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# Isolation and Identification of *Bacillus* sp. with High Protease and Amylase Activity from Sunchang Traditional *Kochujang*

**Sung-Tae Jung[1], Min-Hwa Kim[2], Dong-Hwa Shin[2], and Yong-Suk Kim***

*Research Center for Industrial Development of BioFood Materials, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea*
[1]*Harim Ltd., Iksan, Jeonbuk 570-883, Korea*
[2]*Faculty of Biotechnology (Food Science & Technology Major), Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea*

**Abstract** To improve the quality of traditional *kochujang*, strains with high protease and amylase activity were isolated and identified from Sunchang traditional *kochujang*. Twenty-three strains strongly producing protease and 16 strains strongly producing α- and β-amylase were isolated by using 1% isolated soy protein agar medium and 2% starch agar medium, respectively. Protease activities of the IA7, I5, and IA2 strain were 22.5, 21.2, and 20.6 unit/mL, respectively, and were higher than those of the other strains. Stains with high α-amylase activity included K9 (967.8 unit/mL), K14 (828.3 unit/mL), K13 (662.5 unit/mL), K8 (601.5 unit/mL), and K11 (405.9 unit/mL). The β-amylase activity of the K11 strain was the highest, 34.3 unit/mL, among the isolated strains. Based on morphological, physiological properties, and API 50CHB-kit test for assimilation of 49 carbohydrates, 8 strains selected according to protease, α-amylase, and β-amylase activities were tentatively identified as *Bacillus megaterium* (IA2), *Bacillus subtilis* (IA7, I5), *Bacillus amyloliquefaciens* (K8, K9, K11, and K13), and *Bacillus stearothermophillus* (K14). The IA7, I5, and K11 strains were finally identified as *B. subtilis* (99% ID) based on 16S rDNA sequencing.

**Keywords:** *kochujang*, *Bacillus subtilis*, protease, amylase, identification

## Introduction

*Kochujang*, a fermented red pepper-soybean paste, is a traditional Korean seasoning spice that combines a sweet taste from a starch hydrolyzate, a hot taste of red pepper, and a savory taste from soybean protein hydrolyzates and nucleic acids (1). *Kochujang* has recently attracted interest for its health-promoting properties, including its anti-obesity effect (2,3) and effect of decreasing body weight and serum lipid level (4).

Recently, due to an increase of imported foods, the competitiveness of Korean traditional foods has been weakened. Therefore, strengthening the competitiveness of *kochujang* through the improvement of manufacturing methods and quality, and the development of various products with high quality and functionality was necessary in order to satisfy the demands of a variety of consumers (5).

Factory-made *kochujang* is fermented by the enzymatic action of *Aspergillus oryzae* and yeast, and requires a short fermentation and aging period (6). In the case of traditional *kochujang*, various naturally occurring bacteria and fungi proliferate in the *meju*, and this process requires a long period of time (7). The traditional processes often generate an 'off-flavor' and unacceptable taste because of contaminant microorganisms (8). It is difficult to standardize the flavors of traditional *kochujang*, which could be affected by local weather, environment, and other factors. In order to standardize the flavor and taste of traditional *kochujang*, studies have been conducted on controlled fermentation and the use of enzymes originating from known microflora (*A. oryzae*).

Many studies on the ingredients needed for *kochujang* preparation (8,9) and changes of physicochemical properties during fermentation and aging (10,11) have been conducted. Research on the microorganisms in *kochujang* have been conducted on the preparation of *meju* (13,14), changes in microflora and enzyme activity during fermentation (15), bacteria (16), and yeast distribution (17), and isolation and identification of *Bacillus cereus* (18) in commercial and traditional *kochujang* or *meju*, and screening of gas-producing yeast such as *Saccharomyces* and *Zygosaccharomyces* in traditional *kochujang* (19).

The quality of traditional *kochujang* depends not only on the unique ratio of the ingredients, the preparation method, and the aging conditions, but also on the types of microorganisms present in *meju* and *kochujang*. Therefore, to prepare hygienic *kochujang* with consistent quality, research on the type and properties of microorganisms is required to investigate the fermentation of *kochujang*.

Thus, to prepare hygienic *kochujang* with consistent quality, *Bacillus* sp. with high protease and amylase activity from Sunchang traditional *kochujang* was isolated, and identified it through morphological and biochemical tests and 16S rDNA sequencing.

## Materials and Methods

**Materials** *Kochujang* purchased from Sunchang traditional soy products manufacturer in July 2006 was used for the isolation of microorganisms.

**Screening of the strains with high protease activity** Ten g extracts of *kochujang* were prepared by shaking in

*Corresponding author: Tel: +82-63-270-2570; Fax: +82-63-270-2572
E-mail: kimysmmd@hanmail.net
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100 mL of distilled water, followed by centrifugation at 17,000×g for 10 min (J2-21 Centrifuge; Beckman Instruments, Inc., Palo Alto, CA, USA). The extract was then collected in order to isolate the bacterial strains.

Isolation of the strains with high protease activity was performed by layering of 0.2 mL of the extract onto the surface of 1% isolated soybean protein (ISP, ADM, Decature, IL, USA) agar plates. Each plate was incubated at 37°C for 72 hr. Colonies surrounded by clear zones were selected, and large clear-zone forming colonies were selected again by the toothpick method (20). Isolated colonies were used for the measurement of protease activity.

**Measurement of protease activity** One loopful was taken from each of the selected colonies and inoculated in 10 mL of 1% ISP broth, followed by incubation at 37°C for 72 hr. Supernatant of 1% ISP culture solution that was obtained by centrifuging at 1,159×g for 20 min was used as crude enzyme solution. Aliquots of 1.0 mL of the supernatant were used to measure protease activity; the pH was adjusted to 7.0 by using phosphate buffer (0.2 M, pH 7.2). The pH of 2 mL of 0.6% casein was adjusted to 7.0 and warmed for 2 min at 30°C. The supernatant (0.5 mL) was then added to 2 mL of 0.6% casein with the corresponding pH and reacted at 30°C for 10 min. The reaction was stopped by the addition of 5 mL of 0.4 M trichloroacetic acid, and subsequently filtered. Five mL of 0.4 M $Na_2CO_3$ and 1.0 mL of diluted Folin reagent were added to 1.0 mL of the filtrate and reacted for 30 min at 30°C. The protease activity was then measured as the absorbance at 660 nm using a ultraviolet (UV)-spectrophotometer (UV-1201; Shimadzu Co., Kyoto, Japan), and the units were expressed as the liberation of 1 μM of tyrosine per 1.0 mL of the supernatant (21).

**Screening of the strains with high amylase activity** Isolation of the strains with high amylase activity was performed by the layering of 0.2 mL of the extract, pretreated as for the measurement of protease activity, onto the surfaces of 2% starch (Junsei Chemical Co., Ltd., Tokyo, Japan) agar plates. Each plate was incubated at 37°C for 72 hr. Transparent ring-forming colonies caused by dropping of Gram's iodine (Hayashi Pure Chemical Ind., Ltd., Osaka, Japan) solution were selected, and large transparent ring-forming colonies were selected again by the toothpick method (20). Isolated colonies were used for measurement of α- and β-amylase activity (22).

**Measurement of amylase activity** One loopful was taken from each of the selected colonies and inoculated in 10 mL of 2% starch broth, followed by incubation at 37°C for 72 hr. Supernatant of 2% starch broth culture solution that was obtained by centrifugation at 1,159×g for 20 min was used as crude enzyme solution.

To measure α-amylase activity (23), 1.0 mL of the supernatant was added to a mixture of 1.0 mL of 2% soluble starch (pH 5.0) and 1.0 mL of acetate buffer, and the mixture was heated at 40°C for 30 min. Ten mL of 0.5 M acetic acid was then added to stop the reaction, after which 10 mL of $3.33\times10^{-4}$ N iodine solution was added. The activity of the reaction mixture was measured as its absorbance at 540 nm using a UV-spectrophotometer, and was expressed as units per 1.0 mL of the supernatant.

The β-amylase activity was determined by the dinitrosalicylic acid method (24,25), in which a mixture of 1.0 mL of 2% starch broth and 1.0 mL of acetate buffer was added to 1.0 mL of the supernatant and warmed for 10 min at 30°C. Dinitrosalycylic acid (3 mL) was added to the reaction mixture, and the activity level was determined as the absorbance at 535 nm using the UV-spectrophotometer. Using maltose as the standard, a unit of enzyme activity was expressed as the liberation of 1.0 mg maltose per 1.0 mL of the supernatant.

**Identification of isolated strains** The properties of the 8 isolated strains were investigated by testing the Gram-staining and microscopic observation after cultivation on tryptic soy agar (Difco Laboratories, Detroit, MI, USA) for 24 hr at 37°C. Bergey's Manual of Systematic Bacteriology (26) was used to examine the morphological and physiological properties of the isolated strains. The API 50CHB-kit (bioMerieux Co., Marcy L'Etoile, France), an identification system for microorganisms, was used to investigate and identify the assimilation of the 49 carbohydrates (27).

IA7, I5, and K11 strains, tentatively identified as *B. subtilis* or *Bacillus amyloliquefaciens* in the above tests, were identified by using the 16S rDNA sequencing method. Chromosomal DNA of isolated strains was separated by using Wizard genomic DNA purification kit (Promega Co., Medison, WI, USA). The DNA extracts were used for polymerase chain reaction (PCR) with the universal primers [27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGATACCTTGTTACGACTT-3')] (28). Amplification with 16S rDNA, previously purified with a Wizard SV Gel and PCR clean-up system (Promega Co.), was used for sequencing with a ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The sequence was determined by BLASTN program of GENEBANK data library and analyzed by Clustal X and Mega 2 programs (29).

## Results and Discussion

**Screening and isolation of strains with high protease activity** The protease activities of the isolated strains cultivated in 1% ISP broth medium at 37°C for 72 hr are shown in Fig. 1.

The protease activities of other strains without IA3 (9.4 unit/mL), IA5 (9.8 unit/mL), or IA13 (7.8 unit/mL) were greater than 10 unit/mL. In particular, the protease activities of the IA1 (19.6 unit/mL), IA2 (20.6 unit/mL), IA7 (22.5 unit/mL), IA10 (20.0 unit/mL), IA14 (19.4 unit/mL), and I5 (21.2 unit/mL) strains were higher than those of other strains. The IA2, IA7, and I5 strains were selected for morphological and physiological testing.

Lee *et al.* (30) reported that the protease activities of *A. oryzae*-B and *B. subtilis*-P isolated from traditional, aged *kochujang* were higher than those of *Bacillus licheniformis*-K and *B. subtilis*-G; in addition, the higher the salinity of *kochujang*, the greater the reduction in enzyme activities. The results found for protease activities in our results were higher than those of Kim *et al.* (31), who reported that protease activities of the Sunchang 1 and Park 2 strains, identified as *Bacillus* sp., and isolated from traditional

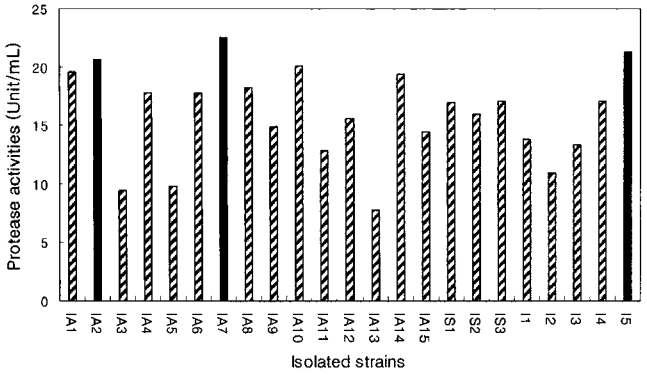



**Fig. 1. Protease activities of strains isolated from Sunchang traditional *kochujang*.**

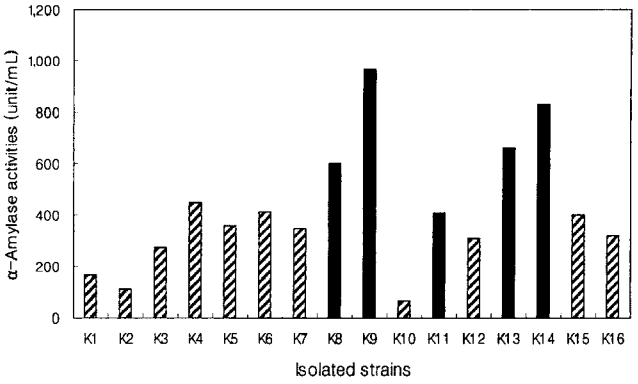



**Fig. 2. α-Amylase activities of strains isolated from Sunchang traditional *kochujang*.**

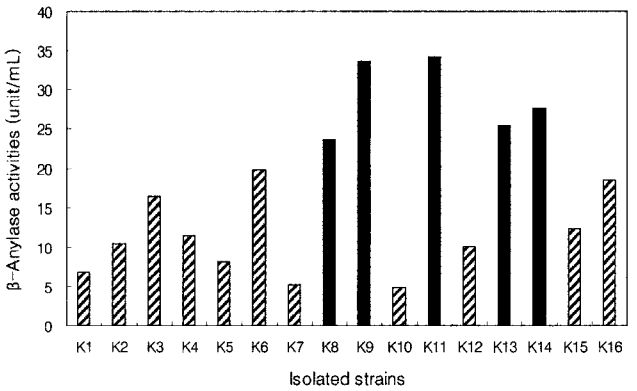



**Fig. 3. β-Amylase activities of strains isolated from Sunchang traditional *kochujang*.**

*kochujang meju*, were 4.76 and 5.34 unit/mL, respectively. In this study, 3 strains (IA2, IA7, and I5 strain) showing high protease activities were selected for the investigation their morphological and physiological properties.

**Screening and isolation of the strains with high amylase activities** The amylase activities of the isolated strains cultivated in 2% starch broth medium at 37°C for 72 hr are shown in Fig. 2 and 3.

Among the isolated strains, the α-amylase activities of 8 strains were greater than 400 unit/mL (Fig. 2). The α-amylase activity of the K9 strain was the highest (967.8 unit/mL), and those of other strains showed activities follows: K14 (828.3 unit/mL), K13 (662.5 unit/mL), K8 (601.5 unit/mL), K4 (448.5 unit/mL), K6 (410.8 unit/mL), K11 (405.9 unit/mL), and K15 strain (401.1 unit/mL).

Among the isolated strains, the β-amylase activities of 8 strains were greater than 15 unit/mL (Fig. 3). The β-amylase activity of the K11 strain was the highest (34.3 unit/mL), and those of other strains showed activity as follows: K9 (33.7 unit/mL), K14 (27.7 unit/mL), K13 (25.4 unit/mL), K8 (23.6 unit/mL), K6 (19.8 unit/mL), K16 (18.5 unit/mL), and K3 strain (16.4 unit/mL).

Lee *et al.* (30) reported that the amylase activities of microorganisms isolated from traditional, aged *kochujang* showed differing levels of activity in the following order: *Bacillus subtilis*-P > *B. licheniformis*-K > *B. subtilis*-G. Kim *et al.* (31) reported that the α-amylase activities of the Sunchang 1 and Park 2 strains, identified as *Bacillus* sp. and isolated from traditional *kochujang meju*, were 13.99 and 11.54 unit/mL, respectively, while the β-amylase activities were 23.25 and 9.89 unit/mL, respectively, higher than those of other strains. However, the α- and β-amylase

**Table 1. Morphological and physiological properties of the strains isolated from *kochujang***

| Characteristics | Bs[1)] | Ba[2)] | IA2 | IA7 | I5 | K8 | K9 | K11 | K13 | K14 |
|---|---|---|---|---|---|---|---|---|---|---|
| Morphological characteristic | | | | | | | | | | |
| Rod-shaped | +[3)] | + | + | + | + | + | + | + | + | + |
| Gram staining | + | + | + | + | + | + | + | + | + | + |
| Spore formation | + | + | + | + | + | + | + | + | + | + |
| Physiological characteristics | | | | | | | | | | |
| Catalase | + | + | + | + | + | + | + | + | + | + |
| Acid from D-mannitol | + | + | + | + | + | + | + | + | + | + |
| Acid from D-glucose | + | + | + | + | + | + | + | + | + | + |
| Utilization of citrate | + | + | -[4)] | + | + | - | - | + | - | - |
| Utilization of propionate | - | - | - | - | - | - | - | - | - | - |
| Nitrate reduced to nitrite | + | + | + | + | + | + | + | + | + | + |
| Voges Proskauer test | + | + | + | + | + | + | + | + | + | + |

[1)]Data on *Bacillus subtilis* in Bergey's manual of systematic bacteriology (24).
[2)]Data on *Bacillus amyloliquefaciens* in Bergey's manual of systematic bacteriology (24).
[3)]Positive.
[4)]Negative.

**Table 2. Carbohydrate utilization of the isolated strain using an API 50CHB-kit**

| Carbohydrates | Bs[1)] | Ba[2)] | Isolated strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | IA2 | IA7 | I5 | K8 | K9 | K11 | K13 | K14 |
| Glycerol | +[3)] | + | + | + | + | + | + | + | + | - |
| Erythritol | -[4)] | - | - | - | - | - | - | - | - | - |
| D-Arabinose | - | - | - | - | - | - | - | - | + | - |
| L-Arabinose | + | + | + | + | + | + | + | + | + | - |
| Ribose | + | + | + | + | + | + | + | + | + | - |
| D-Xylose | + | + | + | + | + | + | + | + | + | - |
| L-Xylose | - | - | - | - | - | - | - | - | - | - |
| Adonitol | - | - | - | - | - | - | - | - | - | - |
| β-Methyl-D-xyloside | - | - | - | - | - | - | - | - | - | - |
| Galactose | + | - | + | + | + | - | - | - | - | - |
| D-Glucose | + | + | + | + | + | + | + | + | + | + |
| D-Fructose | + | + | + | + | + | + | + | + | + | + |
| D-Mannose | + | + | + | + | + | + | + | + | + | + |
| L-Sorbose | - | - | - | - | - | - | - | - | - | - |
| Rhamnose | + | - | - | + | + | - | - | - | - | - |
| Dulcitol | - | - | - | - | - | - | - | - | - | - |
| Inositol | + | - | + | + | + | - | - | - | - | - |
| Mannitol | + | + | + | + | + | + | + | + | + | + |
| Sorbitol | + | + | + | + | + | + | + | + | + | + |
| α-Methyl-D-mannoside | - | - | + | - | - | - | - | - | - | - |
| α-Methyl-D-glucoside | + | + | + | + | + | + | + | + | + | + |
| *N*-Acetyl glucosamine | + | + | + | + | + | + | + | + | + | + |
| Amygdalin | - | + | + | - | - | - | + | + | - | - |
| Arbutine | + | + | + | + | + | - | + | + | + | + |
| Esculine | + | + | + | + | + | + | + | + | + | + |
| Salicine | + | + | + | + | + | - | + | + | + | + |
| Cellobiose | ? | + | + | ? | ? | - | + | + | + | - |
| Maltose | + | + | + | + | + | + | + | + | + | + |
| Lactose | - | + | + | - | - | + | + | + | + | + |
| Melibiose | + | - | + | + | + | - | + | - | - | - |
| Sucrose | + | + | + | + | + | + | + | + | + | + |
| Trehalose | + | + | + | + | + | + | + | + | + | + |
| Inuline | + | - | - | + | + | - | - | - | - | - |
| Melezitose | - | - | - | - | - | - | - | - | - | - |
| D-Raffinose | + | + | + | + | + | + | + | + | + | + |
| Amidon | + | + | + | + | + | + | + | + | + | + |
| Glycogen | + | + | + | + | + | + | + | + | + | + |
| Xylitol | ? | - | - | ? | ? | - | - | - | - | - |
| β-Gentiobiose | + | + | + | + | + | + | + | + | + | - |
| D-Turanose | + | - | - | + | + | - | - | - | - | - |
| D-Lyxose | - | - | - | - | - | - | - | - | - | - |
| D-Tagatose | - | - | - | - | - | - | - | - | - | - |
| D-Fucose | - | - | - | - | - | - | - | - | - | - |
| L-Fucose | - | - | - | - | - | - | - | - | - | - |
| D-Arabitol | - | - | - | - | - | - | - | - | - | - |
| L-Arabitol | - | - | - | - | - | - | - | - | - | - |
| Gluconate | + | - | - | + | + | - | - | - | - | - |
| 2-Keto-gluconate | - | - | - | - | - | - | - | - | - | - |
| 5-Keto-gluconate | - | - | - | - | - | - | - | - | - | - |

[1)]Data on *Bacillus subtilis* in API 50CHB-kit (bioMeriux Co.).
[2)]Data on *Bacillus amyloliquefaciens* in API 50CHB-kit (bioMeriux Co.).
[3)]Positive.
[4)]Negative.

activities determined by Kim *et al.* (31) were lower than those found in our study.

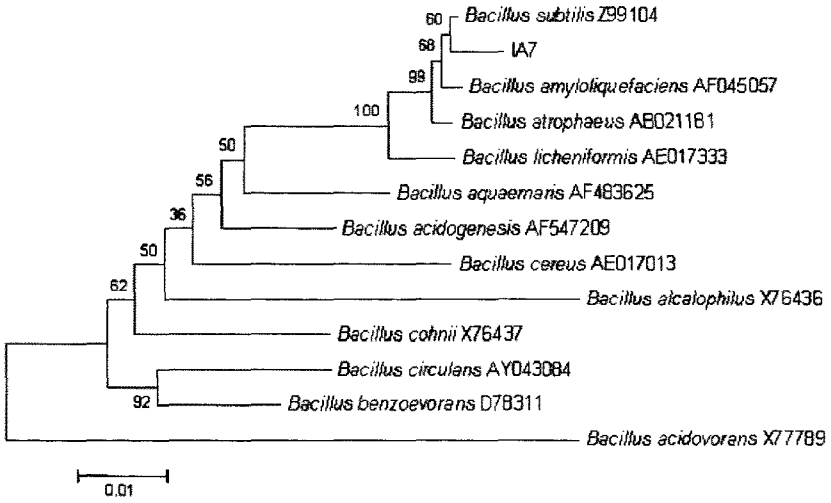
In this study, 5 strains (K8, K9, K11, K1, and K14 strain) showing high α- and β-amylase activities were selected for the investigation their morphological and physiological properties.

**Morphological, physiological, and biochemical properties of the isolated strains** The morphological and physiological properties of the isolated strains showing high protease or α- and β-amylase activities were investigated by Bergey's manual of systematic bacteriology method (26).

Eight strains isolated from Sunchang traditional *kochujang* were Gram-positive, rod-shaped, aerobic, and spore-forming (Table 1). Examination of their physiological properties revealed them to be catalase-positive and Voges-Proskauer response-positive, and the results against other properties without citrate utilization were similar. IA2, K8, K9, K13, and K14 strains did not utilize citrate, and so were

**Table 3. Results of identification based on the biochemical properties of the strains isolated from *kochujang* using an API 50CHB-kit**

| Strains | Significant taxa | % ID | T |
|---|---|---|---|
| IA2 | *Bacillus megaterium* | 50.8 | 0.51 |
| | *Bacillus licheniformis* | 47.8 | 0.61 |
| IA7 | *Bacillus subtilis* | 95.0 | 0.58 |
| I5 | *Bacillus subtilis* | 95.4 | 0.53 |
| K8 | *Bacillus amyloliquefaciens* | 68.3 | 0.25 |
| | *Bacillus subtilis* | 30.9 | 0.26 |
| K9 | *Bacillus amyloliquefaciens* | 79.8 | 0.71 |
| | *Bacillus subtilis* | 11.8 | 0.68 |
| K11 | *Bacillus amyloliquefaciens* | 98.8 | 0.77 |
| K13 | *Bacillus amyloliquefaciens* | 95.7 | 0.26 |
| | *Bacillus stearothermophillus* | 66.1 | 0.48 |
| K14 | *Bacillus subtilis* | 12.8 | 0.23 |
| | *Bacillus amyloliquefaciens* | 11.0 | 0.19 |




>IA7
TATACTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTAC
AGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTT
CAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGC
AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
GCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT
CTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG
GACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGT
TCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTT
TGTAACACCCGAAGTCGGTGAGGTAACCTTTAGGAGCCAGCCGCCGAAGGTGACAGA

**Fig. 4. Phylogenetic tree (upper) and gene sequences (bottom) based on 16S rDNA sequencing, showing the positions of strain IA7 isolated from Sunchang traditional *kochujang*.**

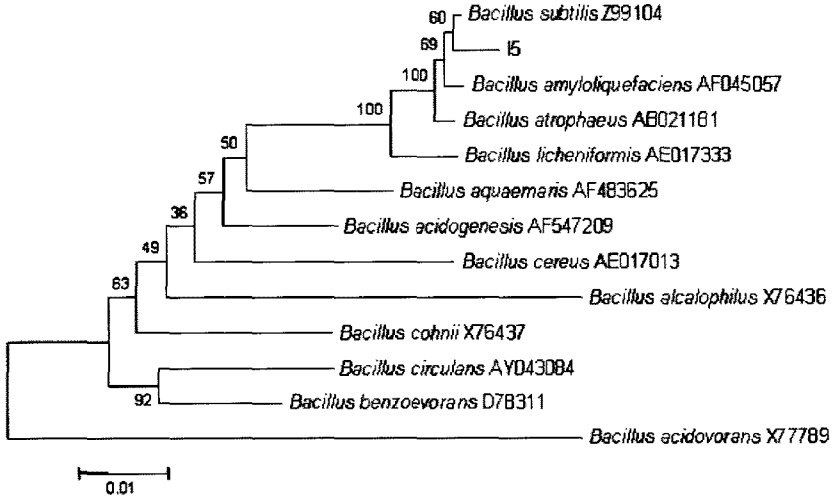
presumed to be *Bacillus polymyxa, B. amyloliquefaciens*, or *Bacillus coagulans* (26). IA7, I5, and K11 strains that utilized citrate were presumed to be *B. subtilis, B. licheniformis,* or *Bacillus megaterium* (26). The isolated strains were presumed to be *Bacillus* sp. based on their morphological and physiological properties.

Table 2 shows the results of an assimilation test of 49 carbohydrates using an API 50CHB-kit. All isolated strains utilized D-glucose, D-fructose, D-mannose, mannitol, and sorbitol, but did not utilize erythritol, L-xylose, or L-sorbose. The IA2 strain differs from the IA7 and I5 strains in terms of its utilization of rhamnose, lactose, inulin, and gluconate, but is similar to the K8, K9, K11, K13, and K14 strains, which show different results for the utilization of galactose and inositol. IA7 and I5 strains showing similar properties in the assimilation test results of 49 carbohydrates were presumed to be identical, and differs from other strains in terms of the utilization of rhamnose, lactose, inulin, and gluconate. K8, K9, K11, and K13 strains had similar properties in the results of an assimilation test of 49 carbohydrates, and the K14 strain differs in the assimilation of glycerol, L-arabinose, ribose, D-xylose, and β-gentibiose.

From the morphological and physiological properties, as well as the assimilation test results for 49 carbohydrates, the strains isolated from Sunchang traditional *kochujang* were tentatively identified as *B. megaterium* (IA2), *B. subtilis* (IA7, I5), *B. amyloliquefaciens* (K8, K9, K11, and K13), and *B. stearothermophillus* (K14) (Table 3).

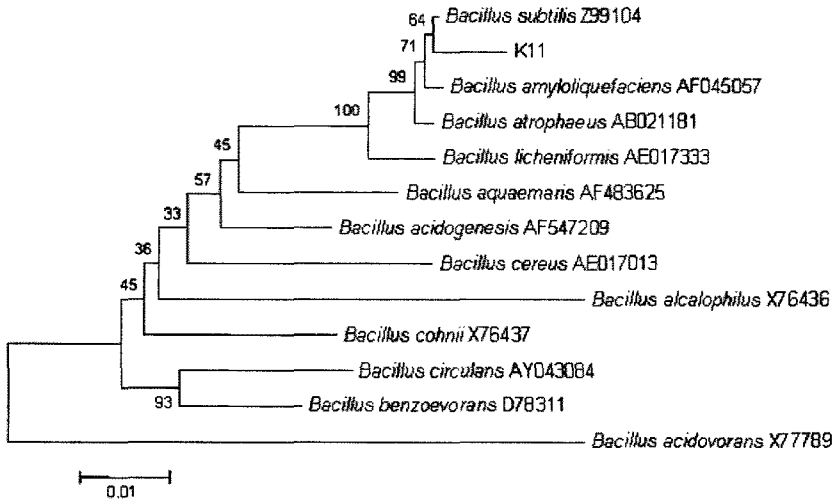
**Identification by 16S rDNA sequencing** IA7, I5, and K11 strains that were regarded as different strains based on their morphological properties, physiological properties, and the results of an assimilation test of 49 carbohydrates were selected for 16S rDNA sequencing.

From the results of the homogeny test conducted by 16S rDNA sequencing, the IA7 (Fig. 4), I5 (Fig. 5), and K11 (Fig. 6) strains show 99% homogeny with *B. subtilis*. IA7 and I5 strains were identical, but K11 strain slightly differs with IA7 and I5 strains in phylogenetic tree. In the case of the IA7 and I5 strains, these results were similar to the




>I5
GCGCGGCTATCTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
GGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTAC
CACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG
ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGT
GCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGG
GCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT
TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAA
TGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGC
TGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGC
ACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGAC
ATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA
CAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTC
TCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGG
ATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGA
GAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTATGAGCCAGCCGCCGAAGGT

**Fig. 5. Phylogenetic tree (upper) and gene sequences (bottom) based on 16S rDNA sequencing, showing the positions of strain I5 isolated from Sunchang traditional *kochujang*.**




>K11
ACAGCGGGGCTATCTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGG
CGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGC
TACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
GCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGA
GCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAAC
AAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT
ACGTGCCAACAGACCGCAGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGT
AAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAG
GGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCG
GTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAC
TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGG
TCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAAT
CTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAAT
CGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTAGGAGCCAGCCGCCG

**Fig. 6. Phylogenetic tree (upper) and gene sequences (bottom) based on 16S rDNA sequencing, showing the positions of strain K11 isolated from Sunchang traditional *kochujang*.**

morphological and physiological properties, and the results of the API 50CHB-kit test, while the results for K11 strain differed. The K11 strain was finally identified as *B. subtilis* based on the results of 16S rDNA sequencing. Finally, the IA7 and I5 strains with high protease activities and the K11 strain with high α- and β-amylase activities isolated from Sunchang traditional *kochujang* were identified as *B. subtilis*.

Lee *et al.* (16) reported that bacteria of 10 genera and 19 species were isolated from traditional *kochujang*, and the bacterial distribution at 12 months of fermentation included 5 genera and 8 species, including 46% *Bacillus* sp. (*B. subtilis*, *B. licheniformis*), 18% *Pasteurella haemolytica*, and 8% *Streptococcus acidominimus*. In addition, 3 strains among 140 strains isolated from *kochujang* using traditional *meju* were identified as *B. subtilis*-P, *B. subtilis*-G, and *B. licheniformis*-K (30). Kim *et al.* (31) isolated the strains with high protease and amylase activity from traditional *kochujang meju* and identified them as *Bacillus* sp.

In conclusion, various types of bacteria were distributed in traditional *kochujang*, but *Bacillus* sp. were the main types of organisms found in the product. The strains isolated in our study were identified as *Bacillus* sp. From these results, *kochujang* with hygienic and identical quality could be made by using selected strains with high amylase and protease activity.

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