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Volatile Components of Korean Soybean Paste Produced by *Bacillus subtilis* PM3

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A strain producing soybean paste flavor was isolated from traditional Korean soybean paste. The isolate was identified as *Bacillus subtilis* PM3. The neutral fraction representing the traditional soybean paste aroma was obtained from the whole volatile components produced by *B. subtilis* PM3 in cooked soybean. Each separated peak from the neutral fraction of gas chromatogram was identified by gas chromatography-mass spectrometry (GC/MS) and Kovat's retention index, and the aromas of each peak were investigated by a sniffing test with the exercise panel. The twenty-nine components, including six character impact compounds and twelve components of flavors of Korean soybean paste, were confirmed. Some regions of gas chromatogram represented the soybean paste odor. It has been confirmed that traditional Korean soybean paste can be manufactured with the strain *B. subtilis* PM3.

Traditional Korean soybean paste is a fermented food having a unique flavor made of soybean. Due to the development of the gas chromatographic technique, studies on the flavors of soybean paste have been made possible. Many studies have been focused on the microorganisms responsible for the main flavors of soybean paste.

Ji et *al.* (9) reported that 39 and 21 components were identified in the flavors of soybean pastes manufactured with traditional Meju and improved Meju, respectively.

The character impact compounds of traditional Korean soybean paste were reported by Kim *et al.* (12).

Song *et al.* (18) isolated strains from traditional soybean paste and analyzed the flavors produced from the isolated strains by the gas chromatographic method. They reported that the soybean paste flavor was synthesized by *Bacillus* sp., the soybean paste-like flavor by *Mucor* sp., and the musky flavor by *Penicillium* sp..

Kwon *et al.* (14) isolated strains of *Bacillus* species from traditional soy sauce and soybean paste, and reported that these strains are involved in the synthesis of traditional Korean soy sauce and soybean paste flavor.

There are, however, few reports available concerning the volatile components synthesized by the isolated strains. The study of the volatile components is essential for the standardizing of the manufacturing process of traditional soybean paste.

The objective of this study was to investigate the characteristics of the flavors of soybean paste fermented by *Bacillus* sp..

We isolated and identified the strain synthesizing the flavors of traditional Korean soybean paste. We conducted gas chromatography(GC)-sniffing tests and identified the components of the neutral fraction representing the traditional soybean paste aroma produced by the isolated strain.

These results lead us to conclude that traditional Korean soybean paste can be manufactured by using the *Bacillus* sp..

MATERIALS AND METHODS

Experimental Materials

Soybean paste fermented traditionally for more than one year was used to isolate strains. Soybean purchased from the Daegu Agricultural Research and Development Agency was used to manufacture the cooked soybean medium.

Isolation, Selection and Identification of the Bacterial Strain

We isolated strains according to the dilution method of diluting soybean paste (5). Each isolated strain were incubated in the cooked soybean medium (Fig. 1) for 40 days at 30℃, and then the strain producing soy sauce and soybean paste flavors was selected. We characte-

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Soybean
|
Soaking : 16 h
|
Steaming : 106℃, 3 h
|
Crude crushing
|
Weighing : 300 g in Erlenmeyer flask
|
Autoclaving : 121℃, 40 min

Fig. 1. Preparation of the cooked soybean medium.

rized the isolate by observing its cultural, morphological and physiological features based on Bergey's manual of systematic bacteriology (13) and the Genus *Bacillus* (4).

Extraction and Fractionation of the Volatile Components

We extracted the volatile components from the soybean medium that had been incubated by the isolate for 40 days at 30℃, and used the uninoculated soybean medium kept for 40 days at 30℃ was used as control.

A simultaneous steam distillation-extraction (SDE) apparatus (17) was used to extract the volatile components.

The soybean medium (700 g) incubated with the isolate was mixed with the same amount of distilled water, and was put in the sample pot. A solvent diethyl ether (100 ml) was added into the solvent pot. The solvent pot was maintained at 40℃. After increasing the temperature of the sample pot to the boiling point, the volatile components were extracted for two hours.

The extracted whole flavor was fractionated by adjusting the pH according to Fujimaki's method (3). The neutral fraction was dried over anhydrous Na_2SO_4 at 4℃ overnight.

GC-Sniffing Test and Identification of the Volatile Components of the Neutral Fraction

The neutral fraction was concentrated to about 2 ml by using a rotary evaporator at the atmospheric pressure, transferred to vials, and then reconcentrated to about 150 μl of final volume by using N_2 gas. The concentrated fraction was used as a sample for the sniffing tests of the components representing each gas chromatographic peak and for identification of the volatile components.

For the GC-sniffing test (21), we used the gas chromatograph that had been designed to allow one part of the volatile components to flow to the column of the detector and other part to flow to the outside of the gas chromatograph. The gas chromatograph used was a Shimadzu GC-8A, and the column used was a CBP-W12-100 (chemically bounded fused silica capillary column). The detector temperature was 240℃, and the oven temperature was increased from 60℃ to 200℃ at the rate of 10℃/min. The carrier gas was N_2(8 ml/min), and the detector was a flame ionization detector.

The identification of volatile components was conducted from the results of a computer library search of the mass spectrum obtained with GC/MS and from Kovat's retention index (8, 16). The GC/MS used was a Finnigan MAT 4510B and column was a CARBOWAX-20M-25M. The injector temperature was 230℃ and detector temperature was 250℃. The oven temperature was kept at 45℃ for 2 min, and then was increased to 220℃ at the rate of 15℃/min.

Then it was kept at the final temperature of 220℃ for 11.4 min. The carrier gas was He (5 ml/min), the electron voltage was 70 eV, and the split ratio was 30:1.

Furthermore, we represented the identified volatile components by comparing the patterns of gas chromatograms for GC-sniffing tests and for identification.

RESULTS AND DISCUSSION

Isolation and Identification of the Bacterial Strain

A strain was isolated from a fixed amount of traditional soybean paste by dilution method. The isolate was inoculated to cooked soybean medium. After culturing at 30℃ for 40 days, the pH of the resultant soybean mixture was 6.65. The colors of surface and inside of the mixture were yellow and dried grass, respectively.

The flavor of the mixture represented the sweet and delicate odor of traditional soybean paste. When a part of the mixture was boiled after adding distilled water, the mixture generated a flavor that represented the mild sweet odor of soybean paste.

Therefore, it may be concluded that the isolate produced the traditional soybean paste odor from the cooked soybean medium.

The characteristics of the isolate are shown in Table 1. The appearances of gram positive, rod shape, and spore formation show that the isolate is a *Bacillus* species.

The isolate was able to produce catalase, amylase and protease, and also able to produce acid from glucose, arabinose, xylose and mannitol. The Voges-Proskauer test of the isolate was positive.

However, the isolate was not able to produce egg-yolk lecithinase and tyrosinase. The isolate showed no abilities to produce gas from glucose, and to use propionate.

In the anaerobic environment, there was no growth of the isolate. Comparing with the characteristics of *Bacillus subtilis* of Bergey's manual (13), there are differences in the utilization of citrate, the formation of indole, growth with lysozyme present, growth at 55℃ and 65℃. Comparing with the characteristics of *Bacillus subtilis* of The Genus *Bacillus* (4), there is difference in the utilization of citrate. From the results as above, the isolate could be identified as a strain of *Bacillus subtilis*

Table 1. Characteristics of the strain isolated and selected from traditional Korean soybean paste.

Gram's stain	+	Diamination of phenylalanine	−
Spore stain	+	Egg-yolk lecithinase	−
Cell diameter >1.0 μm	−	Nitrate reduced to nitrite	+
Spore's round	−	Formation of indole	d
Sporangium swollen	−	Formation of dihydroxyacetone	+
Catalase	+	NaCl and KCl required	−
Anaerobic growth	−	Allation or urate required	−
Voges-Proskauer test	+	Growth at pH 6.8	+
pH in V-P broth <6	+	Growth at pH 5.7	+
pH in V-P broth >7	+	Growth in NaCl 2%	+
Acid from D-glucose	+	Growth in NaCl 5%	+
Acid from L-arabinose	+	Growth in NaCl 7%	+
Acid from D-xylose	+	Growth in NaCl 10%	+
Acid from D-mannitol	+	Growth at 5℃	−
Gas from glucose	−	Growth at 10℃	+
Hydrolysis of casein	+	Growth at 30℃	+
Hydrolysis of gelatin	+	Growth at 40℃	+
Hydrolysis of starch	+	Growth at 50℃	d
Utilization of citrate	−	Growth at 55℃	+
Utilization of propionate	−	Growth at 65℃	d
Degradation of tyrosine	−	Growth with lysozyme present	−

+: Positive, −: Negative, d: Doutable.

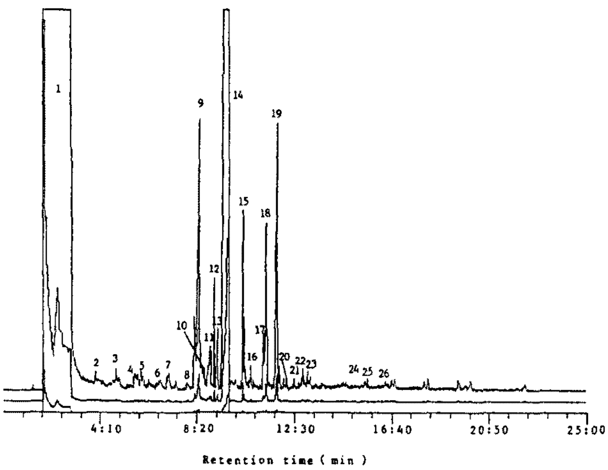

Fig. 2. Gas chromatogram of volatile components in the cooked soybean medium.

Data of sniffing test: Peak No. 9 and 10, sweet soy sauce odor; 11, soy sauce and bean oil odor; 12~14, boiled soy sauce odor; 15, sesame and bean oil odor; 17~19, bran soup odor. Gas chromatographic (GC) conditions: GC, FINNIGAN MAT 4510B; column, CARBOWAX-20M-25M; inj. temp., 230℃; det. temp., 250℃; oven temp., 45℃ for 2 min, 45~220 (15℃/min) and 220℃ for 11.4 min; carrier gas, He (5 ml/min); split ratio; 30:1.

and named *B. subtilis* PM3.

GC-Sniffing Test and Identification of the Volatile Components of the Neutral Fraction

The gas chromatogram of whole volatile components from the cooked soybean medium kept in the incubator for 40 days at 30℃ without inoculation is shown in Fig. 2 and the result of identification is shown in Table 2. The gas chromatogram and identification results of

Table 2. Volatile components in the cooked soybean medium.

Peak No.	Components	Aroma
1	Ethanol	
2	1-Methylhexyl-hydroperoxide*	
3	1-Butanol	
4	3-Methyl-1-butanol	
5	Unknown	
6	1-Ethylbutyl-hydroperoxide*	
7	Unknown	
8	Unknown	
9	1-Octen-3-ol	sweet soy sauce
10	Unknown	
11	Unknown	soy sauce and bean oil
12	2,2'-Bi-1,3-dioxolane*	
13	Benzaldehyde	boiled soy sauce
14	1-Octanol	
15	2-Furanmethanol	sesame and bean oil
16	4-Hydroxy-benzaldehyde	
17	Nitro-benzene	
18	1-Decanol	bran soup
19	2,4-Decadienal	
20	9-Isopropylidene-bicyclo[3.1.0] nonan-2-one*	
21	(Phenoxymethyl)-benzene*	
22	Nonadecanol	
23	Methyl-carbamic acid, phenyl ester*	
24	Unknown	
25	Nitric acid, nonyl ester	
26	1-Iodo-tetradecane*	

* Tentatively identified.

the volatile components of the neutral fraction produced by *Bacillus subtilis* PM3 under the above conditions are shown in Fig. 3 and Table 3, respectively.

The identified volatile components of the neutral fraction produced by *Bacillus subtilis* PM3 are total of 29 different compounds, which can be classified based on the functional group as follows: three hydrocarbons (xylene, naphthalene, etc.), four esters (ethyl acetate, nonyl nitrate, etc.), five aldehydes (pentanal, hexanal, benzaldehyde, benzeneacetaldehyde, etc.), eight alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, 3-penten-1-ol, 1-octen-3-ol, 1-octanol, benzeneethanol, nonadecanol, 1-eicosanol), three acids (acetic acid, nonanedioic acid, hexadecanoic acid), three ketones (1-methyl-2-pentanone, 2-nonanone, etc.), two sulfur containing compounds (dimethyl disulfide, dimethyl trisulfide), and one furan compound (2-furanmethanol), respectively.

The acid components were considered to be introduced into the neutral fraction during the process of fractionation by contamination.

Of the identified components, six components such as dimethyl disulfide, xylene, 3-methyl-1-butanol, dimethyl trisulfide, benzaldehyde and naphthalene were

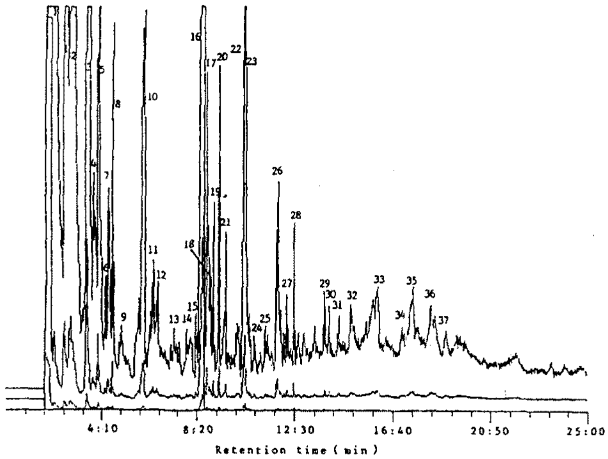

Fig. 3. Gas chromatogram of the neutral fraction obtained from whole volatile components produced by *Bacillus subtilis* PM3.

Data of sniffing test: Peak No. 10, unpleasant odor; 11 and 12, weak soy sauce odor; 16~18, sweet soy sauce odor; 19~23, sesame and soybean paste odor; 26, bran soup-like odor; 27 and 28, floral odor. GC conditions were same as described in Fig. 2.

character impact compounds in the flavor of Korean soybean paste manufactured with the traditional and the improved Meju (12), and three components such as benzaldehyde, 2-furanmethanol and benzeneacetaldehyde were character impact compounds in the flavor of Korean soy sauce manufactured with the traditional and the improved Meju (11).

The identified volatile components in this study were compared with the reported components of traditional Korean soy sauce and soybean paste, and of Japanese Shoyu and Miso. Some identified volatile components were reported to be present also in traditional Korean soy sauce and soybean paste. Eleven components of ethyl ester, 1,3,5-cycloheptatriene, 3-methyl-1-butanol, dimethyl trisulfide, acetic acid, benzaldehyde, 2-furanmethanol, benzeneacetaldehyde, naphthalene, benzeneethanol, and nonadecanol were present in traditional Korean soy sauce (10). Twelve components of ethyl acetate, hexanal, 2-methyl-1-propanol, 3-methyl-1-buthanol, acetic acid, 1-octen-3-ol, benzaldehyde, 2-furanmethanol, benzeneacetaldehyde, naphthalene, benzeneethanol, and nonadecanol were present in traditional Korean soybean paste (10). In addition, it was found that some identified components were also present in Japanese Shoyu and Miso. Sixteen components were present in Japanese Shoyu (22, 23). Those were ethyl acetate, pentanal, dimethyl disulfide, hexanal, 2-methyl-1-propanol, xylene, 3-methyl-1-butanol, dimethyl trisulfide, acetic acid, 1-octen-3-ol, benzaldehyde, 2-furanmethanol, benzeneacetaldehyde, naphthalene, benzeneethanol, and hexadecanoic acid. Eleven of the sixteen components are also present in the volatile components of Japanese Miso (6, 7, 19) also in Japanese Shoyu (22, 23). Those are ethyl acetate, pentanal, hexanal, 2-methyl-1-propanol, 3-methyl-1-butanol, acetic acid, benzaldehyde, 2-furanmethanol, benzeneacetaldehyde, naphthalene, and benzeneethanol. 2,4-Decadienal was present only in the volatile components of Japanese Miso.

When the result was compared with the volatile components of the control cooked soybean medium, eight components listed above were also found in the control.

Those were 3-methyl-1-butanol, 1-octen-3-ol, benzaldehyde, 1-octanol, 2-furanmethanol, 2,4-decadienal, nonadecanol, and nonyl nitrate.

The results of the GC-sniffing test for the volatile components of the control are shown in Fig. 2. The region of the peak No. from 9 to 10 represented sweet soy sauce-odor that contained 1-octen-3-ol and an unidentified component. The peak No. 11 represented soy sauce and bean oil-odor that contained an unidentified component. The region of the peak No. from 12 to 14 represented boiled soy sauce-odor that was identified as 2,2'-bi-1,3-dioxolane, benzaldehyde, and 1-octanol, respectively. In the cooked soybean medium, it is considered that the soy sauce-odor from the GC-sniffing test may be synthesized by the chemical reaction.

Fig. 3 shows the results of the GC-sniffing test on the volatile components produced by *Bacillus subtilis* PM3. There are several peaks of soy sauce and soybean paste-odor. The region of the peak No. from 11 to 12 represented weak soy sauce-odor, which contained 3-penten-1-ol and an unidentified component. The region of the peak No. from 16 to 18 represented sweet soy sauce-odor, which contained 1-octen-3-ol, 2-nonanone and nitric acid nonyl ester. The region of the peak No. from 19 to 23 represented sesame and soybean paste-odor, which were identified to contain an unidentified component, benzaldehyde, 1-octanol, 2-furanmethanol and benzeneacetaldehyde.

The benzaldehyde, one of the components for the flavor of soybean paste produced by *Bacillus subtilis* PM3, was reported to be a character impact compound of Korean soy sauce and soybean paste (11, 12). The 2-furanmethanol, one of the components for the flavor of the soybean paste produced by *Bacillus subtilis* PM3, was also detected as a major component of the soybean that was boiled for eight hours (19), and reported to be synthesized by Maillard reaction (lactose-casein browning system) (2).

The benzeneacetaldehyde, one of the components for the soybean paste flavor produced by *Bacillus subtilis* PM3, is an important volatile component of cocoa and can be synthesized by Maillard reaction (glucose-phe-

Table 3. Volatile components of the neutral fraction produced by *Bacillus subtilis* PM3.

Peak No.	Components	Aroma	TKSS	TKSP	Shoyu	Miso
1	Acetic acid, ethyl ester		+	+	+	+
2	Pentanal				+	+
3	Unknown					
4	2-Methyl-2-pentanone					
5	1,3,5-Cycloheptatriene*		+			
6	Dimethyl disulfide				+	
7	Hexanal			+	+	+
8	2-Methyl-1-propanol			+	+	+
9	Xylene				+	
10	3-Methyl-1-butanol	unpleasant	+	+	+	+
11	3-Penten-1-ol	weak soy sauce				
12	Unknown					
13	Unknown					
14	Dimethyl trisulfide		+		+	
15	Acetic acid		+	+	+	+
16	1-Octen-3-ol			+	+	
17	2-Nonanone	sweet soy sauce				
18	Nitric acid, nonyl ester					
19	Unknown					
20	Benzaldehyde		+	+	+	+
21	1-Octanol	sesame and soybean paste				
22	2-Furanmethanol		+	+	+	+
23	Benzeneacetaldehyde		+	+	+	+
24	Unknown					
25	Naphthalene		+	+	+	+
26	2,4-Decadienal	bran soup				+
27	Unknown	floral				
28	Benzeneethanol		+	+	+	+
29	1,5-Bis(1,1-dimethylethyl)-3,3-dimethyl-bicyclo[3.1.0]hexan-2-one*					
30	Unknown					
31	Nonanedioic acid					
32	[R-(Z)]-12-(Acetyloxy)-9-octadecanoic acid, methyl ester*					
33	Hexadecanoic acid				+	
34	Nonadecanol*		+	+		
35	1-Eicosanol*					
36	Unknown					
37	Octadecanoic acid, phenylmethyl ester*					

TKSS: Traditional Korean soy sauce, TKSP : Traditional Korean soybean paste, + : Present, * : Tentatively identified.

nylalanine system). The 3-methyl-1-butanol, represented the unpleasant odor, is an important compound of green and beany odor (1), and can be reduced gradually by boiling soybean for more than three hours. Besides the synthesis of volatile components by chemical reaction, 3-methyl-1-butanol was reported to be produced also by *Pseudomonas putrifaciens* and *Achrobacter* sp. (15).

Consequently, the bacterial strain that is responsible for the flavor of traditional Korean soybean paste was isolated and identified as *Bacillus subtilis* PM3. Many components of the neutral fraction, produced by *Bacillus subtilis* PM3 from the cooked soybean medium, contained the volatile component of traditional Korean soy sauce and soybean paste, and Japanese Shoyu and Miso. The volatile components were involved in the character impact compounds of the flavor of Korean soy sauce and soybean paste manufactured with the traditional and the improved Meju. From the result of the GC-sniffing test, several peaks on chromatogram represented soy sauce and soybean paste odor. Therefore, it may be suggested that traditional Korean soybean paste can be manufactured by using the *Bacillus subtilis* PM3 strain.

In the future study, detailed studies on the volatile components of traditional Korean soybean paste, on the flavor synthetic mechanism by *Bacillus subtilis* PM3, and on the optimal conditions for fermentation by the strain, are thought to be required.

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