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Molecular identification and antimicrobial activities of *Bacillus subtilis* MS-01 isolated from Indonesian ethnic food *lemea*

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Abstract. *Sariyanti M, Adri PM, Wibowo RH, Nasution AA, Hidayah T, Sipriyadi. 2025. Molecular identification and antimicrobial activities of* Bacillus subtilis *MS-01 isolated from Indonesian ethnic food* lemea. *Biodiversitas 26: 1069-1074.* The community in Bengkulu Province has a variety of traditional foods produced through fermentation, one of which is a bamboo shoot-based dish known locally as *lemea*. *Bacillus subtilis*, found in fermented foods, shows great potential as a probiotic due to its ability to inhibit harmful bacteria. Given its genetic diversity, molecular identification is crucial to differentiate between strains. This study represents the first isolation and identification of *Bacillus* strains from *lemea*, an ethnically fermented bamboo shoot from the Rejang tribe community in Bengkulu, Indonesia. This study aimed to evaluate the antibacterial activity and perform molecular identification analysis using the 16S ribosomal RNA gene region. The *Bacillus* isolate was identified using observations of macroscopic and microscopic morphological Lactic Acid Bacteria (LAB). Inhibition tests of *Bacillus* isolate were conducted using pathogenic bacteria, namely *Escherichia coli*, *Salmonella typhimurium*, and *Enterococcus faecalis*. The isolate demonstrated an exceptionally strong inhibitory effect, particularly against *E. faecalis*. Molecular identification involved polymerase chain reaction amplifying the 16S rRNA gene, producing an amplicon of approximately 1,300 base pairs. Sequence analysis via BLAST showed 99.84% similarity to *B. subtilis* strain X2, SSR17, NIBSM OsG1, BaBc-1, and strain LMV in GenBank. Phylogenetic analysis confirmed the isolate's classification within the order Bacillales and family Bacillaceae. In conclusion, *B. subtilis* MS-01 was successfully isolated and identified, exhibiting antibacterial potential against both Gram-negative and Gram-positive pathogenic bacteria, with a particularly noteworthy effect against *E. faecalis*, thereby suggesting its potential as a probiotic with potent antimicrobial properties.

Keywords: 16S rRNA gene, antibacterial, *Bacillus subtilis*, *lemea*, molecular

Abbreviations: BLAST: Basic Local Alignment Search Tool; rRNA: ribosomal RNA; MRS: de Man, Rogosa, and Sharpe; MHA: Mueller Hinton Agar; MEGA: Molecular Evolutionary Genetics Analysis; NCBI: National Center for Biotechnology Information; CaCO3: Calcium carbonate

INTRODUCTION

Bengkulu is known for traditional food produced through the fermentation of bamboo shoots, called *lemea*. This food is a specialty of the Rejang Tribe, one of the oldest tribes in Bengkulu (Kurnia et al. 2020). Fermented foods foster the growth of mycorrhizae, which contain antibacterial compounds, including bacteriocins, that function as probiotics. The diverse range of fermented foods contributes substantially to food security, which can be derived from bacteria or fungi originating from microorganisms present in the gastrointestinal tract. Several bacteria commonly used as probiotic candidates include *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Leuconostoc*, and *Enterococcus* (Tingirikari et al. 2024).

Most probiotic bacteria are members of gut bacteria, and some have been developed into food products to promote gut health by maintaining the balance of gastrointestinal bacteria. Probiotics enhance innate immunity and modulate inflammation, reduce pathogens that disrupt the digestive system, also cancer and allergy prevention (Galdeano et al. 2019). Probiotics work by competing with pathogens for the nutrients necessary for their growth and replication. Research has demonstrated that probiotics, including *Lactobacillus rhamnosus* strain GG and *L. plantarum*, can prevent the adhesion of enteropathogenic *Escherichia coli* within the gastrointestinal tract (Pourabedin and Zhao 2015). Competitive exclusion of pathogens includes reducing luminal pH, competing for nutrient sources, and producing bacteriocins or bacteriocin-like substances. Probiotic mechanisms also involve enzymatic activity and the synthesis of volatile fatty acids, including Short-Chain Fatty Acids (SCFAs) and Branched-Chain Fatty Acids (BCFAs) play roles in maintaining energy homeostasis and regulating peripheral tissue functions (Plaza-Diaz et al. 2019).

One of the bacteria commonly found in fermented foods is *Bacillus* sp., which plays a key role in the breakdown of organic components in food ingredients (Liu et al. 2024). *Bacillus* is a group of Gram-positive, bacillus-shaped, motile, facultatively aerobic bacteria, some of which are anaerobic. This bacterial genus can be found in diverse ecological niches, such as soil, seawater, feces, and dairy food (Gray et al. 2019; Golnari et al. 2024). These can produce high amounts of lactic acid (Harirchi et al. 2022). According to Kumar et al. (2022), fermented foods made from bamboo shoots predominantly contain *Bacillus* spp. bacteria. *Bacillus* spp. is considered one of the probiotic candidates due to its various properties, such as ease of cultivation, non-toxicity, heat resistance, and the ability to produce various enzymes. *Bacillus* species also produce short-chain organic acids with antibacterial properties. *Bacillus* can produce antibacterial compounds that inhibit pathogens' growth, such as *Escherichia coli* and *Salmonella* sp. Amylase and protease enzymes, polypeptides, extracellular polysaccharides, and lipopeptides with antibacterial activity are some of the key components found in fermented foods containing *Bacillus* spp. bacteria (Li et al. 2023). These bacteria can also inhibit the growth and development of pathogenic bacteria by producing extracellular products such as subtilin, protease-resistant isocoumarin, naphthol-AS-BI-phospholidase, surfactin, coagulin, amino coumarin, and iturins. *Bacillus subtilis*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus. cereus*, *Bacillus pumilus*, and *Bacillus licheniformis* are types of *Bacillus* spp. bacteria that can be utilized as probiotics (Hong et al. 2005).

Given the diversity of *Bacillus* genera, it is essential to accurately identify specific *Bacillus* species in *lemea*. This study employs a meticulous methodology, amplifying the gene encoding ribosomal RNA (rRNA) in lactic acid bacteria was amplified using specific primers derived from the 16S rRNA gene sequence. The 16S rRNA gene is widely utilized for genotype identification because of its exceptional precision in bacterial taxonomy (Bartoš et al. 2024), which is a key component of our research. This gene, with its high degree of conservation among species belonging to the same genus, makes it a reliable tool for bacterial characterization (Suardana 2014). Previous studies on fermented bamboo shoot foods have identified various *Bacillus* species, including *B. subtilis*, *Bacillus tequilensis*, *Bacillus safensis*, and *Bacillus nakamurai*, based on phenotypic traits and 16S rRNA sequencing (Kumar et al. 2022). However, this study is the first to examine *Bacillus* species in *lemea* using the 16S rRNA gene. This pioneering research aimed to explore the antibacterial properties and molecular identification of the 16S rRNA gene from *Bacillus* sp. isolate BCL01 in *lemea*.

MATERIALS AND METHODS

Sample collection

A total of 10 g of *lemea* collected at 72 h of fermentation from the Lebong Regency in Bengkulu, Sumatra, Indonesia, were aseptically transported to the Microbiology Laboratory of the Biology Department, Universitas Bengkulu, for processing and microbial isolation. The sample was aseptically transferred into a separate flask containing 90 mL of sterile 0.1% peptone water and thoroughly homogenized. Subsequently, 0.1 mL aliquots from suitable dilutions were spread onto de Man, Rogosa, and Sharpe (MRS) agar plates and incubated at 30°C for 48 hours. The use of MRS agar medium is due to its classification as a selective medium specifically designed for growing bacteria that produce lactic acid. This medium contains peptone, beef extract, yeast extract, dextrose, Tween 80, ammonium citrate, magnesium sulfate, manganese sulfate, dipotassium phosphate, and sodium acetate.

Procedures

Morphological and biochemical analysis

The bacterial isolate was purified by repeated streak plating on MRS agar three times and further examined for cellular morphology, colonial, and staining characteristics. Biochemical tests included catalase tests, carbohydrate fermentation tests (glucose, sucrose, and lactose), urease activity, and citrate utilization (Khushboo et al. 2023). Motility was tested using a semisolid nutrient agar medium.

Antibacterial activity test

The inhibition test of the *Bacillus* sp. bacterial isolate was conducted using pathogenic bacteria, such as *E. coli*, *Salmonella typhi*, and *Enterococcus faecalis*. The *Bacillus* sp. bacterial isolate was cultured in Mueller Hinton broth medium, then incubated at 37°C for 24 h. The supernatant and pellets were separated and tested against pathogenic bacteria using a paper disc method. The pellet was dissolved using sterile aquadest before being inoculated. A volume of 20 µL supernatant, or pellet, was transferred to paper discs, placed on Mueller Hinton agar medium inoculated with *E. coli*, *S. typhi*, and *E. faecalis*, and then incubated at 37°C for 24 hours. Before the test, the turbidity of the bacteria culture was measured (OD = 0.3). The presence of clear zones around the *Bacillus* colonies was used to confirm positive results. These clear zones were measured using calipers, and the inhibition zone index was calculated. The diameter of the inhibition zones was classified following the criteria outlined by Davis and Stout (1971) and Ouchari et al. 2019.

Genomic DNA extraction

DNA was extracted using the Presto Mini gDNA Bacteria Kit (Geneaid). A total of 1.5 mL of the bacterial culture was centrifuged at 14,000-16,000 RCF in a microcentrifuge tube for 1 minute. Gram+ Buffer was added to the centrifuge tube. Lysozyme (0.8 mg/200 µL) was added to the Gram+ Buffer and vortexed to dissolve the lysozyme. Next, 200 µL of Gram+ Buffer containing lysozyme was transferred to the sample tube (1.5 mL microcentrifuge tube) and suspended again by vortexing or pipetting. The suspension was incubated at 37°C for 30 min, with vortexing every 10 minutes. Afterward, 20 µL of Proteinase K (pre-mixed with double-distilled H_2O) was added and mixed by vortexing. Incubation continued at 60°C for at least 10 min until the lysate became clear/homogeneous. During incubation, the tube suspension was vortexed every 3 min.

Following incubation, a total of 200 µL of GB Buffer was added to the microcentrifuge tube containing the sample and homogenized by vortexing for 10 sec, then incubated at 70°C for 10 min. During this process, a lysate, the residual fluid from the lysis process, is formed. The elution Buffer (200 µL per sample) was also incubated at 70°C until DNA elution. A total of 200 µL of absolute ethanol was added to the sample until the lysate became clear/homogeneous. The GD column was placed in a 2 mL collection tube, and the mixture was transferred to the GD column and centrifuged at 14,000-16,000 RCF for 2 min. The supernatant was removed, and the GD column was transferred to a fresh 2 mL collection tube.

A total of 400 µL of W1 Buffer was added to the GD column, centrifuged at 14,000-16,000 RCF for 30 sec and the supernatant was discarded. Next, 600 µL of Wash Buffer (pre-mixed with ethanol) was added to the GD column, followed by centrifugation at 14,000-16,000 RCF for 30 sec. The supernatant was discarded, and the column was dried by centrifuging again at 14,000-16,000 RCF for 3 min. The dried GD column was moved to a 1.5 mL microcentrifuge tube. Subsequently, 100 µL of Elution Buffer was applied to the column matrix, and the tube was centrifuged at 14,000-16,000 RCF for 30 seconds to dissolve the DNA pellet. The isolated DNA was then stored at -20°C.

Amplification 16S rRNA region

Amplification of the 16S rRNA gene was conducted using two specific primers: forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al. 1998). The PCR reaction mixture consisted of GoTaq Green Master Mix 2X 5 mL, 63f primer 0.5 µL, 1387r primer 0.5 µL, DNA template 1 µL, and nuclease-free water 3 µL, resulting in a total reaction volume of 10 µL. The PCR protocol included an initial pre-denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with a final extension step at 72°C for 7 minutes. After PCR, the amplicons were separated by electrophoresis on a 1% agarose gel at 50 volts for 45 minutes. The results were visualized using an Axygen Gel Documentation System after staining the gel with Ethidium Bromide.

Data analysis

The Sanger method was used to amplify the sequence of the products at PT. Genetika Science Indonesia Laboratory, followed by data analysis. Qualitative descriptive methods were used to observe molecular similarities and to construct a phylogenetic tree using Molecular Evolutionary Genetics Analysis version 11 (MEGA). The sequences of the bacterial isolates were compared with data registered in the National Center for Biotechnology Information (NCBI) database using BLAST. The BLAST results provided the closest relatives and strain types of each species retrieved from the NCBI GenBank database. Following the sequencing process, a phylogenetic tree using the neighbor-joining method was generated with 1000 bootstrap replicates (Tamura et al. 2021).

RESULTS AND DISCUSSION

Morphological and biochemical characteristics

The isolation of Lactic Acid Bacteria (LAB) from *lemea* resulted in 127 colonies, and selection was carried out based on morphological observations, which identified 3 bacterial isolates. The isolates were cultured on nutrient and MRS agar, and the colony characteristics were examined. The MS-01 isolate was the first colony with characteristics as shown in Table 1, then identified as *Bacillus* isolate. On nutrient agar, the colonies appeared cream-colored, circular with smooth edges, and flat in elevation. Calcium carbonate ($CaCO_3$) was added to MRS agar to reveal a clear zone around the colonies, indicating the production of lactic acid by the bacteria (Figure 1.A), and Gram staining indicated the presence of rod-shaped Gram-positive bacteria (Figure 1.B). The clear zone resulted from a reaction between $CaCO_3$ in the media and lactic acid produced by the bacteria, which led to the formation of calcium lactate, which dissolved in the media (Lingga et al. 2023). Lactic acid production has traditionally been associated with non-spore-forming bacteria, known as LAB. However, aerobic spore-forming bacteria, which belong to the *Bacillus* genus, also produce lactic acid. These findings significantly expand our understanding of lactic acid production in bacteria.

Table 1. Morphological and biochemical characteristics

Test parameters	Result
Morphology	
Shape	Circular
Pigmentation	Cream
Edge	Entire
Elevation	Flat
Surface	Smooth
Gram stain	Positive, rod-shaped
Biochemical	
Catalase	+
Urease	-
Citrate	-
Fermentation of carbohydrates	
Glucose metabolism	+
Lactose metabolism	+
Sucrose metabolism	+
Motility	+

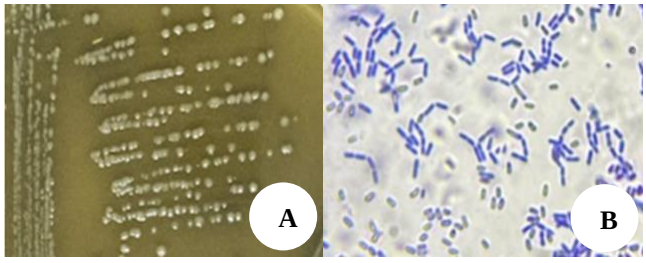

Figure 1. Morphology of MS-01 isolate. A. Culture on MRS agar; B. Gram staining

Table 2. The antibacterial activity of *Bacillus* sp. MS-01 isolate

Isolate	Diameter of inhibition zone (mm)±SD		
	Escherichia coli	***Salmonella typhimurium***	***Enterococcus faecalis***
MS-01	6.68 ± 0.98	4.05 ± 0.25	12.98 ± 0.03

The biochemical test results were positive for catalase and negative for urea and citrate. The catalase test characterized the ability of the isolate to convert hydrogen peroxide into water and oxygen, thus indicating its oxygen metabolism (Prayitno et al. 2020). Fitri et al. (2023) stated that the urease and citrate tests are used to determine whether bacteria utilize urea and citrate as energy sources. These strains are Gram-positive, catalase-positive, motile, and capable of nitrate reduction. They exhibit a morphology of long bacillus chains, clusters, or pairs, with oval or spherical spores located centrally or at their ends. Additionally, they were able to hydrolyze glucose, xylose, arabinose, mannose, and starch. A positive result was indicated by a color change in the medium from green to yellow (Prihatiningsih et al. 2020).

Inhibitory potential of *Bacillus* sp.

The results of antibacterial activity tests using *Bacillus* sp. isolates are shown in Table 2 and Figure 2. The results showed that MS-01 exhibits strong inhibition. Antibacterial activity is thought to be produced by the secondary metabolites of the associated bacteria in the form of bioactive compounds that are similar to those in their hosts. The pathogenic bacteria used in the tests included *E. coli*, *S. typhimurium*, and *E. faecalis*, which were obtained from the Microbiology Laboratory collection of the Biology Department at University of Bengkulu. These pathogenic bacteria are capable of colonizing the human intestine and leading to gastrointestinal infections. *Escherichia coli*, a Gram-negative bacterium to become pathogenic to humans when present in excessive amounts. It is known to cause diarrhea and infect the digestive system, particularly the intestines (Pokharel et al. 2023). *S. typhimurium*, another Gram-negative bacterium, is a common cause of food poisoning, can weaken the immune system, and may lead to typhoid fever in humans. Meanwhile, *E. faecalis* is a Gram-positive pathogen that, upon colonization in the human body, has the potential to cause chronic infections (Fiore et al. 2019).

Bacillus spp. may inhibit the growth of pathogenic bacteria through competition, growth promotion, and antibiosis. *Bacillus* spp. produce compounds that are toxic to competing bacteria, and evidenced by a clear inhibition zone on a suitable medium agar. This indicates that *Bacillus* spp. can inhibit the growth of other bacteria. The size of the clear zone produced by bacteria depends on the ability of the tested bacteria. The larger the clear zone, the greater the bacteria's capability to inhibit the growth of pathogenic bacteria. Differences in inhibition diameter among bacteria are caused by the ability of various bacterial isolates to produce inhibitory compounds (Sanam et al. 2022). Antibacterial testing was also conducted on the supernatant and pellet, which were obtained through centrifugation. According to Cirat et al. (2024), the inhibition zone is formed due to the production of bioactive compounds by LAB, which can damage the cell wall structure of pathogenic bacteria.

16S rRNA amplification and sequence analysis

The amplification of *Bacillus* isolate using the 16S rRNA gene resulted in DNA bands of approximately 1,300 base pairs in length (Figure 3). The 16S rRNA gene was employed to assess the taxonomy, phylogeny, and genetic distance between bacterial species. This gene is highly conserved among prokaryotes but contains hypervariable regions that are useful for bacterial identification. According to Clarridge 2004, the 16S rRNA gene can be used to determine species similarity. A similarity rate of 97% indicates that the bacteria are similar at the genus level, whereas a 99% similarity rate suggests similarity at the species level (Johnson et al. 2019).

The presence of *Bacillus* spp. in fermented bamboo shoots has been previously reported in a study by Kumar et al. (2022). This study conducted molecular sequencing analysis using the 16S rRNA gene and identified the isolates as belonging to the genus *Bacillus* (*Bacillus safensis*, *B. tequilensis*, *B. siamensis*, *B. nakamurai*, and *B. subtilis*) and *Enterobacter*. However, no *Lactobacillus* species were found in the analysis. Additionally, in fermented foods, other bacteria such as LAB have also been reported. A study by Jeyaram et al. (2010) revealed that molecular analysis using the 16S rRNA gene identified the majority of isolates as belonging to the genus *Bacillus*, followed by LAB.

DNA sequences were analyzed by the BioEdit program and aligned using the MEGA 11 program. The neighbor-joining method with 1,000 bootstrap iterations was used to construct a phylogenetic tree, as shown in Figure 4. This analysis was used to assess the genetic relationships between species related to *Bacillus subtilis* MS-01.

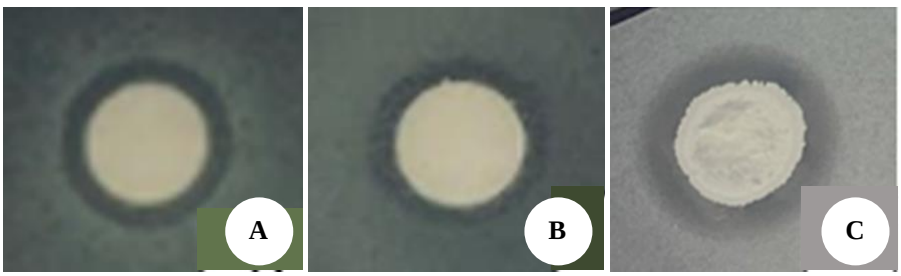

Figure 2. The antibacterial activity of *Bacillus* sp. MS-01 isolate. Against: A. *E. coli*; B. *S. typhimurium*; C. *E. faecalis*

Table 3. BLASTN results for the *Bacillus subtilis* MS-01 isolate

Isolate code	Access number	Homolog	Score-max	Total score	Query cover	E value	% Identity
MS-01	MT373564.1	*Bacillus subtilis* strain X2	2344	2344	100%	0.0	99,84%
	MH889071.1	*Bacillus subtilis* strain SSR17	2344	2344	100%	0.0	99,84%
	KY927393.1	*Bacillus subtilis* strain NIBSM OsG1	2344	2344	100%	0.0	99,84%
	MK254686.1	*Bacillus subtilis* strain BaBc-1	2344	2344	100%	0.0	99,84%
	MG760438.1	*Bacillus subtilis* strain LMV	2344	2344	100%	0.0	99,84%

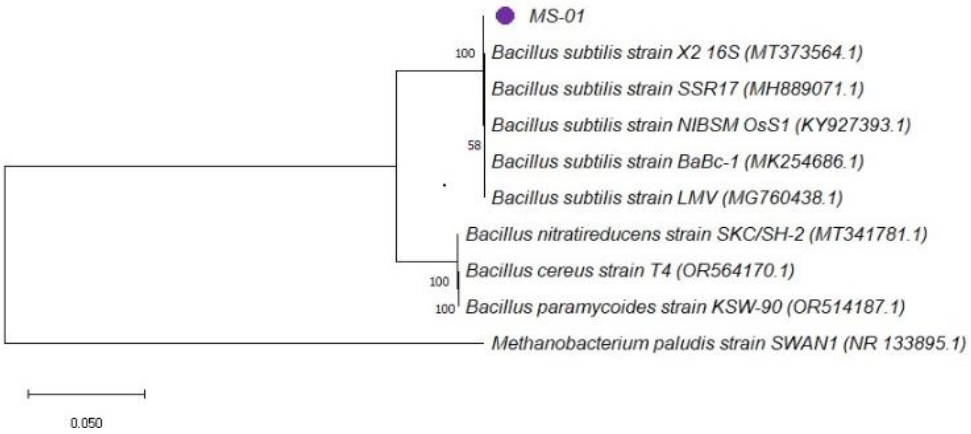

Figure 4. Phylogenetic analysis of *Bacillus subtilis* MS-01

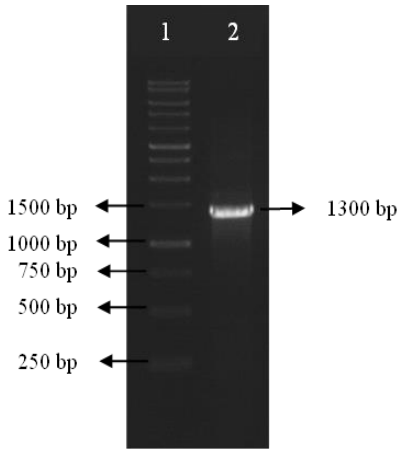

Figure 3. 16S rRNA gene of *Bacillus* sp.: 1: marker 1 kb; 2: 16S rRNA amplicon

Table 3 presents the BLASTN results for the *B. subtilis* MS-01 isolate, which shared the highest similarity (99.84%) with *B. subtilis* strain X2, *B. subtilis* strain SSR17, *B. subtilis* strain NIBSM OsG3, *B. subtilis* strain BaBc-1, and *B. subtilis* strain LMV. *Bacillus* strain X2 was identified as *Bacillus subtilis* subsp. *stercoris*, which produces extracellular endoxylanase and was isolated from the soil in Northeast India. This aerobic culture is Gram-positive and endospore-forming (Dhruw et al. 2020).

In a study by Kumar et al. (2020), *B. subtilis* strain NIBSM OsG3 isolated from the grain tissues of rice (*Oryza sativa* L.) showed antagonistic activities against *E. coli* and was able to ferment inulin. *B.subtilis* plays an important role in several fermented foods, including Natto (Hosoi and Kiuchi 2008) and Daqu (Li et al. 2014). The role of *Bacillus* spp. as a source of industrial enzymes has been well-reviewed elsewhere (Danilova and Sharipova 2020). The amylolytic system of Bacillus species includes glucan-branching enzymes, extracellular amylases, including α-amylases, β-amylases, pullulanase, and glucoamylases, and intracellular oligosaccharide hydrolases. Most type strains of the *B. cereus* and *B. subtilis* groups produce multiple amylases. Thermostable amylases of *Bacillus* are also a key element of daqu fermentations. Gamma-polyglutamic acid (γ-PGA) is produced by *Bacillus* spp. to form part of the mucilage of natto and cheongguk-jang fermented soybeans found in Japan and Korea, respectively. The stringy consistency of natto is mainly due to γ-PGA produced by *B. subtilis*. The production of γ-PGA from natto starters was reduced in a medium containing NaCl greater than 3%. A salt-tolerant *B. subtilis* strain was found in a cheonggukjang fermentation, which also produces γ-PGA (Li et al. 2023).

In conclusion, *Bacillus subtilis* MS-01 has been successfully isolated and identified molecularly and exhibits antibacterial potential against both Gram-negative and Gram-positive pathogenic bacteria, showing a strong inhibition zone against *E. faecalis*. Molecular characterization through the 16S rRNA gene suggested 99.84% similarity with other *B. subtilis* strains.

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