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Isolation and Identification of *Bacillus cereus* from Fermented Red Pepper-Soybean Paste (*Kochujang*), and Its Heat Resistance Characteristics

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Abstract To isolate *Bacillus cereus* presenting at a level of 5 log CFU/g in *kochujang*, a primary dilution (10^{-1}) of *kochujang* was heated at 85°C for 5 min. Two isolated strains Voges-Proskauer positive colony (KBC) and a negative colony (KBM) were identified as *B. cereus* and *Bacillus mycoides*, respectively, by biochemical test and 16S rDNA sequencing. $D_{100°C}$-Values of KBC and KBM strains was 8.37 and 7.08 min, respectively. When spores of KBC strain were inoculated to *kochujang* at the level of 4-5 log spores/g, the number of spores was no significant difference ($p<0.05$) for each sample from 1 up to 60 day of aging. When *kochujang* was inoculated with 4 log spores/g and heated at 85°C for 15 min, the number of spores was similar to that of unheated *kochujang*. Therefore, we estimated that *B. cereus* isolated from *kochujang* resistant on the heat treatment (85°C, 15 min) and its heat resistance characteristics could be used to count the number in *kochujang*.

Keywords: *kochujang*, isolation, *Bacillus cereus*, D-value, heat treatment

Introduction

Bacillus cereus can be isolated from various food product and cereal grain environments (1-3), and is a potential food poisoning organism. Some *B. cereus* strains are known to be capable of producing emesis and diarrhea. The population dosage of the organism which can generate infection is in the range of 5-6 log spores/g (4,5).

Several methods for isolation of *B. cereus* from foods have been attempted. Recently, a colony blot immunoassay (6) and a filtration method to detect *B. cereus* spore in raw milk have been reported (7), but the direct counting method using MYP agar supplemented with polymyxin B sulphate and egg yolk solution is most commonly used (8,9). MYP agar is a selective medium, but is limited in distinguishing *B. cereus* from other *Bacillus* strains e.g., *B. thuringiensis*, *B. mycoides*, *B. anthracis*, and *B. pseudomycoides*, that may be found on fermented food. Also, other *Bacillus* sp. is known to grow on MYP agar and to produce lecithinase (10).

Various methods for the inactivation of *B. cereus* spores have been introduced in the past several years. Bactericidal effect of plant extracts (11) and reduction in cooked rice (12) of *B. cereus* were studied. In addition, the effects of pH and sodium chloride on the heat resistance of the *B. cereus* spore were studied in food and in heating media. Acidifying the heating medium decreased the D-value (13), but sodium chloride in the heating medium tended to protect spores of *B. cereus* against the heat (14). Although interactions between elevated temperature, pH, and sodium chloride are frequently reported, the report fail to evaluate the characteristics of the *B. cereus* spore isolated from low-acid food containing high salt levels.

Kochujang, a fermented red pepper-soybean paste, is a spicy condiment in Korea that is characterized by a hot, sweet, and spicy taste. Current research studies on *kochujang* concentrate on the biological functions (15,16), physicochemical characteristics (17), and microorganism content of the product (18,19). Because the quality of *kochujang* is known to be affected by microorganisms, many reports for microbiological contamination of *kochujang* have been simulated. The major bacterium related with fermentation of *kochujang* is *Bacillus* sp. (20,21). *Kochujang* (pH 4.7-4.8) contains high salt (12-13%) and numerous aerobic cells (about 7-8 log CFU/g). In addition, *B. cereus* was detected at the level of 1-5 log CFU/g with 24.4% of detection ratio in *kochujang* (22). However, recently Korean governments regulate the number of *B. cereus* (no more than 4 log CFU/g) in *kochujang* (23).

The objectives of this study were (i) to establish a basic condition for the isolation of *B. cereus* from *kochujang* prepared by the traditional methods of the Sunchang area, (ii) to determine heat resistance of the *B. cereus* which survived in unfavorable conditions (high salinity and low pH), and (iii) to evaluate the growth of *B. cereus* in *kochujang* that has been inoculated with fixed spore suspensions.

Materials and Methods

***Kochujang* preparation** Red pepper 'Dabok' and soybean 'Baektae' produced in the autumn of 2005 (Sunchang, Jeonbuk, Korea) were used for this study. Glutinous rice, a product of Donggye, Sunchang, and domestic sun-dried salts were used for the preparation of *kochujang*.

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The mixing ratio for preparing *kochujang* according to the traditional methods of the Sunchang area is given in Table 1. Glutinous rice was soaked overnight, then ground and mixed with malt extract and sugar for 1 hr at 60°C, and filtered. *Kochujang* was dispensed into sterilized glass bottles in 100 g units, and capped to limit contamination by airborne organisms. The packed *kochujang* was fermented at 25°C for 60 day followed by analysis of pH, salt content, presumptive *B. cereus*, and the total number of viable aerobic cells.

For the *kochujang* preparation for test of the growth of *B. cereus* in *kochujang*, duplicate glutinous rice mixtures were inoculated with *B. cereus* spores (a spore suspension diluted to 7 log spore/mL with sterile distilled water). Two inoculums levels were prepared for testing with target values of 4 and 5 log spore/g, respectively. Each spore dilution was inoculated to malt digested syrup and homogenized in blender (BagMixer, Interscience, St. Nom, France) for 3 min. Malt digested syrup inoculated with the spores was mixed with half of each ingredient, homogenized, and remixed with the remaining ingredients. The inoculated *kochujang* preparations were fermented at 25°C for 60 day and the number of *B. cereus* and total viable cell counted as per controls.

Measurement of presumptive and confirmed *B. cereus* Ten g of *kochujang* was homogenized in a blender with 90 mL of 0.004% phosphate buffer solutions for 3 min and serially diluted to 10^{-3}. Enumeration and isolation of *B. cereus* were performed by layering of 0.2 mL on to surface of MYP agar (Oxoid, Basingstoke, Hampshire, UK) plates (Ø 16 cm) supplemented with polymyxin B sulphate and egg yolk solution. Each MYP plate was incubated at 30°C for 12-15 hr. Colonies surrounded by precipitate zones were presumed to be *B. cereus* and were isolated. To isolate the low level of *B. cereus* spores 10 mL of the primary dilution was heated at 85°C for 5 min and cooled in an ice water bath before further serial dilution, which was followed by surface plating on to MYP agar plates. Isolated colonies were subcultured in nutrient agar slants at 35°C for 24 hr and subsequently confirmed as *B. cereus* by the Voges-proskauer reaction test (VP), nitrate reduction, glucose assimilation, Gram-staining, and the shape and position of the endospore. In addition to the analyses of oxidase activity, rhizoid growth was evaluated and starch degradation performed (24). The total number of aerobic cells was determined using appropriate dilutions (1 mL) that were plated on nutrient agars (Merck, Darmstadt, Germany) which were then incubated at 35°C for 24 hr followed by counting.

Identification of isolated *B. cereus* strains Biochemical test kit (50CHB; Biomerieux, Marcy l'Etoile, France) and 16S rDNA sequencing were used to identify the isolated strains. For the biochemical tests, each of the strains was extracted from the nutrient agar slants using sterile cotton swabs and a mixture of 1 mL sterile saline solution. The extracted microbe solution was placed in a medium for identification, mixed sufficiently, inoculated onto a strip, and cultured at 35°C. The strip was read after 24 and 48 hr. For the sequencing of 16S rDNA, chromosomal DNA of each strain was extracted with a Wizard genomic DNA purification kit (Promega Co., Madison, WI, USA) and amplified by PCR using 2 universal primers: 27F (5'-AGA GTTTGATCATGGCTCAG-3') and 1492R (5'-GGATAC CTTGTTACGACTT-3') (25). The amplified PCR products were purified using Wizard SV Gel and PCR clean-up system (Promega Co.). The purified products were sequenced by an ABI PRISM 3700 DNA Analyzer (Foster City, CA, USA), and the resulting sequences were aligned with the Genebank sequences using Clustal X. The sequences were analyzed phylogenetically using the Mega 2 program.

Preparation of spore suspensions The VP positive and VP negative strains isolated from the *kochujang* were used as follows. Two strains were cultivated at 37°C for 24 hr in nutrient broth (Oxoid, Basingstoke, Hampshire, UK). The cultured cells were dispensed on nutrient agar plates supplemented with $MnSO_4$ 40 mg/L and $CaCl_2$ 100 mg/L (26), and incubated at 37°C for 5 day. Sporulation was verified by daily microscopic examination. When at least 90% sporulation was estimated to be reached, spores were collected from the surface of the agar with sterilized cotton swabs, suspended in sterile distilled water and washed three times by centrifugation (10,000×g for 10 min). The pellet was kept at 4°C during 12 hr in order to reduce the number of vegetative non-sporulated bacteria, and the pellet resuspended at a concentration of 10 log spores/mL level. The final suspension was distributed in sterile Eppendorf microtubes and kept at 4°C.

Heat treatment of the *kochujang* and spore suspensions Initial dilutions (10^{-1}) of *kochujang* and spore suspensions (about 5 log spore/mL) were prepared for heat treatment. One mL of each sample was injected into sterilized glass capillary tubes. The capillary tubes were flame-sealed, and submerged in a thermostate controlled water bath at 55, 65, 75, 85, 90, or 95°C for 5 min. After the heat treatment, the tubes were cooled in ice-water bath. The tubes were diluted serially for successive decimal levels using ice-cold sterile water. Appropriate dilutions were used to determine the total aerobic cell count and presumptive *B. cereus* count. Heat treatments were done for triplicate samples.

To isolate *B. cereus* from the inoculated *kochujang*, dilutions of the inoculated *kochujang* were heated at 85°C for 15 min. Spore suspensions were heated at 85, 90, 95, and 100°C as described previously. Appropriate dilutions were layered on to nutrient agar plates and incubated at 35°C for 24 hr. The D-values were calculated from the negative reciprocal of slopes of survival curves (coefficient of correlation $R^2 \geq 0.98$) as the time required to decrease the population by one $\log_{10}$ cycle. The z-values were determined as the increment in temperature required to reduce the D-value by one $\log_{10}$ cycle.

Statistical analyses Analysis of variance was calculated using release 8.1 of the statistical computer program SAS (SAS Institute Inc., Cary, NY, USA). Duncan's multiple range test was used to determine significant differences among the means when significant effects were observed ($p<0.05$). All tests were repeated 3 times.

Results and Discussion

Measurement of the microorganisms in the raw ingredients Table 1 shows the microbial counts in the raw ingredients. The total numbers of aerobic cells detected were 5.12, 8.58, and 3.83 log CFU/g in the red pepper powder, *meju* powder (*koji*), and malt-digested syrup, respectively. Presumptive *B. cereus* strains were detected 6.4±0.09 log CFU/g in *meju* powder. Detection of *B. cereus* in red pepper was reported at 98.1±25.4 CFU/g by the MPN method (27), but could not be quantified by the direct plating method. From these results, it could be said that *meju* powder should be considered as the major source of total aerobic cells and presumptive *B. cereus* strains that are detected in *kochujang*.

Changes of microorganisms, pH, and salt content in *kochujang* Changes of the microorganisms in *kochujang* during fermentation for 60 day are shown in Table 2. The initial population is 7.82 log CFU/g for total aerobic cells and 5.10 log CFU/g for presumptive *B. cereus*. The total numbers of aerobic cell were slightly decreased with fermentation time, but the presumptive *B. cereus* strains did not change. Although the total aerobic cells showed a significant difference ($p<0.05$) after 60 day of fermentation, the difference in the level of reduction was very little.

To enumerate *B. cereus*, 100 colonies of presumptive *B. cereus* were subjected to the confirmation procedure. All colonies tested were positive for nitrate reduction and the glucose assimilation test, but the VP test was negative. For more effective isolation, we suggested that a VP test be performed on presumptive *B. cereus* colonies, and then other confirmation tests be applied only for VP positive colonies.

Table 1. Mixing ratio of raw ingredients for the preparation of *kochujang* and the microbial counts in the raw ingredients[1)]

Raw ingredient	Ratio (%, w/w)	Total aerobic cell (log CFU/g)	Presumptive *B. cereus* (log CFU/g)
Glutinous rice powder	22.2	NT	ND
Red pepper powder	25.0	5.12±0.07	ND
Meju powder (*koji*)	5.5	8.58±0.23	6.4±0.09
Salt	12.8	NT	ND
Malt digested syrup	5.0	3.83±0.10	ND
Tap water	29.5	NT	ND
Total	100		

[1)]NT, Not tested; ND, not detected in 10^{-1} dilution; mean±SD (n=3).

Generally, *B. cereus* can easily be isolated from most food products with a contamination level kept in the range of 2-6 log CFU/g or mL. Some food categories have on occasion been reported to have high numbers of presumptive *B. cereus* (<4 log CFU/g), and include starches and cooked product such as cucumbers, fresh tomatoes, and rice (28). Foods included in the high level (>3 log CFU/g) were unhusked rice (9), cucumber (27), pasteurized milk (29), and spices (30). However, in the present study for a 10^{-3} dilution of *kochujang* the *B. cereus* proved to be undetectable. We expected that *kochujang* was naturally contaminated with a low level of *B. cereus* strains, and was poor media compare to other foods such as cereal grain or milk for the growth of *B. cereus*. There were no differences in the pH and salt content up to 60 day of fermentation. The results indicated that a high salt content (12.7%) and low pH (4.7) affected the growth of microorganisms in the *kochujang*.

Characteristics for isolation of *B. cereus* strains To isolate *B. cereus* presenting at a low level in *kochujang*, there is need to lower dilutions ($<10^{-3}$). As *kochujang* contained aerobic cells of 7-8 log CFU/g (Table 2), it was difficult to isolate *B. cereus* colonies that could be expected below a 3 log CFU/g level. However, the primary dilution of *kochujang* was heated to decrease likely background microflora interfering with the isolation of *B. cereus* (85°C for 5 min), and the VP positive colony, that were regarded as *B. cereus*, and VP negative colony were isolated, respectively.

The outward characteristics of the VP positive colonies on the MYP media were a 1-2 mm creamy white zone with an 8-9 mm precipitation zones, but VP negative colonies yielded a 6-7 mm pink zone with an 8-9 mm precipitation zone. When each colony isolated from the original MYP media was subcultured on subsequent MYP media where there was no interference from other microorganisms, the VP positive colony with a creamy color evidenced a regular edge and eosin pink wide precipitation zone compared to the VP negative colony (Fig. 1).

The characteristics of the VP positive colony (KBC) and the VP negative colony (KBM) are shown in Table 3. Confirmation tests for the KBC and KBM strains were positive for lecithinase production, acid production from mannitol, nitrate reduction, and the anaerobic assimilation of glucose. Also, 2 strains were Gram-positive, oxidase negative, and catalase positive and failed to swell the sporangium. The position of the endospore in the KBC strain was central or subterminal, while in the KBM strain the endospore was subterminal. Rhizoid growth (Fig. 1)

Table 2. Changes of microbial count, pH, and salt content in *kochujang* during fermentation at 25°C[1)]

Fermentation time (day)	pH	Salt content (%)	Total aerobic cell (log CFU/g)	Presumptive *B. cereus* (log CFU/g)	Confirmed *B. cereus* (log CFU/g)
0	4.76±0.01$^{A2)}$	12.74±0.05^{A}	7.82±0.02^{A}	5.10±0.04^{A}	ND
20	4.77±0.01^{A}	12.72±0.03^{A}	7.65±0.01^{B}	5.10±0.02^{A}	ND
40	4.71±0.01^{A}	12.71±0.01^{A}	7.57±0.03^{C}	5.02±0.07^{A}	ND
60	4.77±0.01^{A}	12.75±0.02^{A}	7.57±0.01^{C}	4.99±0.05^{A}	ND

[1)]Mean±SD (n=3); ND, not detected at 10^{-3} dilution.
[2)]Means with the same capital alphabet in column were not significantly different at $p < 0.05$.

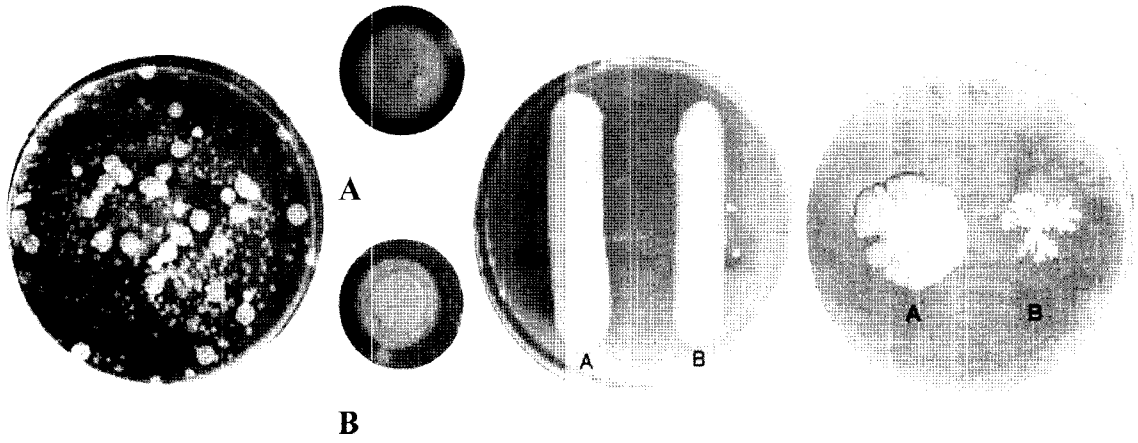

Fig. 1. Comparison of presumptive *B. cereus* colonies isolated from heated *kochujang* (5 min at 85°C). Left, results in culture on MYP agar for 12 hr at 35°C; center, result in subculture on MYP agar; right, rhizoid growth. A; Vogas-proskauer positive colony (KBC), B; Vogas-proskauer negative colony (KBM).

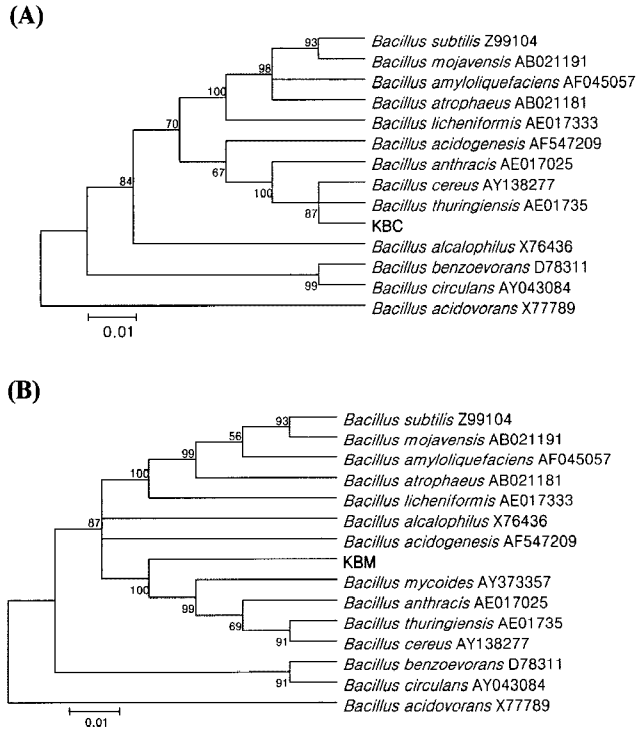

Fig. 2. Phylogenetic tree based on the 16S rDNA sequences showing the position of strain KBC (A) and KBM (B) isolated from *kochujang*.

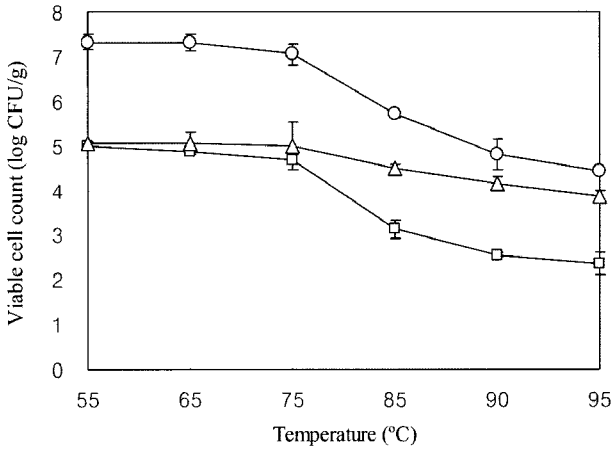

Fig. 3. Survival curves for microorganisms present in *kochujang* and spore suspensions of *B. cereus* (KBC strain) isolated from *kochujang* treated at different temperatures for 5 min. ○, Total viable cells; △, *B. cereus* (KBC strain); □, presumptive *B. cereus*.

was negative in the KBC strain, but positive in the KBM strain, and starch degradation were a weak positive in KBC and negative in KBM. From these results, we confirmed the strain KBC to be *B. cereus*, whereas the KBM strain was difficult to distinguish from *B. laterosporus* and *B. mycoides*. Positive rhizoid growth is the definitive characteristic of *B. mycoides* (10), however, VP and starch degradation negativity are the charac-teristics of *B. laterosporus* (24). The 2 strains were further characterized and identified using biochemical test and the 16S rDNA sequence analysis (Fig. 2) whereby the KBC and KBM were clearly determined to be *B. cereus* and *B. mycoides*, respectively.

Heat resistance characteristics of the isolated strains The D- and z-values for spores of the 2 *Bacillus* strains (strain KBC and KBM) isolated from *kochujang* are shown in Table 4. D-Values ranged from 45.05 ($D_{85°C}$) to 8.37 min ($D_{100°C}$) for strain KBC and 39.82 ($D_{85°C}$) to 7.08 min ($D_{100°C}$) for strain KBM. The D-value of strain KBC, with 28.0 ($D_{90°C}$), 15.70 ($D_{95°C}$), and 8.37 min ($D_{100°C}$), was higher than that reported by other researchers. Sarrias *et al.* (9) and Valero *et al.* (27) reported $D_{90°C}$-values ranging from 1.4 to 22.0 min for *B. cereus* isolated from different sources. Also $D_{95°C}$-values (1.83-4.63 min) for *B. cereus* ATCC 4342 and $D_{100°C}$-values (3.6-5.9 min) for *B. cereus* isolate from spices have been reported (31,32). In general, the presence of sodium chloride in the heating medium increases heat resistance, an effect that varied among strains (14). We speculate that a high salt content in *kochujang* may increase the heat resistance of micro-organisms that are isolated from *kochujang* e.g., the z-values of KBC and KBM strains were 20.53 and 18.55°C, respectively. In addition, their coefficients (R^2) were estimated at 0.99 (KBC) and 0.98 (KBM), respectively.

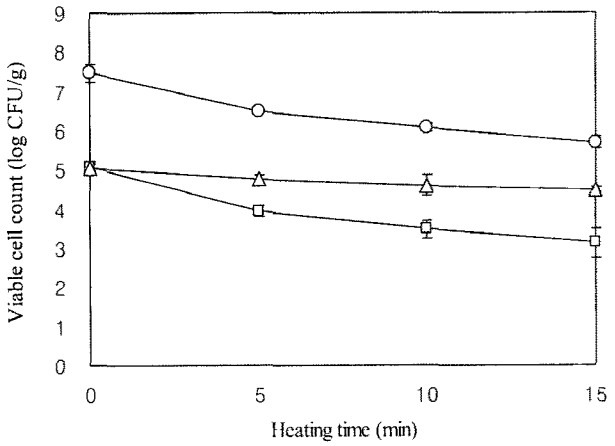

Fig. 4. Survival curves for microorganisms present in *kochujang* and spore suspensions of *B. cereus* (KBC strain) isolated from *kochujang* treated for different time intervals at 85°C. ○, Total viable cells; △, *B. cereus* (KBC strain); □, presumptive *B. cereus*.

Determination of optimal heating conditions for the isolation of *B. cereus* from *kochujang* Figure 3 shows the change in total aerobic cells and presumptive *B. cereus* in heated *kochujang* and spore suspension of *B. cereus* (KBC) after exposure to heated distilled water (5 min each) over the temperature range 55-95°C. In the heated *kochujang*, total aerobic cells and presumptive *B. cereus* resulted in a 2.89 and 2.63 log CFU/g reduction by increasing temperature from 55 to 95°C, respectively, while the heated KBC strain resulted in a 1.19 log CFU/g reduction. Between the temperature of 75 and 85°C, the numbers of total aerobic cells and presumptive *B. cereus* were reduced by 1.35 and 1.56 log CFU/g, but the numbers of the KBC strain were only slightly reduced by 0.49 log CFU/g. Therefore, the optimal heating temperature was determined to be 85°C.

When each test sample was heated for 5, 10, and 15 min at 85°C, the numbers of total aerobic cells and presumptive *B. cereus* decreased 1.78 and 1.97 log CFU/g by heat treatment for 15 min, respectively, but the number of the KBC strain decreased only 0.56 log CFU/g (Fig. 4). Consequently, heat resistance of the KBC strain appeared to be stronger than that of the microorganisms present in the *kochujang*. These results are particularly useful in isolating *B. cereus* from *kochujang* that contains a high level of other microorganisms. Thus, 85°C was a suitable temperature to decrease the background microflora which ordinary interferes with the isolation of *B. cereus*.

Changes in the population of *B. cereus* by inoculation to *kochujang* To create plates that would contain 15-150 typical colonies, each *kochujang* was inoculated with *B. cereus* (KBC) at level of 4 and 5 log spore/g by dilution at 10^{-2} and 10^{-3}, respectively. According to the results we have outlined, *kochujang* was inoculated with 4 log spore/g of *B. cereus* and heated at 85°C for 15 min to decrease the background population of microflora.

The change in population of KBC strain is highlighted in Table 5. The number of KBC strain measured during fermentation was no significant difference ($p<0.05$) at each sample level (4 and 5 log spore/g). *Kochujang* usually has an unfavorable condition for the growth of *B. cereus* because of the low pH and high salt concentration (Table 2). Some researchers have reported that the minimum pH supporting growth of *B. cereus* is 4.90 in the broth (33) and 4.35 in food (34), but Valero *et al.* (35) found no growth at pH 5.0 and 16°C up to 60 day period. For the concentration of sodium chloride, Mossel *et al.* (36) suggested 10% NaCl was inhibitory for *B. cereus*, whereas Mahakarnchannakul and Beuchat (37) reported that only 2.3-3.0 log reduction in mashed potatoes supplement 4% sodium chloride at 10°C within 7 day. We observed, however, that *B. cereus* survived in *kochujang* but failed to reproduce. Also, when *kochujang* was inoculated at 4 log spore/g of *B. cereus* and heated at 85°C for 15 min, the number of KBC strain was

Table 3. Characteristics of 2 strains isolated from *kochujang* on the basis of their biochemical and physiological properties and DNA sequences

Test	Strains[1)]	
	KBC	KBM
Confirmation test		
Lecithinase production	+	+
Acid production from mannitol	+	+
Voges-proskauer reaction	+	-
Nitrate reduction	+	+
Anaerobic utilization of glucose	+	+
Supplement test		
Oxidase test	-	-
Catalase test	+	+
Rhizoid growth	-	+
Starch degradation	+	-
Biochemical test		
% ID	99.6	90.9
T index	0.88	0.82
Significant taxa	*Bacillus cereus*	*Brevibacillus laterosporus*
16S rDNA sequencing		
% Similarity	99.0	99.0
Significant taxa	*Bacillus cereus*	*Bacillus mycoides*

[1)]KBC, Positive colony in Voges-proskauer reaction test; KBM, negative colony in Voges-proskauer reaction test; +, positive; -, negative.

Table 4. Heat resistance characteristics of 2 strains isolated from *kochujang*[1)]

Strain[2)]	D-Value (min)				z-Value (°C)	R^2
	$D_{85°C}$	$D_{90°C}$	$D_{95°C}$	$D_{100°C}$		
KBC	45.05±2.07[A]	28.00±1.24[A]	15.70±1.92[A]	8.37±0.42[A]	20.53	0.99
KBM	39.82±1.32[B]	21.32±1.66[B]	10.86±0.50[B]	7.08±0.07[B]	18.55	0.98

[1)]Means with the same capital alphabet in column were not significantly different at $p<0.05$; mean±SD (n=3).
[2)]KBC, Positive colony in Voges-proskauer reaction test; KBM, negative colony in Voges-proskauer reaction test.

Table 5. Changes of *B. cereus* (log CFU/g) in *kochujang* inoculated with an isolated strain (KBC) during fermentation for 60 day at 25°C

Inoculum (log spore/g)	Dilution	Heat treatment	Fermentation time (day)[1)]			
			0	20	40	60
4	10^{-2}	85°C/15 min	4.29±0.11^{A}	4.30±0.02^{A}	4.21±0.05^{A}	4.32±0.04^{A}
		Not treated	4.29±0.26^{A}	4.32±0.01^{A}	4.28±0.01^{A}	4.31±0.06^{A}
5	10^{-3}	Not treated	5.28±0.04^{A}	5.40±0.06^{A}	5.41±0.07^{A}	5.37±0.08^{A}

[1)]Means with the same capital alphabet in row were not significantly different at $p<0.05$; mean±SD (n=3).

unchanged compared with unheated *kochujang*, thereby indicating no effect on the total number. Using a similar method, te Giffel *et al.* (30) applied heat (80°C, 10 min) and counted *B. cereus* in various food products. Therefore, we estimated that the heat treatment (85°C, 15 min) is a useful added step to improve the isolation of *B. cereus* that may be present at low levels in traditional *kochujang*.

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