

Identification and Distribution of *Bacillus* Species in *Doenjang* by Whole-Cell Protein Patterns and 16S rRNA Gene Sequence Analysis

Kim, Tae-Woon, Young-Hoon Kim, Sung-Eon Kim, Jun-Hwa Lee, Cheon-Seok Park, and Hae-Yeong Kim*

Institute of Life Sciences and Resources, and Graduate School of Biotechnology, Kyung Hee University, Yongin 446-701, Korea

Received: February 6, 2010 / Revised: April 4, 2010 / Accepted: April 5, 2010

Many bacteria are involved in the fermentation of *doenjang*, and *Bacillus* species are known to perform significant roles. Although SDS–PAGE has been frequently used to classify and identify bacteria in various samples, the microbial diversity in *doenjang* has not yet been investigated. This study aims to determine the identity and distribution of dominant *Bacillus* species in *doenjang* using SDS–PAGE profiles of whole-cell proteins and 16S rRNA gene sequencing. Reference *Bacillus* strains yielded differential SDS–PAGE banding patterns that could be considered to be highly specific fingerprints. Grouping of bacterial strains isolated from *doenjang* samples by whole-cell protein patterns was confirmed by analysis of their 16S rRNA gene sequences. *B. subtilis* was found to be the most dominant strain in most of the samples, whereas *B. licheniformis* and *B. amyloliquefaciens* were less frequently found but were also detected in several samples. The results obtained in this study show that a combined identification method using SDS–PAGE profiles of whole-cell proteins and subsequent 16S rRNA gene sequence analysis could successfully identify *Bacillus* species isolated from *doenjang*.

Keywords: *Doenjang*, *Bacillus*, SDS–PAGE, 16S rRNA gene sequence, whole-cell proteins

Fermentation of *doenjang*, a traditional Korean fermented soybean paste, is carried out by various microorganisms. It is produced from *meju* and includes natural bacterial strains such as *Bacillus* species and molds of *Aspergillus*, *Mucor*, and *Rhizopus* species [10]. Many researchers have reported that *B. subtilis* and *B. licheniformis* are the dominant organisms in *doenjang* and that they play important roles during fermentation [5, 9, 14, 17, 18]. Identification of bacterial flora by phenotypic methods has been used in the majority of previous studies, but it is regarded as time-

consuming and limited in terms of both its discriminatory ability and accuracy. The genus *Bacillus* includes a variety of phenotypically heterogeneous species exhibiting a wide range of nutritional requirements, physiological and metabolic diversities, and DNA base compositions. To date, partial 16S rRNA gene sequences and rRNA gene restriction patterns have been used for the rapid identification and classification of *Bacillus* species and related genera [16]. Recently, identification of the bacterial flora involved in *doenjang* fermentation based on 16S rRNA gene sequences has proven to be a useful strategy [1]. However, some molecular methods become laborious when a large number of samples require screening. Whole-cell protein pattern analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) offers certain advantages: it is a fairly fast and easy method of identifying a large number of strains when performed under highly standardized conditions, and it has an adequate level of taxonomic resolution at the species and subspecies levels.

SDS–PAGE protein pattern analysis has been successfully used to identify various types of bacteria, including *Listeria* species [11] and lactic acid bacteria, and to solve problems in the identification of closely related species in the genera *Lactobacillus* and *Leuconostoc* [4, 6, 8, 12, 13]. However, the usefulness of this method for the identification of *Bacillus* species isolated from *doenjang* has not yet been assessed. In this paper, we screened and identified *Bacillus* species in *doenjang* using SDS–PAGE of whole-cell proteins and 16S rRNA gene sequencing.

Reference *Bacillus* strains were obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) (Fig. 1). These organisms were cultured in LB broth (Difco, Detroit, MI, U.S.A.) at 30°C for 18 h. Six *doenjang* samples were obtained from several households and local markets in Korea. To obtain bacterial isolates, each sample was suspended and diluted in sterilized water and then spread onto the surface of an LB agar plate. The plates were incubated under aerobic

*Corresponding author

Phone: +82-31-201-2660; Fax: +82-31-204-8116;
E-mail: hykim@khu.ac.kr

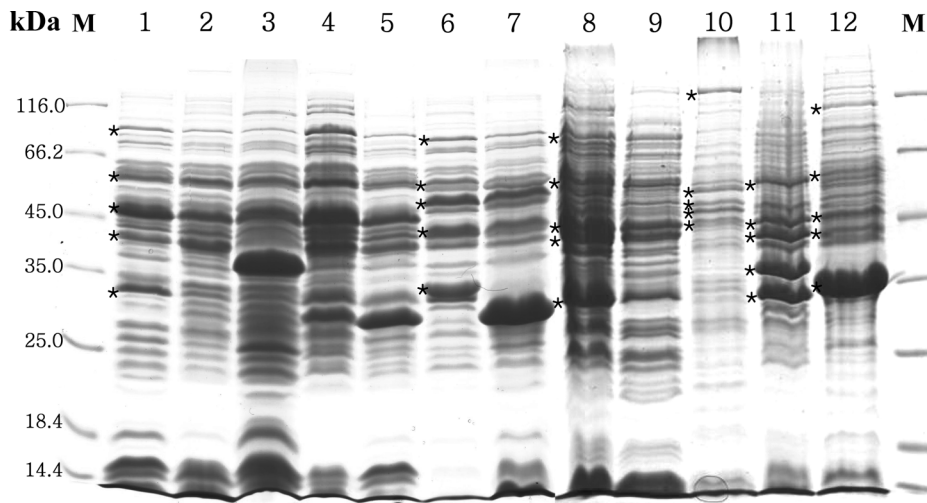


Fig. 1. SDS-PAGE profiles of whole-cell proteins from reference *Bacillus* species.

Lanes: M, protein molecular mass markers (kDa); Lane 1, *Bacillus subtilis* KCTC 3014; 2, *B. subtilis* KCTC 1028; 3, *B. subtilis* subsp. *subtilis* KCTC 3135; 4, *B. subtilis* subsp. *spizizenii* ACTC 6633; 5, *B. subtilis* subsp. *spizizenii* KCTC 3705; 6, *B. pumilus* KCTC 3348; 7, *B. pumilus* KCTC 13101; 8, *B. licheniformis* KCTC 1918; 9, *B. licheniformis* KCTC 1030; 10, *B. amyloliquefaciens* KCTC 1660; 11, *B. sonorensis* KCTC 13198; 12, *B. atrophaeus* KCTC 3701.

conditions for 1–2 days at 30°C to allow for colony development. Colonies obtained from appropriately diluted samples were randomly selected. The morphologies of the colonies and cells of the isolates were observed under a light microscope at 1,000× magnification after Gram-staining for preliminary identification. SDS-PAGE of whole-cell proteins was carried out as described by Kim *et al.* [8]. Strains were incubated aerobically overnight at 30°C in 5 ml of LB broth and centrifuged at 12,000 ×g for 3 min at 4°C. Each sample pellet was washed twice with deionized water and suspended in 50 µl of 50 mM Tris-HCl buffer (pH 8.0). Fifty mg of glass beads (diameter, 425 to 600 µm; Sigma, St. Louis, MO, U.S.A.) was added to the tubes, and the bacteria were vortexed for 5 min. The sample pellet was then resuspended by adding an equal volume of sample buffer [2× SDS sample buffer: 25 ml of 4× Tris-HCl/SDS (pH 6.8), 20 ml of glycerol, 4 g of SDS, 2 ml of 2-mercaptoethanol, 1 mg of bromophenol blue, and water to bring the volume to 100 ml]. For protein denaturation, samples were heated for 5 min at 95°C. The cell debris was settled by centrifugation, and the supernatant was collected for analysis on a 12% SDS-polyacrylamide vertical slab gel. After electrophoresis, the gel was stained for 2 h with 0.05% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, VA, U.S.A.) and destained with 10% acetic acid and 30% methanol solution for 2 h. The destained gels were scanned for further analysis. Cluster analysis of denaturing gradient gel electrophoresis profiles was performed with the numerical taxonomic system using a multivariate statistics program (NTSYSpc, version 2.02j; Exeter Software, Setauket, NY, U.S.A.). For

each strain, a data record was constructed in which each band of particular molecular weight was represented as either being present (value 1) or not present (value 0). Jaccard's similarity coefficient was estimated using NTSYSpc software. Jaccard's coefficients are defined as $a/(a+b+c)$, where a is the number of positive matches (*i.e.*, bands common to two samples), and b and c refer to the number of bands present only in samples 1 and 2, respectively. Unweighted pair group method using arithmetic averages (UPGMA) clustering was performed using the SHAN option of NTSYSpc, and a dendrogram representing the relationship between all individuals tested was derived from the TREE option [15]. To prepare cells for DNA extraction, the cultivated strains were collected by centrifugation at 14,000 ×g for 5 min at 4°C. The resulting pellet was then subjected to extraction using a Genomic DNA Prep Kit (SolGent, Daejeon, Korea). The yield and quality of DNA were analyzed electrophoretically on 1% (w/v) agarose gels. The extracted DNA was then used as a PCR template for the amplification of 16S rRNA genes with the universal primer pair 5'-AGAGTTTGATCCTGGCTCAG-3' (27F, forward) and 5'-GGCTACCTTGTTACGACTT-3' (1492R, reverse). The 16S rRNA gene was amplified by PCR with a thermal cycler (Astec PC808, Fukuoka, Japan) for 30 cycles of penetration at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 90 s, followed by a 10 min final incubation at 72°C. The anticipated product of approximately 1.5 kb was analyzed by agarose gel electrophoresis, then purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, U.S.A.), and then ligated into pGEM-T Easy Vector (Promega, Madison, WI, U.S.A.) and transformed

Table 1. Molecular mass distribution of major bands of reference strains in SDS–PAGE.

Band (kDa)	Species					
	<i>B. subtilis</i>	<i>B. pumilus</i>	<i>B. licheniformis</i>	<i>B. amyloliquefaciens</i>	<i>B. sonorensis</i>	<i>B. atrophaeus</i>
Band 1	89	80	78	118	55	95
Band 2	60	57	55	53	45	60
Band 3	48	50	46	50	42	47
Band 4	41	42	43	48	37	43
Band 5	32	31	33	46	33	35

into *Escherichia coli* DH5 α competent cells. The recombinant plasmids were purified using a plasmid purification kit (Nucleogen, Seoul, Korea) and digested with *Eco*RI to confirm the presence of the insertion. The nucleotide sequences of the plasmids were determined using an ABI PRISM Dye Terminator Sequencing Kit and ABI PRISM 377 Sequencer (Perkin-Elmer, Norwalk, CT, U.S.A.) according to the manufacturer's instructions, using the primers T7 (forward) and SP6 (reverse).

SDS–PAGE of Whole-Cell Proteins

Colonies that developed from higher dilutions plated on LB agar were randomly picked for analysis. The numbers of isolates selected from each *doenjang* sample ranged from 31 to 33. The grouping and identification of strains isolated from different samples were performed by analysis of whole-cell proteins using SDS–PAGE. First, the patterns of some reference *Bacillus* species were analyzed. The results, shown in Fig. 1, indicated that SDS–PAGE was able to clearly discriminate almost all of these species from one another. The protein profiles for each reference species showed specific band patterns, with differences being visible among *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. amyloliquefaciens*, *B. atrophaeus*, and *B. sonorensis*. The

apparent molecular masses of major bands for *B. subtilis* were 89, 60, 48, 41, and 32 kDa. *B. licheniformis* was characterized by a reference pattern containing major bands with apparent molecular masses of 78, 55, 46, 43, and 33 kDa. Bands of 118, 53, 50, 48, and 46 kDa were the main components of the pattern of *B. amyloliquefaciens*. The typical protein pattern of each reference species was characterized by the presence of bands with different molecular masses (Table 1). The dendrogram of reference strains derived from the UPGMA clustering of correlation coefficients of the SDS–PAGE profiles is shown in Fig. 2. The highest correlation values were obtained only between the same species, and clear overall differences among different species were observed. The 194 isolates were classified into different groups based on whole-cell protein patterns, and then these patterns were compared with those of the reference strains. Some of these patterns are presented in Fig. 3 along with their reference strains. The isolate represented in lane 2 in Fig. 3 showed a whole-cell protein pattern similar to that of the reference strain *B. subtilis* KCTC 3014 in lane 1. Accordingly, this isolate was designated *B. subtilis*. In addition, the isolates represented in lanes 4, 6, 8, and 10 were determined to be *B. licheniformis*, *B. amyloliquefaciens*, *B. pumilus*, and *B. sonorensis*,

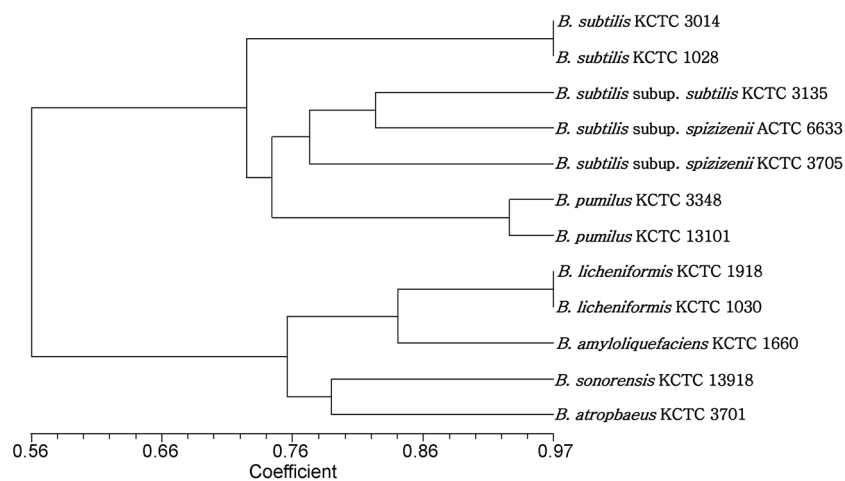


Fig. 2. Cluster analysis of profiles obtained from whole-cell protein patterns. Similarity was analyzed using UPGMA clustering.

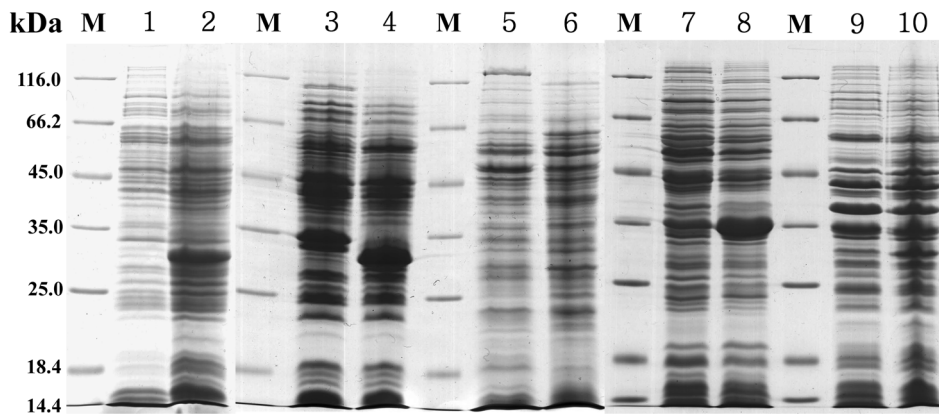


Fig. 3. Comparison of SDS–PAGE profiles of whole-cell proteins between reference strains and isolates from *doenjang* samples. Lanes: M, protein molecular mass markers (kDa); 1, *Bacillus subtilis* KCTC 3014; 2, GK27 strain isolated from *doenjang*; 3, *B. licheniformis* KCTC 1918; 4, SK21 strain isolated from *doenjang*; 5, *B. amyloliquefaciens* KCTC 3014; 6, KK13 strain isolated from *doenjang*; 7, *B. pumilus* KCTC 3348; 8, CK08 strain isolated from *doenjang*; 9, *B. sonorensis* KCTC 13918; 10, AK19 strain isolated from *doenjang*.

respectively (Fig. 3). Although the protein profile results could be used to distinguish isolates present in *doenjang*, their patterns did not perfectly match those of the reference strains (Fig. 3). This distinction might have resulted from differences in habitat that could affect phenotypic characteristics, even in bacteria of the same genotype.

To confirm the reliability of results obtained by analysis of whole-cell protein patterns, isolates *B. subtilis* GK27, *B. licheniformis* SK21, *B. amyloliquefaciens* KK13, *B. pumilus* CK08, and *B. sonorensis* AK19 were further examined using 16S rRNA gene sequencing. Their sequences showed more than 99% identity with those of some *Bacillus* species available in the GenBank database (National Center for Biotechnology Information, Bethesda, MD, U.S.A.): *B. subtilis* EF101729, *B. licheniformis* FJ435674, *B. amyloliquefaciens* AB301017, *B. pumilus* GU568192, and *B. sonorensis* EF433411.

Distribution of *Bacillus* Species in *Doenjang*

The SDS–PAGE results corresponded well with those determined by the analysis of 16S rRNA gene sequences. Of the 194 isolates, 97 were *B. subtilis*, 31 were *B. licheniformis*,

17 were *B. amyloliquefaciens*, 6 were *B. pumilus*, and 20 were identified as *B. sonorensis* (Table 2). Twenty-three additional isolates could not be assigned to any known bacterial taxon; these strains remain unidentified. *B. subtilis* was found to be the most dominant strain in most samples, whereas *B. licheniformis* and *B. amyloliquefaciens* were less frequently found but were also detected in several samples.

Many studies on the distribution and characteristics of *Bacillus* species in *doenjang* have been reported [2, 3, 7, 18]. Yoo *et al.* [18] found *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* to be the three dominant species in *doenjang*, a result that is similar to that of the present study. We observed differences in the distribution and number of *Bacillus* species among the samples tested that may be associated with the quality of the *doenjang*. Identification of *Bacillus* species based on a molecular method such as 16S rRNA gene sequencing has proven to be useful [1], but it is regarded as time-consuming and laborious for the treatment of a large number of strains. The analysis of whole-cell protein patterns allowed us to differentiate between isolates from *doenjang* easily and simultaneously.

Table 2. Distribution of *Bacillus* species isolated from *doenjang*.

Sample	Number of <i>Bacillus</i> species					
	<i>B. subtilis</i>	<i>B. licheniformis</i>	<i>B. amyloliquefaciens</i>	<i>B. pumilus</i>	<i>B. sonorensis</i>	Unknown
A (n=31) ^a	16 (51.6%)	2 (6.5%)	1 (3.2%)		4 (12.9%)	8 (25.8%)
B (n=32)	13 (40.6%)	12 (37.5%)	7 (21.9%)			
C (n=33)	17 (51.5%)			5 (15.2%)		11 (33.3%)
D (n=32)	17 (53.1%)	3 (9.4%)	7 (21.9%)	1 (3.1%)		4 (12.5%)
E (n=33)	20 (60.6%)	11 (33.3%)	2 (6.1%)			
F (n=33)	14 (42.4%)	3 (9.1%)			16 (48.5%)	

^an indicates number of isolates.

The results obtained in this study showed that a combined identification method using SDS–PAGE patterns of whole-cell proteins and subsequent 16S rRNA gene sequence analysis could successfully identify *Bacillus* species isolated from *doenjang*. The identification of *Bacillus* species based on whole-cell protein patterns by SDS–PAGE has not been previously reported. This result suggests that such an approach will be useful for grouping and identifying *Bacillus* species in *doenjang*, as a complementary identification method.

Acknowledgment

This work was supported by a grant from the Kyung Hee University in 2010 (KHU-20100158).

REFERENCES

1. Cho, K. M. and W. T. Seo. 2007. Bacterial diversity in Korean traditional soybean fermented foods (*Doenjang* and *Ganjang*) by 16S rRNA gene sequence analysis. *Food Sci. Biotechnol.* **16**: 320–324.
2. Choi, M. R., H. S. Lim, Y. J. Chung, E. J. Yoo, and J. K. Kim. 1999. Selective cytotoxic effects of *doenjang* (Korean soybean paste) fermented with *Bacillus* strains on human liver cell lines. *J. Microbiol. Biotechnol.* **9**: 504–508.
3. Chung, K. S., J. Y. Kim, S. W. Hong, and B. K. Lee. 2006. Isolation of bacteria producing a B-cell-specific biological response modifier found in Korean fermented soybean paste. *J. Microbiol. Biotechnol.* **16**: 126–135.
4. Dicks, L. M. T., H. J. J. Van Vuuren, and F. Dellaglio. 1990. Taxonomy of *Leuconostoc* species, particularly *Leuconostoc oenos*, as revealed by numerical analysis of total soluble cell protein patterns, DNA base compositions, and DNA–DNA hybridizations. *Int. J. Syst. Bacteriol.* **40**: 83–91.
5. Ji, W. D., S. H. Yang, M. R. Choi, and J. K. Kim. 1995. Volatile components of Korean soybean paste produced by *Bacillus subtilis* PM3. *J. Microbiol. Biotechnol.* **5**: 143–148.
6. Jin, J., S. Y. Kim, Q. Jin, H. Eom, and N. S. Han. 2008. Diversity analysis of lactic acid bacteria in *takju*, Korean rice wine. *J. Microbiol. Biotechnol.* **18**: 1678–1682.
7. Kim, D. H., H. P. Song, K. Y. Kim, J. O. Kim, and M. W. Byun. 2004. A correlation between fibrinolytic activity and microflora in Korean fermented soybean products. *J. Korean Soc. Food Sci. Nutr.* **33**: 41–46.
8. Kim, T. W., S. H. Jung, J. Y. Lee, S. K. Choi, S. H. Park, J. S. Jo, and H. Y. Kim. 2003. Identification of lactic acid bacteria in *kimchi* using SDS–PAGE profiles of whole cell proteins. *J. Microbiol. Biotechnol.* **13**: 119–124.
9. Kim, T. W., J. H. Lee, S. E. Kim, M. H. Park, H. C. Chang, and H. Y. Kim. 2009. Analysis of microbial communities in *doenjang*, a Korean fermented soybean paste, using nested PCR–denaturing gradient gel electrophoresis. *Int. J. Food Microbiol.* **131**: 265–271.
10. Lee, S. S. 1995. *Meju* fermentation for a raw material of Korean traditional soy products. *Korean J. Mycol.* **23**: 161–175.
11. Park, S. H., S. H. Jung, H. J. Kim, Y. H. Chung, and H. Y. Kim. 2006. Classification of meat-based *Listeria monocytogenes* using whole-cell protein patterns and serotyping analysis. *Food Sci. Biotechnol.* **15**: 324–327.
12. Pot, B., C. Hertel, W. Ludwig, P. Descheemaeker, K. Kersters, and K. H. Schleifer. 1993. Identification and classification of *Lactobacillus acidophilus*, *L. gasseri*, and *L. johnsonii* strains by SDS–PAGE and rRNA-targeted oligonucleotide probe hybridization. *J. Gen. Microbiol.* **139**: 513–517.
13. Sánchez, I., S. Seseña, and L. Palop. 2003. Identification of lactic acid bacteria from spontaneous fermentation of ‘Almagro’ eggplants by SDS–PAGE whole cell protein fingerprinting. *Int. J. Food Microbiol.* **82**: 181–189.
14. Shin, S. Y., Y. B. Kim, and T. J. Yu. 1985. Flavor improvement of soybean pastes by the addition of *Bacillus licheniformis* and *Saccharomyces rouxii*. *Korean J. Food Sci. Technol.* **17**: 8–14.
15. Song, B. K., M. M. Clyde, R. Wickneswari, and M. N. Normah. 2000. Genetic relatedness among *Lansium domesticum* accessions using RAPD markers. *Ann. Bot.* **86**: 299–307.
16. Xu, D. and J. C. Côté. 2003. Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S–23S ITS nucleotide sequences. *Int. J. Syst. Evol. Microbiol.* **53**: 695–704.
17. Yoo, S. K., S. M. Kang, and Y. S. Noh. 2000. Quality properties on soybean pastes made with microorganisms isolated from traditional soybean pastes. *Korean J. Food Sci. Technol.* **32**: 1266–1270.
18. Yoo, S. K., W. H. Cho, S. M. Kang, and S. H. Lee. 1999. Isolation and identification of microorganisms in Korean traditional soybean paste and soybean sauce. *Korean J. Appl. Microbiol. Biotechnol.* **27**: 113–117.