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Cultivable Microbial Diversity in Domestic Bentonites and Their Hydrolytic Enzyme Production

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We have isolated and identified 72 bacterial strains from four bentonite samples collected at the mining areas located in Gyeongsangbuk-do, Republic of Korea, and measured their hydrolytic enzyme (α-amylase, protease, and cellulase) activities to identify the isolates with industrial-use potential. Most of the isolates belonged to the Bacillaceae, with minor portions being from the Paenibacillaceae, Micrococcaceae, and Bacillales Family XII at the family level. Of the strains isolated, 33 had extracellular α-amylase activity, 30 strains produced cellulase, and 35 strains produced protease. Strain MBLB1268, having the highest α-amylase activity, was identified as *Bacillus siamensis* (0.38 ± 0.06 U/ml). *Bacillus tequilensis* MBLB1223, isolated from Byi33-b, showed the highest cellulase activity (0.26 ± 0.04 U/ml), whereas *Bacillus wiedmannii* MBLB1197, isolated from Zdb130-b, exhibited the highest protease activity (54.99 ± 0.78 U/ml). These findings show that diverse bacteria of the Bacillaceae family adhere to and exist in bentonite and are potential sources of industrially useful hydrolytic enzymes.

Keywords: **Microbial diversity, bentonite, hydrolytic enzyme**

Clay minerals are classified as kaolinite and montmorillonite minerals, as a class of layered silicates formed from chemical weathering of other silicate minerals on the surface of the earth [1]. Clay minerals with varying amounts of iron, magnesium, alkali metals, alkaline earths, and other cations are present in the interlayer space or in the lattice structure. They have well-known adsorbent properties due to their layered form, with a high internal surface area, high cation-exchange capacity, and good swelling properties in the presence of water [2]. In the Korea Food and Drug Administration, six kinds of clay minerals (diatomaceous earth, kaolin, bentonite, acid clay, talc, and perlite) are listed in the Food Additive Code, and they are used in filtration aids in the beverage, brewing, and fermentation industries [3].

Bentonite is a kind of montmorillonite, and it contains various elements such as potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), and aluminum (Al^+) [4]. Bentonites have been investigated for the adsorption of heavy metals and molecular species in a variety of environments and industries and have been identified as low-cost adsorbents [5]. In addition to studies on the physicochemical

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properties of clay minerals, some studies on the influence of some food-derived microorganisms have been carried out. Bentonite exhibited a growth inhibition effect only on *Escherichia coli* and *Staphylococcus aureus* strains among foodborne pathogens, which include *S. aureus*, *E. coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Pseudomonas fluorescens* [6]. As a result of the mineral-bacterial interaction system between *Bacillus litoralis* SWU9 and bentonite, the ions of bentonite such as Ca^{2+} and Mg^{2+} act as a buffer to prevent the decrease of pH and enhance microbial activity and consumption of glucose [7]. Recently, a previous study examined the effect of adding six kinds of bentonite (including bentonite modified with different cations including Na^{+}, K^{+}, and Mg^{2+}) on *kimchi* fermentation [8]. The cation exchange capacity and dissolution of exchanged elements of clay minerals affect pH and salt concentration during *kimchi* fermentation, thus affecting *kimchi* microflora. Notably, *Leuconostoc* sp. was significantly increased in the clay mineral treatment group compared to that in the non-treatment group. However, though there are studies on the effect of microorganisms on bentonite as described, there has been no research on microorganisms present in bentonite and their biological activities.

In this study, we isolated microorganisms in domestic bentonites to investigate cultivable microbial diversity. Furthermore, their hydrolytic enzyme activities including α-amylase, cellulase, and protease, were evaluated to provide basically scientific information and explore industrial applicability of bentonite-derived microorganisms.

Four bentonite samples were collected at the Gampo-40, Youngil-33, Daebo-130, and Guryong-130 mining areas (denoted Bgp40-b, Byi33-b, Zdb130-b, and Bdb130-1, respectively) located in Gyeongsangbuk-do, Republic of Korea (Table 1). Each of bentonite samples (1 g) was crushed and suspended in distilled water (10 ml) to determine pH and NaCl concentration (%, w/v) by pH meter (Fisher Science Education, USA) and HI 96821 Refractometer (HANNA instruments, USA), respectively. All of bentonite samples showed 0–0.02% (w/v) NaCl concentration. In addition, most of bentonite samples exhibited the slightly high pH range (9.0–10.1) except the bentonite Bdb130-b sample of which pH was determined to be 3.27 (Table 1). The chemical composition of bentonite samples was analyzed using energy dispersive X-ray fluorescence spectrometer (Rigaku NEX SG) [9], resulting that major chemical components of all bentonite samples were SiO_2 with 52.4–62.6 wt.%, followed by Al_2O_3 with 13.4–16.4 wt.% (data not shown). The crushed bentonite samples (5 g) were also suspended in tryptic soy broth (TSB) and De Man, Rogosa, and Sharpe (MRS) medium. The suspensions were spread on TSB and MRS agar plates and incubated at 37℃ and 30℃, respectively, for 1 week. After incubation, the colonies were transferred to the same fresh medium at least three times to obtain a pure colony. To identify the isolated strains, 16S rRNA and 28S rRNA sequence analyses of bacteria and yeast, respectively, were performed as follows: genomic DNA was extracted and used as a template for PCR using HiYield™ Genomic DNA Mini Kit (RBC, Taiwan). The universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA genes of the isolated strains, which were sequenced by Macrogen Co., Ltd. The 16S and 28S rRNA gene sequences were obtained and assembled as described previously by Roh *et al.* [10] using the SeqMan program (DNA star). SILVA (http://www.arb-silva.de/aligner) was used to align the 16S rRNA gene sequences of the isolated strains and related species [11]. The phylogenetic neighbors were identified using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and pairwise alignments were performed using the Ezbiocloud (http://www.ezbiocloud.net/eztaxon/) [12]. The evolutionary distances were determined using the Kimura two-parameter model [13]. The Phylogenetic tree of the

Table 1. List of domestic bentonite samples.

Number	Sample ID	Mining area	Region	pH	NaCl (%)
1	Bgp40-b	Gampo-40	35°40'46.74"N 129°26'37.57"E	10.1	0
2	Byi33-b	Youngil-33	35°58'07.83"N 129°27'05.75"E	9.0	0
3	Zdb130-b	Daebo-130	36°00'58.02"N 129°32'02.24"E	9.03	0
4	Bdb130-1	Guryong-130	36°00'55.08"N 129°32'02.90"E	3.27	0.02

Table 2. Microbial isolation from domestic bentonite samples.

Family \ Samples	Bgp40-b	Byi33-b	Bdb130-1	Zdb130-b
Bacillaceae (66)	13	29	12	12
Bacillales Family XII (1)	1	0	0	0
Paenibacillaceae (3)	0	0	3	0
Micrococcaceae (2)	1	0	0	1
Total (72)	15	29	15	13

16S rRNA gene sequences of the isolated strains and related taxa was constructed using MEGA7 program [14] based on the neighbor-joining (NJ) algorithm [15].

A total of 72 microorganisms were isolated from the four bentonite samples through culture-dependent methods (Table 2, Table S1). Almost all the isolates belong to the *Bacillaceae* family, while some belong to the *Bacillales* Family XII, *Paneibacillaceae*, and *Micrococcaceae* families (Fig. 1). Because the four bentonite sampling sites were different, there was a small difference in the diversity of the microorganisms; however, the *Bacillaceae* family was predominant. The most abundant microorganisms were isolated from Byi33-b, all belonging to the *Bacillaceae* family. *Bacillus toyonensis* and *B. wiedmannii* were the most distributed among the *Bacillaceae* family in the four bentonite samples. Both *B. toyonensis* and *B. wiedmannii* belong to the *B. cereus* group, which are facultatively anaerobic and spore-forming bacteria known to be distributed in various environments [16]. The members of the *B. cereus* group are also known as food-spoilage organisms, but no toxin production and cytotoxicity were seen in these two strains [17]. In particular, spores of *B. toyonensis* enhanced their health and growth performances by add-

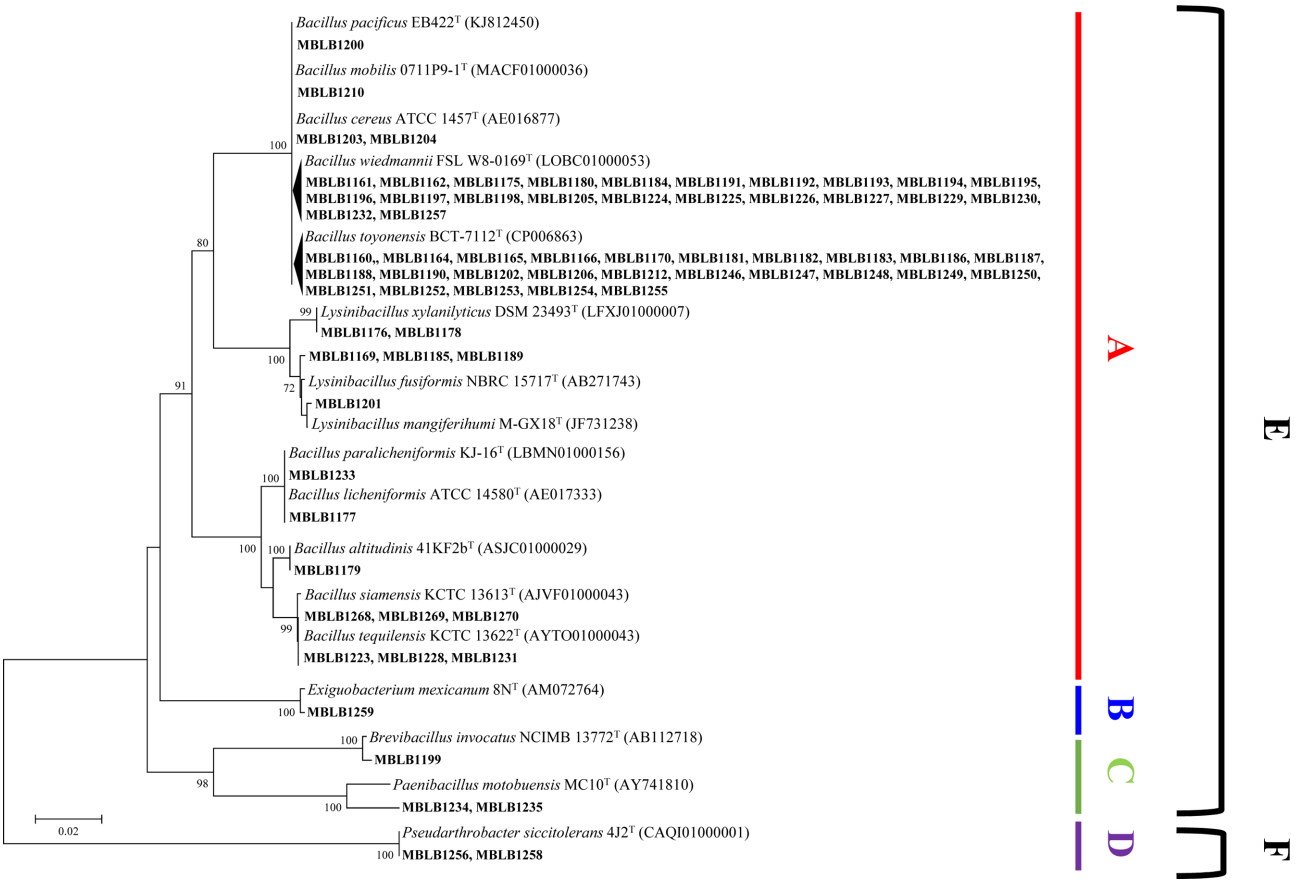

Fig. 1. Neighbor-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences showing the position and relationship of strains isolated from domestic bentonite samples and other related taxa. Numbers at nodes indicate bootstrap values (>70%) calculated based on the NJ. Bar, 0.02 substitutions per nucleotide position. *Bacillaceae* (A), *Bacillales* Family XII (B), *Paenibacillaceae* (C), and *Micrococcaceae* (D) represent family level, while *Bacillales* (E) and *Micrococcales* (F) for order level.

ing to livestock feed [18]. In the Bdb130-1 sample, only three *Paenibacillaceae* were isolated, which were identified as *Brevibacillus invocatus* and *Paenibacillus motobuensis*. Interestingly, two isolated strains MBLB1234 and MBLB1235 clustered with *P. motobuensis* MC10^T with 97.8% sequencing similarities (Table S1, Fig. 1). According to the recent study reporting that 98.65% of 16S rRNA gene sequence similarity could be as the threshold to determine a novel species [19], these two strains could be therefore considered new species within the genus *Paenibacillus*. The 16S rRNA gene sequence of strain MBLB1234 was deposited in GenBank with accession number MG333459 and the further study on the polyphasic taxonomic analyses for this strain MBLB1234 will be conducted to report this strain to be novel *Paenibacillus* species. The *Micrococcaceae* family isolates from Bgp40-b and Zdb130-b were identified as *Pseudarthrobacter siccitolerans* and the only *Bacillales* Family XII isolated from Bgb40-b was identified as *Exiguobacterium mexicanum*. In addition, *B. altitudinis*, *B. cereus*, *B. licheniformis*, *B. mobilis*, *B. pacificus*, *B. paralicheniformis*, *B. siamensis*, *B. tequilensis*, *Lysinibacillus*

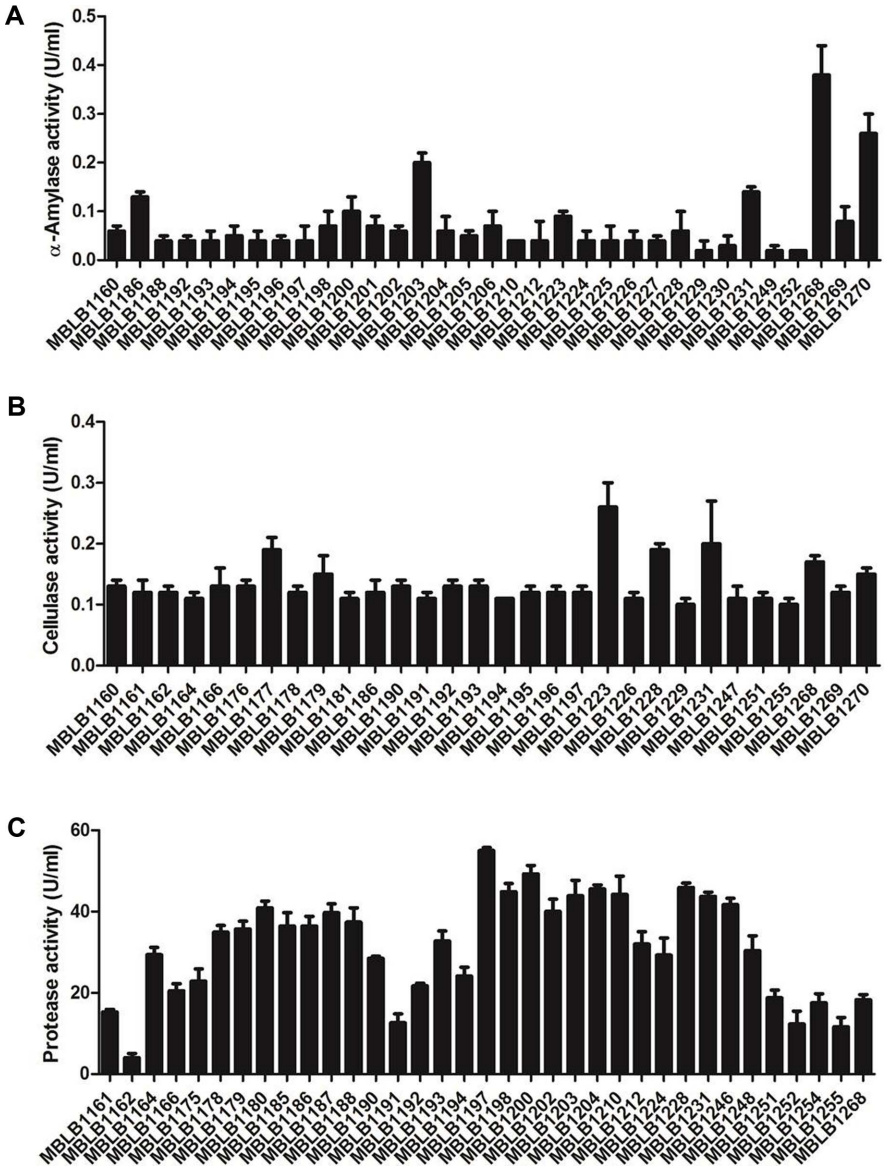

Fig. 2. Hydrolytic enzyme activities including α-amylase (A), cellulase (B) and protease (C) of the strains isolated from domestic bentonite samples.

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fusisormis, *L. mangiferiumii*, *L. xylanilyticus*, and *P. siccitolerans* were also isolated from the four bentonite samples. Microorganisms such as that from the *Bacillaceae* family can produce a variety of hydrolytic enzymes, including α-amylase, cellulase, and protease, which are potentially of great industrial value [20]. Accordingly, the activities of the corresponding hydrolytic enzymes produced by the isolated strains from bentonite were thereafter investigated.

The activities of α-amylase, cellulase, and protease in the isolated bacteria were screened through the plate assay method. To select for the bacteria with α-amylase activity, TSB with 0.2% starch agar was used. The 72 strains isolated from clay minerals were streaked on 0.2% starch agar plates and incubated at 37℃ for 3 days. The culture plates were flooded with Lugol's iodine solution, and the presence of a halo around the colonies was used as the marker to detect amylase-producing isolates. Carboxy methyl cellulose (CMC) agar was used to isolate cellulase-producing bacteria. The 72 strains isolated from clay minerals were streaked on CMC agar plates and incubated at 37℃ for 3 days. Congo red (0.1%) solution was added to the plates for 15 min and then rinsed with 1 M NaCl [21]. Cellulase activity was detected based on the clearing zones that formed around the bacterial colonies on CMC agar plates. Bacteria with protease activity were isolated on skim milk agar. A single colony of each isolate was cultivated on skim milk agar plates and then incubated at 37℃ for 2 days. A positive reaction for protease activity was indicated by a halo around the colony. Finally, the positive strains were compared qualitatively for their protease production areas on skim milk agar plates. The bacteria showing high ratios of clearing zone width to colony width were selected as potential high-yield protease producers.

A total of 72 strains isolated from bentonite samples were selected for the activity of each enzyme. Amylase activity was detected in 33 strains, cellulase activity was detected in 30 strains, and protease activity was detected in 35 strains (Fig. 2). The activity of each enzyme in the selected strains was confirmed. The activities of α-amylase and cellulase in the primary selected isolates was measured by the DNS (3,5-dinitrosalicylicacid) method. This method is a quantitative measurement of the amount of reducing sugars liberated from starch and CMC. To quantify the α-amylase activity, isolates were cultured in nutrient broth (NB) for 24 hours at 37℃. Then, the cultures were centrifuged (13,000 rpm, 15 min, 4℃) to obtain a supernatant containing the crude enzyme. A volume (200 μl) of the enzyme solution was mixed with 200 μl of 0.05 M sodium phosphate buffer with 0.5% starch and was allowed to react for 30 min at 37℃. To measured cellulase activity, primary isolates were cultured in LB broth with 1% CMC for 48 h at 37℃. Then, the cultures were centrifuged (13,000 rpm, 15 min, 4℃) to obtain a supernatant. A volume (200 μl) of 0.2 M sodium phosphate buffer with 1% CMC was mixed with 200 μl of supernatant and was allowed to react for 30 min at 50℃. After the reaction, 900 μl of DNS solution was added to the mixture; it was subsequently boiled at 100℃ for 10 min, and then cooled at room temperature for 20 min. After this process, 500 μl of mixture was diluted with 500 μl distilled water to measure its optical density (OD) at 540 nm in a 24-well plate. Enzymatic activity was defined as the amount of enzyme that produced 1 μmol of glucose per minute. Quantification of protease activity for the isolates was performed based on a previously reported method with modification [22]. The isolated strains were cultivated in 5 ml TSB at 37℃, with shaking at 180 rpm for 48 h, and supernatants were obtained by centrifugation (13,000 rpm, 15 min at 4℃). The culture supernatants (100 μl) were added to mixtures of 200 μl of 0.05 M sodium phosphate buffer (pH 7.0) and 300 μl of 0.6% casein. After incubation at 37℃ for 30 min, the reaction was quenched by adding 0.6 ml of 0.4 M trichloroacetic acid; centrifugation at 13,000 rpm for 10 min was performed subsequently. The supernatant (200 μl) was added to a mixture of 200 μl of 0.4 M Na_2CO_3 and 200 μl of 1 N phenol, followed by incubation at 40℃ for 30 min. Finally, the protease activity was investigated by measuring absorbance at 660 nm. One unit of protease activity is defined as the amount of protease that liberates 1 μg of tyrosine per minute under experimental conditions.

α-Amylase activity was detected in 13 strains in Byi33-b, in 10 strains in Bdb130-1, in 8 strains in Zdb130-b, and in 2 strains in Bgp40-b. MBLB1268 and MBLB1270, which were isolated from Byi33-b and exhibited the highest α-amylase activities, were identified to be *B. siamensis* (Fig. 2A). Bacterial α-amylases have potential applications in the food, fermentation, textile, and paper industries, and most bacterial α-amy-

lases have been produced from bacteria of the genus *Bacillus* [23]. A previous study showed that *B. siamensis* D2-2, which was isolated from low-salt soybean paste (*Doenjang*), displayed various enzyme activities and did not produce toxin genes and biogenic amines. The researchers suggested that the isolated strain D2-2 could be used as a potential starter culture to produce high-quality low-salt *Doenjang* [24]. *B. siamensis* MBLB1268 isolated from bentonite also has cellulase and protease activities, as well as high amylase activity, like the D2-2 strain. Therefore, *B. siamensis* MBLB1268 may potentially be a useful starter culture for food fermentation.

Cellulase has application potential in a variety of industries, including food, brewery and wine, industrial waste to chemical raw materials, animal feed, fiber and laundry, pulp and paper, and agriculture [25]. The strains with cellulase activity were the most abundant in Byi33-b (12 strains), followed by Zdb130-b (9 strains), Bdb130-1 (5 strains), and Bgp40-b (4 strains). The MBLB1223, MBLB1231, and MBLB1228 isolates showed the highest cellulase activities (Fig. 2B). All of these strains were isolated from Byi33-b and identified as *B. tequilensis*. Previous studies have shown that *B. tequilensis* isolates from various environments not only possess xylanase activity, but cellulase activity as well [26]. Interestingly, *B. tequilensis* MBLB1223, MBLB1231, and MBLB1228 have cellulase activities ranging from 0.19 to 0.26 U/ml, similar to that of *B. subtilis* NS7 [27]. The cellulase activity of *B. subtilis* NS7 increased up to 6 times by optimization of culture conditions, such as temperature and pH, and media, such as carbon and nitrogen sources. *B. tequilensis* strains isolated from bentonite may have industrial potential.

Various proteases from bacteria of the genus *Bacillus* have been used as industrial enzymes for removing proteinaceous stains in the detergent industry and producing bioactive peptides in the food and pharmaceutical industries [28]. Similar to other enzymatic activities (α-amylase and cellulase), protease-active strains were selected most frequently in Byi33-b (13 strains), and a similar number of protease-active strains were isolated in the other bentonite samples (7, 8, and 7 strains in Bdb130-1, Zdb130-b, and Bgp40-b, respectively). There were more protease-active strains isolated from the bentonite samples than amylase and cellulase-active strains. The MBLB1197 strain showed the highest protease activity (55.0 ± 0.8 U/ml) among the strains from the four bentonite samples (Fig. 2C). *B. wiedmannii* FSL W8-0169^{T}, which exhibited the highest similarity to MBLB1197 based on 16S rRNA sequence, was isolated from raw milk stored in a silo at a dairy powder processing plant in the northeastern region of USA and was found to contain a protease gene that degrades casein [16].

Kang *et al*. [8] observed the effect of bentonite on *kimchi* fermentation. Bentonite decreased the pH after fermentation by increasing the amount of organic acids produced by the microorganisms during *kimchi* fermentation. This suggests that fermentation sugars such as glucose were more highly produced by strains with various hydrolytic enzyme activities isolated from bentonite. The Byi33-b sample had the highest abundance of strains with α-amylase, cellulase, and protease activities among the bentonite samples. In contrast, Bgp40-b showed the least abundance of strains with various enzyme activities. Starter culture strains of fermented food must have various enzyme activities such as α-amylase, cellulase, and protease [29]. Byi33-b, which is rich in strains with various enzyme activities, has potential for use in the fermentation process.

In summary, the diverse members of *Bacillales* order were isolated from several bentonite samples collected at mining areas located in Gyeongsangbuk-do, Republic of Korea. In addition, the isolated strains were revealed to present high hydrolytic enzyme activities for α-amylase, cellulase, and protease which could be useful for various industrial fields including food and pharmaceutical industries. Finally, this study could provide the basic microbial information in bentonite as one of clay minerals and industrial applications by bentonite-derived functional microorganisms.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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