was light brown, whereas the soy sauce prepared by serial fermentation, salting, and ripening was dark brown, as shown in Fig. 4 (upper). The color of the soy sauce was slightly darker in proportion to the fermentation period based on the spectral analysis shown in Fig. 3 (lower). Light absorbance of soy sauce at 430 nm increased proportionally to the fermentation period and was also proportional to free AA and organic acid contents. This result may be a clue that thermophilic fermentation of the DFSM slurry is useful to produce ingredients for soy sauce but that ripening of the fermented DFSM under a salting condition may not be a factor that influences soy sauce quality according to color of and nutritional ingredients in the DFSM extract.

Organoleptic character of the soy sauce

The aroma and flavor of the soy sauce made from the test soy sauce may have been significantly different from traditional Korean soy sauce based on the organoleptic test shown in Table 5. The panelists clearly distinguished differences in aroma and flavor of the test soy sauce from the reference soy sauces. This result may have been caused by the character of traditional Korean soy sauces. Traditional Korean soy sauce is made using meju that has been naturally fermented without quality control. The meju must be contaminated by various unwanted microorganisms during fermenting in open air, and the various unwanted metabolites may accumulate in the meju body (Yokotsuka 1961). The quality of soy sauce is opportunistically different each time depending on when and where the meju is prepared. The aroma and flavor of traditional Korean soy sauce are negatively influenced by various unwanted metabolites (Jung et al. 2012; Kim et al. 2011a, b). In particular, the unwanted metabolites impart a specific smell to traditional Korean soy sauce. All panelists detected that specific smell from the traditional Korean soy sauce but not from the test soy sauce. This is a why the panelists preferred the soy

sauce made from the test soy sauce rather than traditional Korean soy sauce.

Conclusion

Boiled soy beans are traditionally crushed and shaped to be a tetrahedral or globular structure to make meju in Korea. The solid meju bound with rice straw is incubated by hanging in the air. This is the major reason why meju becomes contaminated with various unwanted microorganisms. Various microorganisms growing in the meju may be the cause for production of various metabolites. Some of these metabolites may be useful ingredients, whereas other may be unwanted in the soy sauce. The solid meju is gradually dried from the outside to the inside during fermentation and finally completely dried in air. The solid meju culture employed in the process to prepare traditional Korean soy sauce is a major factor that extends the period and process of soy sauce preparation and negatively influences soy sauce quality. Solid fermentation to prepare the meju may not be essential to prepare soy sauce because biochemical hydrolysis of soy protein and metabolite production from carbohydrates contained in soybean can also be generated in submerged cultures. Using thermophilic bacteria and applying submerged cultivation to ferment soybean appears to be a key point to effectively control bacterial contamination and significantly reduce the period for soy sauce preparation. Directly preparing soy sauce from DFSM or soybean is possible using the submerged fermentation process developed in this study.

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