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Levan-Producing *Bacillus subtilis* BS 62 and Its Phylogeny Based on Its 16S rDNA Sequence

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Abstract A viscous substance producer strain BS 62, which was isolated from conventional Chungkookjang, was examined for its productivity of levansucrase and levan during soybean fermentation at 37°C. After one day of cultivation, the enzyme activity reached the highest level, 8 units ml^{-1}. Extracts of fermented soybeans were precipitated by ethanol and hydrolyzed by either 0.1 N HCl or invertase, and the hydrolyzates were analyzed using thin layer and ion chromatographies. Fructose was the only sugar detected. This suggests that fructose was derived from the levan produced by the strain BS 62 during soybean fermentation. The aerobic, endospore-forming bacterium BS 62 was identified as a *Bacillus subtilis* sp., based on the composition of its cellular fatty acids and phylogeny, which was determined by its 16S rDNA sequence.

Key words: Levan, soybean fermentation, *Bacillus subtilis*, Chungkookjang, 16S rDNA

It has been suggested that traditional soybean-fermented foods, such as Doenjang (soybean paste) and Chungkookjang in Korea and Natto in Japan, contain certain biologically active materials. Fibrinolytic enzymes have been isolated from *Bacillus* sp. from Chungkookjang [8], Doenjang [15, 18], as well as from Natto [30]. Thrombin inhibitors in Doenjang have been investigated [13]. Inhibitory peptides for an angiotensin-converting enzyme have been isolated from commercial Doenjang [27, 29]. Doenjang is manufactured by a few *Bacillus* sp., and their extracts have been investigated for the *in vitro* selective cytotoxic effect on human liver cell lines [3]. The daily intake of soybean paste soup has been found to significantly reduce standardized mortality rates related to gastric cancer in 29 Japanese health center districts [10].

Levan (fructose polymer linked via β-2, 6 linkages) is an attractive substance because of its antitumor [2], antiallergy, and cell membrane permeation activities [16]. Levan exhibits a direct effect on Lewis lung carcinoma cells *in vitro*, and tumor cells incubated with levan show a pronounced decrease in their oncogenic properties [19]. The physico-chemical properties of viscous substances extracted from Chungkookjang have been investigated [17]. The viscosity and thermal property of levan have been investigated by a viscometer [12]. It has been previously reported that levan is produced by the reaction of levansucrase in sugar cane or sucrose-added medium [6, 26], and that *B. licheniformis* produces levan from sucrose, although it can grow on glucose, fructose, or sucrose [20].

In this report, a bacterial strain, BS 62, was selected from among various viscous substance-producing isolates from conventional Chungkookjang to elucidate levan existence in fermented soybean, which was prepared by using the selected strain without addition of sucrose. In addition, the strain was investigated for its taxonomy and phylogeny based on its cell wall fatty acid and 16S rDNA sequence.

Materials and Methods

Strains and Media

The bacterial strains employed in this study were bacilli isolated from the fermented soybean food, conventional Chungkookjang. To isolate aerobic, endospore-forming bacteria, one gram of each collected sample was suspended in a Nutrient broth (Difco) in a glass tube, boiled for 10 min, and then streaked on a Nutrient agar plate using a sterile loop. After incubation at 37°C, the single colonies formed were isolated and purified. The strains were maintained both as lyophilized cultures and as sporulated cultures [9] on agar slopes containing 0.02 mM $MnSO_4$.

Biotin Requirement and Viscous Substance Production

The medium [7] used to determine the biotin requirement and viscous substance production consisted of citric acid 2 g, peptone 12 g, glycerol 2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, K_2HPO_4

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0.5 g, NH_4Cl 7 g, and $FeCl_3 \cdot 6H_2O$ 0.04 g, per litre of H_2O, pH 6.8 [7]. The strains were streaked on the agar plates with and without 100 μg l^{-1} biotin, and cultured at 37°C for 1 day. The biotin requirement was estimated by observing the bacterial growth on the plate and the amount of viscous substance around the colonies. The ropiness of the viscous substance was also compared based on the length of the thread formed when using a sterile toothpick. The plates were kept in a refrigerator for 3 days and the tests repeated 3 times.

Assay of Levansucrase

The strain BS 62 seed-cultured in Nutrient broth was inoculated and incubated for overnight in soybean that had been soaked in water and autoclaved at 121°C for 30 min. Thirty milliliters of distilled water was added to 10 g of fermented soybeans and agitated for 1 h to extract levansucrase. The mixture was centrifuged at 6,000 ×g for 15 min and the supernatant obtained was used as the crude enzyme solution.

The reaction mixture, containing 10% sucrose, 50 mM phosphate buffer, pH 7.0, and the crude enzyme solution in a total of 2 ml, was incubated at 30°C. After 30 min incubation, the reaction mixture was boiled and the glucose released was determinated using the method of Somogyi-Nelson [22]. One unit of levansucrase was defined as the enzyme amount that released 1 μmol of glucose per min under the above conditions. Because levansucrase catalyzes formation of free fructose and oligosaccharides in the presence of sucrose, the assay procedure was based on the determination of free glucose [31].

TLC Analysis of Sugars

The fermented soybeans were extracted with 10 volumes of distilled water for 1 h, and centrifuged. The supernatant obtained was precipitated by adding three volumes of ethanol. The precipitate was collected by centrifugation and dissolved in a minimum amount of distilled water. The solution was dialyzed against water overnight in a cold room and then freeze-dried. The dried sample was dissolved in distilled water to give a concentration of 1% and then digested with 0.1 N HCl at 100°C for 2 h.

The sugar components in the hydrolysate were analyzed qualitatively by ascending thin-layer chromatography (TLC) on a silicagel plate using n-butanol-pyridine-water (8:1:1) as the solvent. The plate was sprayed with an anisaldehyde-H_2SO_4 reagent and then heated at 110°C for 20 min.

The sugar components were determined by ion chromatography using a Dionex instrument equipped with pulsed amperometric detector. The column was equilibrated with 0.2 M NaOH (75%) and H_2O (25%), with a flow rate of 0.75 ml per min.

Enzymatic Degradation by Invertase

The ethanol-precipitated and lyophylized material was obtained from the fermented soybeans, and hydrolyzed with invertase to examine the products (Fig. 3). The reaction mixture containing 10 units of invertase and 1 mg of the lyophylized material in 1 ml of 100 mM phosphate buffer (pH 6.0) was incubated at 30°C.

Invertase, which liberates fructose units exowisely from the nonreducing end of fructan molecules and hydrolyzes the β-2, 6 linkage, was also used to analyze the sugar composition and the linearity of levan [11].

Fatty Acid Analysis of the Cell Wall

The fatty acid composition of the cell wall was analyzed by the Microbial Identification System (MIDI Inc., U.S.A.) with a Sherlock software. Fatty acids were separated and esterified to determine the composition according to the method of Miller and Berger [20, 21]. The methylesters were analyzed by gas chromatographic separation on a 25 m×0.2 mm ultra 2.5% phenylmethyl siloxane capillary column (Hewlett-Packard 19091B-102, U.S.A).

Sequence Analysis of 16S rDNA

The chromosomal DNA was isolated using a method described elsewhere [33]. The amplification of the 16S rDNA was conducted using two primers according to Stackebrandt and Liesack [28], 5'-GAGTTTGATCCTGGCTCAG-3' (position 9 to 27, in *E. coli* 16S rRNA numbering) and 5'-AGAAA-GGAGGTGATCCAGCC-3' (position 1542 to 1525, in *E. coli* 16S rRNA numbering). A PCR was performed in a final reaction volume of 100 μl which contained 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 2.5 U of Taq DNA polymerase. The reaction was run for 35 cycles in a DNA thermal cycler (Model 480, Perkin-Elmer Co., U.S.A.), employing the thermal profile according to Yoon *et al.* [33]. The cloning and sequencing of the amplified 16S rDNA were performed by methods described elsewhere [14, 25], using T3 promoter primer (5'-ATTAACCCTCACTAAAG-3'), T7 promoter primer (5'-AATACGACTCACTATAG-3'), and certain other primers [34].

The 16S rDNA sequence of strain BS 62, as determined in the present study, has been deposited in the GenBank, NCBI data library under the accession number AB 016721.

Phylogenetic Analysis

The 16S rDNA sequence of strain BS 62, as determined in this study, was aligned using the CLUSTAL W software [32]. The sequences of representative species of the genus *Bacillus* and related taxa were cited using the GenBank Database. The values of 16S rDNA similarity were calculated from the alignment and the evolutionary distances were calculated using a Kimura two-parameter correction. A phylogenetic tree was constructed using the neighbor-joining method [24] based on the calculated distance matrix.

Table 1. Biotin requirement and viscous substance productivity of isolates.

Isolates (Strain No.)	Biotin requirement	Viscous substance productivity[a]	Ropiness[b]
11	N	+	++
12	R	+++	+
32	N	+	++
41	R	+++	+
42	R	+	++
51	R	+++	++
52	R	++	+
61	N	+	+
BS 62	R	+++	+++
71	R	+++	+++
72	R	+++	-
73	N	++	+

N: Biotin is not required for growth. R: Biotin is required for growth. [a]Productivity was measured as amount of viscous substance around colonies on agar plate, as described in Materials and Methods. [b]Ropiness was measured as length of thread formed by the sticky material using sterile toothpick. +++, High level; ++, Moderate level; +, Low level; -, not detected.

RESULTS

Biotin Requirement and Viscous Substance Productivity

Viscous substance-producing bacilli, which were also aerobic, endospore-forming bacteria, were isolated from the fermented soybean food, Chungkookjang. The biotin requirement as a growth factor and the viscous substance productivity of the isolates were investigated (Table 1), because all the viscous substance producers, *Bacillus subtilis* strains, required biotin as a growth factor [7]. Among the twelve strains which produced viscous substances, eight strains required biotin (biotin$^-$), whereas four strains, Nos. 11, 32, 61, and 73, did not (biotin$^+$). Two strains, BS 62 and 71, both of which required biotin, produced large amounts of viscous substances and also shared morphological similarities. Accordingly, one strain, BS 62, was selected for further study.

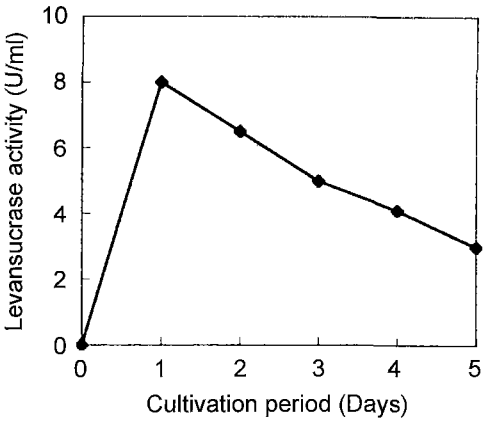

Fig. 1. Change in levansucrase activity during Chungkookjang fermentation when using strain BS 62 at 37°C.
The enzyme activities of the culture filtrates were measured as described in the Materials and Methods.

Levansucrase Production by Strain BS 62

Steamed soybeans soaked overnight and autoclaved at 121°C for 30 min were inoculated with the strain BS 62. During fermentation at 37°C, the levansucrase activities were analyzed relative to time intervals (Fig. 1). After one day of cultivation, the enzyme activity reached to the highest level, 8 units ml^{-1}; thereafter, the level gradually decreased.

Confirmation of Levan Products

The sugar components in the acid hydrolyzates of the levan products were analyzed by TLC and the results are shown in Fig. 2. Only one kind of sugar, which comigrated with fructose, was detected. In a further analysis by ion chromatography, as shown in Fig. 3, only a fructose peak was found in the chromatogram of the acid hydrolyzate. The enzyme hydrolyzates were prepared by reacting the lyophilized matter with invertase at 30°C for 24 h, and then analyzed by ion chromatography. Here also, a single peak was observed, which was identified as fructose previously. The hydrolysis product of levan from *Bacillus polymyxa* was reported to consist entirely of fructose [5].

Composition of the Cellular Fatty Acids

As shown in Fig. 4 and Table 2, the cellular fatty acids of strain BS 62 were composed of four major fatty acids, anteiso-$C_{15:0}$ (36.7%), iso-$C_{15:0}$ (24.6%), iso-$C_{17:0}$ (16%), and anteiso-$C_{17:0}$ (10.3%). The major fatty acid profile of *B. subtilis* BD99 [4] is anteiso-$C_{15:0}$ (40%), iso-$C_{17:0}$ (20%),

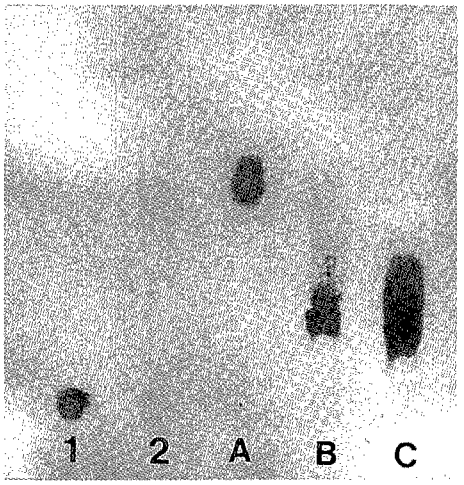

Fig. 2. TLC of the acid hydrolyzate of levan prepared from soybean culture of strain BS 62.
One percent of the levan preparation was digested with 0.1 N HCl at 100°C for 2 h, then the reaction products were analyzed by TLC. 1, Control; 2, Acid hydrolyzate; A, Fructose; B, Glucose; C, Sucrose.

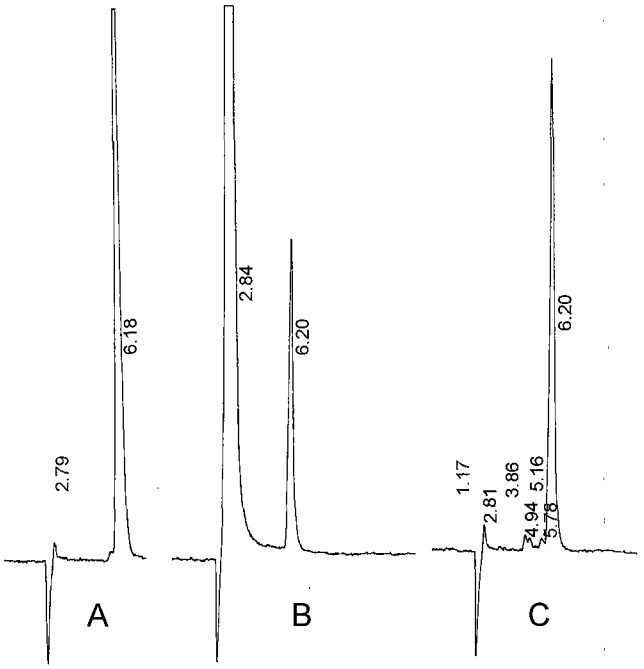

Fig. 3. Ion chromatogram of reaction products from levan.
One mg of the levan preparation was incubated with 10 units of invertase (20 μg) in 0.2 ml of the 0.1 M phosphate buffer, pH 6.0, at 30°C for 24 h, then the reaction products were analyzed for sugar by ion chromatography. A, Fructose as the standard; B, Reaction mixture with invertase; C, Acid hydrolyzate.

and iso-$C_{15:0}$ (15%), showing a similar pattern to the strain BS 62. Accordingly, these results suggested that the strain BS 62 is chemotaxonomically close to *B. subtilis* BD99.

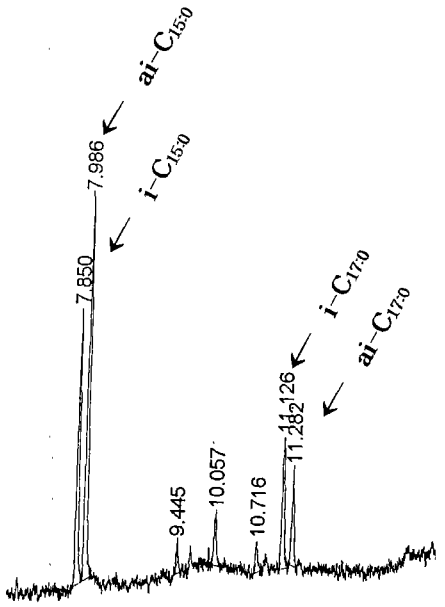

Fig. 4. Gas-liquid chromatogram of cellular fatty acids from strain BS 62.

Table 2. Fatty acid composition of total membrane lipid extracts from strain BS 62 and *Bacillus subtilis* BD99.

Fatty acid (s)[a]	Fatty acids (%) in total membrane lipid extracts from:	
	BS 62	BD99[b]
Iso-$C_{15:0}$	24.6	15
Anteiso-$C_{15:0}$	36.7	40
Iso-$C_{16:0}$	2.9	5
n-$C_{16:0}$	6.0	5
Iso-$C_{17:1}$ ω10c	3.4	1
Iso-$C_{17:0}$	16.0	20
Anteiso-$C_{17:0}$	10.3	1

[a]Fatty acids are abbreviated such that the number of carbon atoms precedes the colon and the number of double bonds follows the colon. The prefixes anteiso and iso represent the type of branched-chain structure. [b]Fatty acid composition of *Bacillus subtilis* BD99 obtained from reference [4].

Sequence of 16S rDNA

The 16S rDNA sequence was analyzed to determine which species matched strain BS 62 with the highest homology among those bacilli cited in the GenBank. The phylogenetic tree constructed using the neighbor-joining method is shown in Fig. 5. The sequence data (GenBank accession No. AB 016721; the full-length 16S rDNA sequence of strain BS 62 consists of 1,502 bp) were aligned to construct a phylogenetic tree. The phylogenetic position of strain BS 62 was then compared with certain *Bacillus* species and related taxa in a dendrogram. In the phylogenetic tree, strain BS 62 was closest to *B. subtilis* DSM 10 and part of a robust monophyletic cluster with *B. mojavensis* IFO 15718, *B. vallismortis* DSM 11031, *B. amyloliquefaciens* ATCC 23350, and *B. atrophaeus* JCM 9070. The levels of sequence similarity of strain BS 62 within the monophyletic cluster was greater than 99.3% (Table 3). The sequence of strain BS 62 was almost identical to that of *B. subtilis* DSM 10 (99.9%). Based on the cellular fatty acid composition and 16S rDNA sequence, strain BS 62 would appear to be a strain of *B. subtilis*.

DISCUSSION

Biotin has been found to be essential for the growth of all strains belonging to *B. subtilis* (*natto*) which were isolated from commercial products of Natto [7]. In the current study, most biotin$^-$ strains produced large amounts of viscous substances, except strain No. 42 which produced only a little. Strain No. 73 of biotin$^+$ produced a considerable amount of viscous substances. Among the Chungkookjang bacilli, the biotin requirement was related to the productivity of viscous substances. Since poly(γ-glutamic acid) (γ-PGA) has been reported as a component of the viscous substances in Natto [7], strain BS 62 was examined for its productivity of γ-PGA, which was confirmed (data not shown).

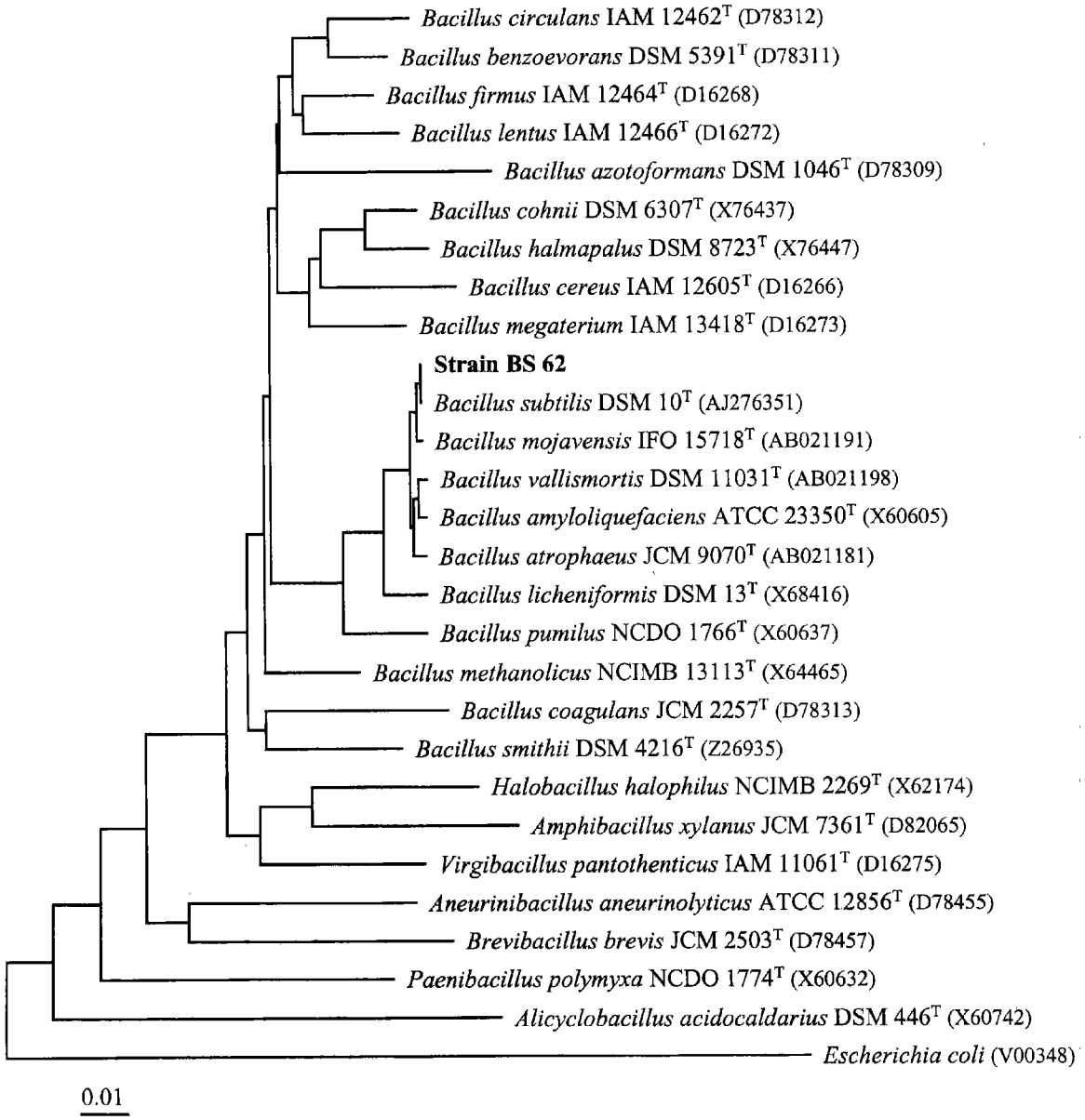

Fig. 5. Phylogenetic tree based on the 16S rDNA sequence.
The diagram shows positions of strain BS 62, type strains of certain *Bacillus* species, and representatives from some other related taxa. Scale bar represents 0.01 substitution per nucleotide position.

To elucidate the production of levan, a biologically active material in fermented soybeans, levansucrase activities were measured during soybean fermentation using strain BS 62, and levan prepared from the soybean extracts by ethanol precipitation was analyzed. The only kind of sugar found in the hydrolyzate of the preparation by TLC and ion chromatography was fructose. This suggests that the fructose was derived from levan [5]. Attempts were made to examine whether levan could be produced in a solid soybean medium without addition of sucrose, and the presently described results suggest that levan could be produced during the fermentation of steamed soybeans when using strain BS 62. Therefore, Chungkookjang would appear to contain a certain amount of levan polysaccharide, which confers antitumor activity when it is ingested and digested at a specific molecular weight into the human intestine. Calazans *et al.* [1] plotted the antitumor activities of levans from *Zymomonas mobilis* relative to the viscosity average molecular weight, and obtained results indicating that the antitumor activities depend on the molecular weights. Since Doenjang and Chungkookjang are Korean traditional soybean foods, it is considered that systematic research is necessary to increase the content of such biologically active substances during the fermentation process.

Table 3. Level of 16S rDNA similarity between strain BS 62, type strains of certain *Bacillus* species, and representatives from other related taxa.

Strain	% Similarity in:																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 Strain BS 62																			
2 *Bacillus subtilis* DSM 10[T]	99.9																		
3 *Bacillus mojavensis* IFO 15718[T]	99.6	99.7																	
4 *Bacillus vallismortis* DSM 11031[T]	99.6	99.7	99.5																
5 *Bacillus atrophaeus* JCM 9070[T]	99.2	99.3	99.3	99.5															
6 *Bacillus amyloliquefaciens* ATCC 23350[T]	99.3	99.5	99.4	99.6	99.4														
7 *Bacillus licheniformis* DSM 13[T]	98.3	98.4	98.4	98.2	98.3	98.2													
8 *Bacillus pumilus* NCDO 1766[T]	97.2	97.2	97.0	97.3	97.4	97.1	96.3												
9 *Bacillus cohnii* DSM 6307[T]	94.3	94.3	94.3	94.4	94.6	94.0	94.3	94.9											
10 *Bacillus cereus* IAM 12605[T]	94.0	94.0	94.0	94.0	94.3	93.8	93.9	93.8	95.1										
11 *Bacillus firmus* IAM 12464[T]	95.1	95.1	95.3	94.9	95.1	94.9	95.4	95.0	94.7	94.2									
12 *Bacillus circulans* IAM 12462[T]	93.6	93.7	93.8	93.5	93.3	93.8	93.8	94.0	95.3	93.9	96.4								
13 *Bacillus methanolicus* NCIMB 13113[T]	94.9	95.4	95.5	95.2	95.0	95.3	95.0	94.4	94.8	93.5	96.3	95.3							
14 *Bacillus coagulans* JCM 2257[T]	93.0	93.0	92.8	93.0	93.1	92.8	92.9	92.4	92.6	92.7	93.5	92.3	94.1						
15 *Amphibacillus xylanus* JCM 7361[T]	90.9	90.9	90.9	90.7	90.8	90.8	90.7	91.1	91.5	90.4	91.8	91.8	92.2	91.1					
16 *Virgibacillus pantothenticus* IAM 11061[T]	93.2	93.2	93.2	93.3	93.4	93.3	93.4	93.1	92.9	92.2	93.8	93.9	92.6	91.4	93.1				
17 *Brevibacillus brevis* JCM 2503[T]	89.2	89.0	88.9	88.7	88.8	88.8	88.9	88.3	89.3	88.8	88.9	88.3	89.8	88.5	89.5	88.4			
18 *Paenibacillus polymyxa* NCDO 1774[T]	88.2	88.3	88.3	88.4	88.2	88.4	88.3	89.4	89.2	88.9	89.3	89.7	89.2	87.8	89.1	88.9	88.5		
19 *Alicyclobacillus acidocaldarius* DSM 446[T]	85.1	85.2	85.2	85.5	85.5	85.1	85.6	84.9	85.6	84.9	85.0	84.6	86.6	85.1	84.8	85.9	85.1	85.7	
20 *Escherichia coli*	78.8	78.9	78.8	78.9	78.9	79.5	78.6	79.0	78.3	78.5	79.4	78.7	79.5	79.4	78.7	77.7	79.6	79.8	77.3

[T]Type strain.

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