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Screening and Characterization of Potential *Bacillus* Starter Cultures for Fermenting Low-Salt Soybean Paste (Doenjang)

Hye Hee Jeon[1], Ji Young Jung[1], Byung-Hee Chun[1], Myoung-Dong Kim[2], Seong Yeol Baek[3], Ji Young Moon[3], Soo-Hwan Yeo[3], and Che Ok Jeon[1]*

[1]*Department of Life Science, Chung-Ang University, Seoul 06974, Republic of Korea*
[2]*Department of Food Science and Biotechnology, Kangwon National University, Chuncheon 24341, Republic of Korea*
[3]*Department of Agrofood Resources, National Institute of Agricultural Sciences, RDA, Wanju-gun 55365, Republic of Korea*

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**Corresponding author*
Phone: +82-2-820-5864;
Fax: +82-2-825-5206;
E-mail: cojeon@cau.ac.kr

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The bacterial strains were screened as potential starters for fermenting low-salt doenjang (a Korean traditional fermented soybean paste) using Korean doenjang based on proteolytic and antipathogenic activities under 6.5–7.5% NaCl conditions. Phylogenetic analysis based on 16S rRNA gene sequences showed that they all belonged to the genus *Bacillus*. Proteolytic and antipathogenic activities against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Aspergillus flavus*, as well as fibrinolytic, amylase, and cellulase activities of the 10 strains were quantitatively evaluated. Of these, strains D2-2, JJ-D34, and D12-5 were selected, based on their activities. The functional, phenotypic, and safety-related characteristics of these three strains were additionally investigated and strains D2-2 and D12-5, which lacked antibiotic resistance, were finally selected. Strains D2-2 and D12-5 produced poly-γ-glutamic acid and showed various enzyme activities, including α-glucosidase and β-glucosidase. Growth properties of strains D2-2 and D12-5 included wide temperature and pH ranges, growth in up to 16% NaCl, and weak anaerobic growth, suggesting that they facilitate low-salt doenjang fermentation. Strains D2-2 and D12-5 were not hemolytic, carried no toxin genes, and did not produce biogenic amines. These results suggest that strains D2-2 and D12-5 can serve as appropriate starter cultures for fermenting low-salt doenjang with high quality and safety.

Keywords: Doenjang, *Bacillus*, fermented soybean paste, starter culture, protease, antipathogenic activity

Introduction

Doenjang is a traditional Korean fermented soybean paste that is mainly used as a dipping sauce for vegetables, fish, and meats or as an important ingredient for preparing Korean stews. In Korea, doenjang is generally made by an additional fermentation of the solid material that separates from a mixture of meju (fermented soybean lumps) and ganjang (fermented soy source), which is prepared by soaking meju in solar salt solution (approximately 16–18% (w/v) salts) for approximately 1–2 months [13, 19]. Doenjang has gained considerable attention not only as a nutritious source or flavoring ingredient providing amino acids, fatty acids, minerals, and vitamins [32] but also as a beneficial substance with health-related functional properties such as antioxidant [34], fibrinolytic [7], antimutagenic [35], and anticancer activities [20].

Doenjang is traditionally fermented under uncontrolled conditions in a porcelain pot, which increases the possibility of pathogen contaminations or putrefactions during doenjang fermentation, and this is a major reason why doenjang is traditionally fermented under high-salt conditions [23]. However, high-salt diets are not beneficial to human health because they cause diverse diseases such as hypertension and diabetes [14]. Therefore, Koreans have taken great interest in reducing the salt content of their

diets, and Korean companies have made efforts to reduce the salt contents of doenjang products as well. Currently, some Korean food companies produce low-salt doenjang containing 7–10% salts; however, low-salt doenjang products inherently pose high risks of pathogenic contaminations or putrefactions during the fermentation and sale periods. In addition, traditional doenjang production is accomplished by spontaneous fermentation without the use of starter cultures, which leads to the growth of diverse microorganisms and variations in the quality of doenjang products [15, 26, 30], making it difficult to produce commercial doenjang with uniform quality. Taking these factors into consideration, the use of starter cultures showing key functional properties and antipathogenic activities may be a primary requisite for the commercial production of low-salt doenjang with uniform and high quality.

The aim of this study was to screen starter cultures for their capacity to ferment low-salt doenjang. Because traditional Korean doenjang is fermented with soybeans as the major raw material, protease activity is a primary criterion used for screening doenjang starter cultures [29]. Antipathogenic (antimicrobial) activity may be another important criterion for screening starter cultures to protect low-salt doenjang against pathogenic growth or putrefaction during the fermentation and sale periods [10]. Several previous studies were conducted to screen and develop starter cultures for doenjang fermentation; however, most of these studies were performed in normal culture medium containing less than 2% NaCl [16, 18, 29]. However, proteolytic and antimicrobial activities can differ markedly depending on the salt concentration [11, 15], suggesting that previously screened starter cultures may not show both proteolytic and antimicrobial activities in doenjang containing 7–10% salts. Therefore, in this study, bacterial strains showing both proteolytic and antimicrobial activities at 6.5–7.5% NaCl conditions were screened, and their functional, phenotypic, and food safety-related properties were also tested to evaluate them as potential starter cultures for fermenting low-salt doenjang.

Materials and Methods

Collection of Doenjang Samples and Bacterial Strains

To screen potential starter cultures for fermentation of low-salt doenjang, 43 home-made doenjang samples were collected in sterile plastic tubes from private houses in the Gyeonggi and Gangwon provinces (Republic of Korea). They were immediately transported to our laboratory in iceboxes and stored at 4°C until screening began. In addition, 613 and 27 bacterial strains that had been isolated from doenjang samples in various regions of Korea (provided by Dr. M.D. Kim, Department of Food Science, Kangwon National University, Korea and Dr. S.Y. Baek, Rural Development Administration, Korea, respectively) were also used for screening purposes.

Primary Screening of Bacterial Strains Based on Protease Activities

Potential starter cultures for low-salt doenjang fermentation were primarily screened on the basis of proteolytic activities under a fixed salt concentration. Briefly, approximately 3 g of doenjang was resuspended in 5 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.0), serially diluted 10-fold with PBS buffer, and spread on tryptone soya agar (TSA; Becton Dickinson, USA) supplemented with 7% (w/v) NaCl (TSA-NaCl; final NaCl: 7.5%) and 1% (w/v) skim milk. To screen potential starter cultures from bacterial strains provided by the two institutions mentioned above, the 640 bacterial strains were pooled in groups of 10 strains, diluted 10-fold with PBS buffer, and spread on TSA-NaCl containing 1% skim milk. After the agar plates were incubated at 37°C for 2 days, only bacterial colonies with clear zones exceeding 1 mm were selected for secondary screening, based on antipathogenic activity.

Secondary Screening of Bacterial Strains Based on Antipathogenic Activities

Two representative gram-negative and gram-positive food-borne pathogens, *Escherichia coli* O157:H7 and *Bacillus cereus* ATCC 27348, were used as indicator strains to screen bacterial strains for antipathogenic activities. Briefly, 5 µl of the indicator strains grown overnight in TSB (Becton Dickinson, USA) were added to 5 ml of low-melt TSA (TSB with 2.0% low melting agar (Bio-Rad, USA)) containing 6.5% (w/v) NaCl to a final concentration of approximately 10^6 colony-forming units/ml, which was over-layered on solidified TSA-NaCl. After the top agar had hardened, 1 µl of primarily screened bacterial cells cultivated in TSB overnight was dropped onto the top-agar surface and incubated for 24 h at 37°C. The antipathogenic activities of the bacterial test strains were evaluated based on the inhibition zones formed around bacterial colonies on the top-agar surface, which represented growth inhibition of the indicator strains.

Identification of Bacterial Strains Based on Their 16S rRNA Gene Sequences

Colonies of bacterial strains exhibiting good proteolytic and antipathogenic activities were resuspended in 100 µl of 5% (w/v) Chelex-100 solution (Bio-Rad) and boiled for 10 min to extract crude genomic DNA. PCR amplification and sequencing of 16S rRNA genes from the crude genomic DNA were performed as described previously [27]. The resulting 16S rRNA gene sequences were compared with those of all validated type strains using the Nucleotide Similarity Search program (http://eztaxon-e.ezbiocloud.net/) [24]. The 16S rRNA gene sequences of bacterial strains exhibiting good proteolytic and antipathogenic activities and closely related

taxa were aligned using SINA (the SILVA web aligner; http://www.arb-silva.de/aligner/) [39]. A phylogenetic tree was constructed using the neighbor-joining method available in the PHYLIP software (ver. 3.695) [9].

Quantitative Evaluation of Proteolytic and Antipathogenic Activities

The proteolytic and antipathogenic activities of bacterial strains obtained from the primary and secondary screenings were quantitatively evaluated. Proteolytic activities of bacterial strains were quantitatively evaluated based on the diameter of clear zones formed around bacterial colonies grown on TSA-NaCl with 1% (w/v) skim milk, as described above. Two pathogens, *E. coli* and *B. cereus*, used for the secondary screening, and three additional pathogenic strains, *Staphylococcus aureus* (KCTC 3881), *Listeria monocytogenes* (KCTC 13064), and *Aspergillus flavus* subsp. *flavus* (KACC 41809), were used as indicator strains to quantitatively evaluate the antipathogenic activities of the bacterial strains. Growth inhibition of four indicator strains by the bacterial strains was tested using the top-agar method described above, and their quantitative antipathogenic activities were evaluated after a 16–48 h incubation at 37°C; the incubation time differed, depending on pathogenic bacteria. The antifungal activities of bacterial strains were tested based on the growth inhibition of *A. flavus* on potato dextrose agar (PDA; Becton Dickinson, USA) containing approximately 6.5% NaCl (PDA-NaCl), as described previously [3]. Briefly, bacterial colonies were spotted near *A. flavus* cells on the center of PDA-NaCl plates and growth inhibition of *A. flavus* by the bacterial test strains was evaluated after a 48-h incubation at 37°C.

Evaluation of Fibrinolytic, Amylase, and Cellulase Activities

The fibrinolytic, amylase, and cellulase activities of bacterial strains selected from the primary and secondary screenings were additionally tested. Fibrinolytic activity was tested using a fibrin-plate method [2]. Briefly, fibrin agar plates were prepared using 0.5% (w/v) low-melting agarose and 0.4% (w/v) bovine fibrinogen clotted with 10 NIH U bovine thrombin (Sigma, USA). The bacterial strains were cultivated overnight in TSB supplemented with 7% (w/v) NaCl to be a final 7.5% NaCl concentration, and cell-free supernatants were obtained from the cultures by centrifugation. Cell-free supernatants (10 μl) were loaded on paper disks on the fibrin agar plates, and their fibrinolytic activities were evaluated based on clear zones forming around the paper disks after a 24-h incubation at 37°C. For amylase activity testing, starch agar plates containing 1% soluble starch, 0.2% yeast extract, 0.1% K_2HPO_4, 0.15% $MgSO_4 \cdot 7H_2O$, 7.5% NaCl, and 1.5% agar were prepared, and 1 μl of bacterial cells cultivated overnight in TSB-NaCl was dropped onto the starch agar plates. Amylase activities were assayed using Gram's iodine solution after a 24-h incubation at 37°C. Cellulolytic activity was assayed on carboxymethyl cellulose (CMC) agar (1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.3 g NH_4NO_3, 10.0 g CMC, and 12.0 g agar per liter) containing 7.5% (w/v) NaCl, as described previously [1].

Evaluation of Hemolysis, Endotoxin Production, and Antibiotic Susceptibility

Hemolysis was tested by streaking bacterial cells on TSA containing 5% sheep blood (Thermo Scientific, USA) and their hemolysis abilities were evaluated after the agar plates were incubated for 16 h at 37°C. The production capabilities of *B. cereus* emetic toxins and enterotoxins by the bacterial test strains was assessed by PCR using primer sets targeting enterotoxin genes, as described previously [12, 18, 22]. *B. cereus* ATCC 27348 was used as a positive control. The antibiotic susceptibility of the bacterial strains was evaluated using filter paper disks (6 mm; GE Healthcare Life Sciences, USA) containing the following antibiotics (mg or units per disk): ampicillin (10), polymyxin B (100 U), streptomycin (50), penicillin G (20 U), gentamicin (30), chloramphenicol (100), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15), carbenicillin (100), neomycin (30), or novobiocin (5).

Characterization of Other Phenotypic Properties

The production of biogenic amines (BAs) including histamine, cadaverine, putrecine, and tyramine, by bacterial strains was examined in TSB-NaCl containing 0.2% of their corresponding precursor molecules (histidine, ornithine, lysine, and tyrosine) and 0.005% pyridoxal 5′-phosphate. After a 3-day incubation at 37°C, BAs in culture broths were analyzed with a HPLC system (Agilent, USA), as described previously [28]. Production of Poly-γ-glutamic acid (γ-PGA) by the bacterial strains was tested in PGA broth [42] containing 7.5% NaCl, as described previously [18]. *B. subtilis* NCIMB 3610^T was used as a positive control. Additional enzymatic activities of the bacterial strains were tested using the API ZYM kit (bioMérieux, France), according to the manufacturer's instructions, except that the inocula were prepared by resuspending cells in 7.5% NaCl. The NaCl growth range of the bacterial strains was investigated in TSB containing different NaCl concentrations (0–20% at 1% intervals) at 37°C for 3 days. Acceptable growth temperatures for the bacterial strains was tested by monitoring their growth at different temperatures (5–60°C, 5°C intervals) for 4 days. Acceptable pH values were tested in TSB having different pH values (4.0–10.0, 0.5 unit intervals) at 37°C for 4 days. Anaerobic growth was evaluated on TSA under anaerobic conditions (with 4–10% CO_2), using the GasPak Plus system (BBL, USA) after a 20-day incubation at 37°C.

Results and Discussion

The traditional fermentation approach for doenjang, a nutritious food and flavoring ingredient, under uncontrolled conditions in a porcelain pot increases the chances of contamination by pathogenic and spoilage organisms; hence, it is traditionally fermented under high-salt conditions. However, considering the ill effects of consuming high-salt foods, a healthier and effective approach to doenjang fermentation involving the use of starter cultures is

required. Here, bacterial strains were screened as potential starter cultures for fermenting low-salt doenjang, using homemade Korean doenjang samples, based on their proteolytic and antipathogenic activities under conditions of 6.5–7.5% NaCl.

Screening of Potential Starter Cultures for Doenjang Fermentation, Based on Proteolytic and Antipathogenic Activities

To identify potential starter cultures for fermenting low-salt doenjang, we first screened bacterial strains based on their proteolytic activity on TSA containing 7.5% NaCl, because traditional Korean soybean paste (doenjang) is made from soybeans alone. Two hundred and thirteen bacterial strains showing proteolytic activity were screened. Because Korean doenjang fermentation is normally performed under non-aseptic conditions in a porcelain pot, the probability of microbial contamination during doenjang fermentation is high. In particular, contamination with unwanted microorganisms such as putrefactive or pathogenic microbes has been a primary concern in low-salt doenjang (7–10% NaCl) products, compared with traditional doenjang with high-salt concentrations (16–20% NaCl), suggesting that using starter cultures with antipathogenic activities may be critical for the safe production of low-salt doenjang. Therefore, the antipathogenic activities of 213 bacterial strains showing proteolytic activity were further tested against two representative food-borne pathogens, *Escherichia coli* O157:H7 and *Bacillus cereus* ATCC 27348, on TSA containing 6.5% NaCl. Among them, 43 bacterial strains displayed clear antipathogenic activities. Finally, 10 bacterial strains showing good proteolytic (clear zone diameter >1.0 mm) and antipathogenic activities (inhibition zone diameter >2.0 mm) against *E. coli* or *B. cereus* were selected for further studies.

To date, many studies have been performed to screen starter cultures showing proteolytic and antipathogenic activities for doenjang fermentation; however, their screenings were generally conducted under normal medium conditions such as on nutrient agar or TSA lacking additional NaCl [16, 18, 25, 29]. However, it has been reported that proteolytic and antipathogenic activities differ markedly depending on the salt concentration [11, 15], suggesting that potential starter cultures identified under normal medium conditions may lack these activities under doenjang-fermentation conditions containing over 7% NaCl. Our results also showed that most bacterial strains with proteolytic and antipathogenic activities that were screened on TSA without additional NaCl did not show these activities under high-salt conditions with over 7% NaCl (data not shown). This finding suggested that starter-culture screening should be performed under salt conditions to obtain potential starter cultures with proteolytic and antipathogenic activities during doenjang fermentation. To the best of our knowledge, this is the first study to screen potential starter cultures showing proteolytic and antipathogenic activities under a salt condition of approximately 7% NaCl for doenjang fermentation.

Identification of Bacterial Strains Using 16S rRNA Gene Sequences

Phylogenetic identification of 10 bacterial strains showing good proteolytic and antipathogenic activities was performed based on their 16S rRNA gene sequences. The results showed that all 10 bacterial strains belonged to the genus *Bacillus* (Table 1), but some bacterial strains such as strains D11-21 and D2-2 were distantly clustered with previously validated *Bacillus* species, although they had high 16S

Table 1. Identification of *Bacillus* strains showing good proteolytic and antipathogenic activities, based on their 16S rRNA gene sequences.

Strain	Closest type strain	Sequence similarity (%)	GenBank Accession No.
D2-2	*Bacillus siamensis* KCTC 13613^T	99.93	KT955735
D2-3	*Bacillus subtilis* subsp. *inaquosorum* KCTC 13429^T	99.93	KT955736
JJ-D16	*Bacillus methylotrophicus* subsp. *plantarum* FZB42^T	99.86	KR262842
D11-1	*Bacillus methylotrophicus* KACC 13105^T	100.00	KT955738
JJ-D34	*Bacillus methylotrophicus* KACC 13105^T	99.92	KR262843
D12-5	*Bacillus subtilis* subsp. *subtilis* NCIMB 3610^T	99.93	KT955740
JJ-D51	*Bacillus siamensis* KCTC 13613^T	99.93	KR262844
D18-8	*Bacillus methylotrophicus* KACC 13105^T	99.93	KT955741
D11-21	*Bacillus sonorensis* NBRC 101234^T	99.50	KT958255
YJ12-3	*Bacillus methylotrophicus* subsp. *plantarum* FZB42^T	99.93	KT958254

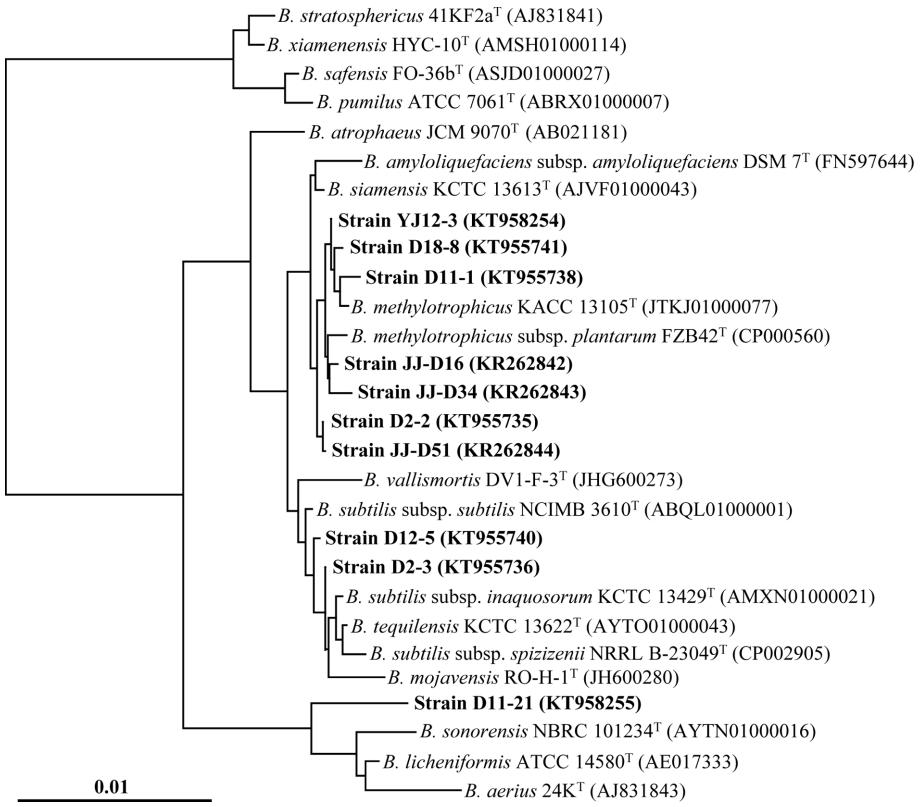

Fig. 1. Phylogenetic analysis of 10 *Bacillus* strains showing high proteolytic and antipathogenic activities, based on their 16S rRNA gene sequences.

Alicyclobacillus acidocaldarius DSM446^T was used as an outgroup (not shown). The scale bar indicates the number of changes per nucleotide position.

rRNA gene sequence similarities (Fig. 1). These findings suggested that we successfully isolated some *Bacillus* strains representing new species within the genus *Bacillus* while screening.

Quantitative Evaluation of the Proteolytic and Antipathogenic Activities

Quantitative analysis of the proteolytic activities of the 10 bacterial strains obtained through primary and secondary screenings reveled that most screened *Bacillus* strains, especially strains D2-2, JJ-D16, JJ-D34, JJ12-5, and YJ12-3, had high proteolytic activities (Table 2 and Fig. S1). The antipathogenic activities of the 10 bacterial strains against two pathogens (*E. coli* and *B. cereus*), which were used for the secondary screening, and three additional pathogens (*S. aureus*, *L. monocytogenes*, and *A. flavus*) were quantitatively evaluated (Table 2 and Fig. S1). The 10 bacterial strains had relatively strong inhibitory activities against all the pathogenic test strains, especially against *L. monocytogenes*. Although they showed different inhibitory spectra depending on pathogenic strain targeted, the 10 bacterial strains tested all belonged to the genus *Bacillus* and had high 16S rRNA gene sequence similarities. For example, strain D2-3 showed high antibacterial activity against *B. cereus*, but showed no inhibitory activity against *S. aureus*. In contrast, strain D12-5 had weak antibacterial activity against *B. cereus*, but strong inhibitory activity against *S. aureus*. These results suggest that the 10 *Bacillus* strains produce different antimicrobial compounds. It has been previously reported that pathogenic bacteria and fungi producing aflatoxin or ochratoxin are present in doenjang samples [23, 37]. Our tests showed that most *Bacillus* strains had inhibitory activities against *A. flavus*, producing aflatoxin, although the inhibitory activities were different depending on the *Bacillus* strains, suggesting that the *Bacillus* strains can inhibit the growth of pathogenic fungi during low-salt doenjang fermentation or sale periods.

Evaluation of Fibrinolytic, Amylase, and Cellulase Activities

Some *Bacillus* strains produce fibrinolytic enzymes such

Table 2. Quantitative assays of proteolytic and antipathogenic activities of 10 *Bacillus* strains selected for starter-culture screening for low-salt doenjang fermentation.

Strain	Proteolytic activity[a]	Antipathogenic activity				
		E. coli O157:H7[b]	*B. cereus* ATCC 27348[b]	*S. aureus* KCTC 3881[b]	*L. monocytogenes* KCTC 13064[c]	*A. flavus* subsp. *flavus* KACC 41809[b]
D2-2	++++	+++	++	+++	++++	+++
D2-3	+	+	++++	–	+++++	+
JJ-D16	+++	++++	+++	++	++++	++
D11-1	+++	+++	+	++	+++	+++
JJ-D34	++++	++	++++	+	+++++	+++
D12-5	+++	+++	+	+++++	+++++	+
JJ-D51	+++	+++	++	++	++++	++++
D18-8	+++	++	++++	+	++++	++++
D11-21	+	+++	++++	+	++	++
YJ12-3	++++	+++	+++	+++	+++	++

[a]Diameter (mm) of clear zones around colonies showing protease activity: +, 1.0–1.5; ++, 1.5–2.0; +++, 2.0–2.5; ++++, >2.5.

[b]Diameter (mm) of inhibition zones around colonies showing antimicrobial activities: –, negative; +, 0.01–1.0; ++, 1.0–2.0; +++, 2.0–3.0; ++++, 3.0–4.0; +++++, >4.0.

[c]Diameter (mm) of inhibition zones around colonies showing antimicrobial activities: –, negative; +, 0.01–2.0; ++, 2.0–4.0; +++, 4.0–6.0; ++++, 6.0–8.0; +++++, >8.0.

as nattokinase that can hydrolyze fibrin clots and, thus, fibrinolytic activity can be an important functional property present in fermented soybean pastes, including doenjang [8, 21, 25]. Therefore, fibrinolytic enzyme activities were tested for each of the 10 *Bacillus* strains screened. Most of the *Bacillus* strains showed strong fibrinolytic enzyme activities (Table 3 and Fig. S2). However, some strains showed low fibrinolytic enzyme activities, and strain YJ12-3 showed no such activity. Fibrinolytic enzymes are proteinases, meaning that, in general, the bacterial strains with high fibrinolytic enzyme activity could also show high proteolytic activity. Although the 10 selected *Bacillus* strains had relatively high proteolytic activities, their proteolytic and fibrinolytic activities differed markedly (Tables 2 and 3). Proteolytic activities were tested by cultivating the *Bacillus* strains on TSA containing 7.5% NaCl, whereas fibrinolytic activities were tested on fibrin agar lacking additional NaCl (low-NaCl condition) after culturing *Bacillus* strains in TSB containing 7.5% NaCl. These results may suggest that the activities of proteolytic or fibrinolytic enzymes that the selected *Bacillus* strains produce differ, depending on the NaCl concentration. Our test also showed that strain YJ12-3 had low protease activity on TSA, whereas it had high protease activity on TSA containing 7.5% NaCl. Most of the *Bacillus* strains also showed strong amylase and cellulase activities (Table 3 and Fig. S2). Based on the proteolytic, antipathogenic, and fibrinolytic activities observed, we selected three *Bacillus* strains (D2-2, JJ-D34, and D12-5) for further study as potential doenjang starter cultures. Their phenotypic characteristics related to soybean fermentation, health-related functionality, and safety were additionally investigated.

Evaluation of Hemolysis, Endotoxin Production, and Antibiotic Susceptibility

Hemolysis by the D2-2, JJ-D34, and D12-5 *Bacillus* strains was tested. None of these strains induced hemolysis on

Table 3. Quantitative assays of fibrinolytic, amylase, and cellulase activities of 10 selected *Bacillus* strains used for starter-culture screening for low-salt doenjang fermentation.

Strains	Fibrinolytic activity[a]	Amylase activity[b]	Cellulase activity[b]
D2-2	++++	+++++	+++++
D2-3	++++	+++	+++
JJ-D16	++	++++	+++++
D11-1	++	++++	+
JJ-D34	+++	+++	+
D12-5	+++	+++	+++++
JJ-D51	++++	+++++	++++
D18-8	+++	++++	+
D11-21	++++	+	+++++
YJ12-3	–	++++	+++++

[a]Diameter (mm) of halo zones around disk: –, negative; +, 0.01–1.5; ++, 1.5–3.0; +++, 3.0–4.5; ++++, >4.5.

[b]Diameter (mm) of halo zones around colony: +, 0.01–1.0; ++, 1.0–2.0; +++, 2.0–3.0; ++++, 3.0–4.0; +++++, >4.0.

sheep blood agar, indicating that they all are γ-hemolytic bacilli. In contrast, *B. cereus* ATCC 14579 (used as a positive control) produced a clear zone around colonies on sheep blood agar, indicative of β-hemolysis. The presence of *Bacillus* emetic toxin and enterotoxin (diarrheal toxin) genes in strains D2-2, JJ-D34, and D12-5 was evaluated using a polymerase chain reaction amplification with one primer set for the emetic toxin gene (*Ces*), encoding cereulide synthetase, and 10 primer sets for enterotoxin genes: four *hbl* genes (*hblA*, *hblB*, *hblC*, and *hblD*), collectively encoding hemolysin BL that causes *B. cereus*-associated diarrhea; three *nhe* genes (*nheA*, *nheB*, and *nheC*), encoding a non-hemolytic enterotoxin; and the *bceT*, *cytK*, and *entFM* genes, encoding enterotoxin T, cytotoxin K, and enterotoxin FM, respectively [18]. PCR analysis showed none of the three *Bacillus* strains carried the *B. cereus* emetic toxin or enterotoxin genes, whereas PCR amplicons for all PCR primer sets were produced with the positive control *B. cereus* ATCC 14579 strain, except for *Ces*. All three *Bacillus* strains were sensitive to neomycin, penicillin G, polymyxin B, streptomycin, chloramphenicol, ampicillin, gentamicin, tetracycline, kanamycin, lincomycin, oleandomycin, and carbenicillin. However, strain JJ-D34 showed novobiocin resistance, whereas strains D2-2 and D12-5 were sensitive to novobiocin. These results suggested that strains D2-2 and D12-5 are more appropriate as potential starter cultures. Thus, strains D2-2 and D12-5 were finally selected as potential starter cultures for low-salt doenjang fermentation.

Characterization of Other Phenotypic Properties

Biogenic amines can be used as food-quality indicators and are frequently found in doenjang, at various concentrations that depend on the fermentation conditions used [5, 41]. BAs are mainly produced by microbial decarboxylase activities during fermentation [4, 30, 40]. We did not observe BA production by strains D2-2 and D12-5. Poly-γ-glutamic acid is a naturally occurring biodegradable, non-immunogenic, and unusual anionic biopolymer consisting of repeating units of glutamic acid and it has been shown that γ-PGA has various functional properties [33]. As several *Bacillus* species are known to produce viscous γ-PGA on their exteriors, the production of γ-PGA by strains D2-2 and D12-5 was tested, which showed both strains also produced γ-PGA on their exterior (data not shown). Additional enzyme activity testing was performed using an API-ZYM kit, which showed that strains D2-2 and D12-5 were positive for alkaline phosphatase, esterase (C8), esterase (C4), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, α-chymotrypsin, acid phosphatase, *N*-acetyl-β-glucosaminidase, α-glucosidase, and β-glucosidase (Table 4). Although soybeans contain substantial amounts of isoflavones (closely related phytoestrogens), the bioavailability of isoflavones is generally low because their glucoside forms in soybeans are not easily absorbed through the stomach [38]. Therefore, the β-glucosidase activity of strains D2-2 and D12-5 during fermentation may contribute to the functional enhancement of doenjang products by increasing the bioavailability of isoflavones. The growth properties of strains D2-2 and D12-5 were tested, which demonstrated that they can grow under doenjang fermentation conditions (Table 4). Strains D2-2 and D12-5 grew in NaCl concentrations up to 16%, and only marginal anaerobic growth was observed. These findings suggested that *Bacillus* strains D2-2 and D12-5 showing proteolytic and antipathogenic activities as well various functional properties can facilitate the fermentation of low-salt doenjang containing 7–10% NaCl.

Although diverse microorganisms, including *Bacillus*, *Leuconostoc*, *Tetragenococcus*, *Enterococcus*, *Staphylococcus*, *Rhizopus*, *Mucor*, and *Aspergillus*, have been identified in doenjang samples during fermentation, it is generally accepted that *Bacillus* species such as *B. subtilis*, *B. amyloliquefaciens*, *B. siamensis*, *B. methylotrophicus*, and *B. licheniformis* play the most important fermentative roles [15, 17, 25, 26, 31]. In addition, *Bacillus* species have been also considered important probiotic microorganisms, conferring health benefits on doenjang products [7, 6, 16, 36]. Taken together, *Bacillus* strains D2-2 and D12-5 were screened as potential starter cultures for low-salt doenjang fermentation on the basis of their proteolytic and antipathogenic activities, functionality,

Table 4. Phenotypic characteristics of strains D2-2 and D12-5.

Characteristic	D2-2	D12-5
Growth at		
NaCl (optimum, %)	0–16 (0–2)	0–16 (0–2)
Temperature (optimum, °C)	15–55 (40–45)	15–55 (40–45)
pH (optimum)	4.5–9.5 (6.0–7.0)	4.5–9.5 (6.0–7.0)
Anaerobic condition	(+)[a]	(+)[a]
Enzyme activity of		
Cystine arylamidase	–	+
β-Glucuronidase	–	+

Both strains were positive for the following enzymes: alkaline phosphatase, esterase (C8), esterase (C4), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, α-chymotrypsin, acid phosphatase, *N*-acetyl-β-glucosaminidase, α-glucosidase, and β-glucosidase. Both strains were negative for the following enzymes: lipase (C14), valine arylamidase, α-galactosidase, β-galactosidase, α-mannosidase, and α-fucosidase. Symbols: +, positive; –, negative.

[a]Weak growth after 20 days.

and safety, and our results suggested that strains D2-2 and D12-5 can be applied to produce commercial low-salt doenjang (containing 7–10% NaCl) with high quality and safety.

It has been generally accepted that most *Bacillus* species are strictly aerobic; however, doenjang is fermented under anaerobic conditions, meaning that *Bacillus* strains D2-2 and D12-5 that have very weak growth under anaerobic condition even after a long incubation time may not display proteolytic and antipathogenic activities and functionality during doenjang fermentation. In addition, most bacteria have different physiological and phenotypic properties depending on their environmental conditions, suggesting that strains D2-2 and D12-5 may have low proteolytic and antipathogenic activities and functionality in doenjang although they show great activities on TSA with 6.5–7.5% NaCl. Therefore, further investigations on the proteolytic and antipathogenic activities and functionalities of strains D2-2 and D12-5 in doenjang are required for their better evaluation as starter cultures for doenjang fermentation. In addition, more studies on microbial communities, metabolites, and sensory characteristics (doenjang taste, flavor, and color) through real applications of strains D2-2 and D12-5 to doenjang fermentation are necessary, which will eventually provide good rationales for the selection of good starter cultures to produce standardized doenjang with functional properties, safety, and tastes.

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