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Characterization of *Paenibacillus* sp. MBT213 Isolated from Raw Milk and Its Ability to Convert Ginsenoside Rb₁ into Ginsenoside Rd from *Panax ginseng*

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Abstract

This study was conducted to isolate and characterize *Paenibacillus* sp. MBT213 possessing β -glucosidase activity from raw milk, and examine the enzymatic capacity on the hydrolysis of a major ginsenoside (Rb₁). Strain MBT213 was found to have a high hydrolytic ability on ginsenoside Rb₁ by Esculin Iron Agar test. 16S rDNA analysis revealed that MBT213 was *Paenibacillus* sp. Crude enzyme of MBT213 strain exhibited high conversion capacity on ginsenoside Rb₁ into ginsenoside Rd proven by TLC and HPLC analyses. The API ZYM kit confirmed that *Paenibacillus* sp. MBT213 exerted higher β -glucosidase and β -galactosidase activity than other strains. Optimum pH and temperature for crude enzyme were found at 7.0 and 35°C in hydrolysis of ginsenoside Rb₁. After 10 d of optimal reaction conditions for the crude enzyme, ginsenoside Rb₁ fully converted to ginsenoside Rd. Ginseng roots (20%) were fermented for 14 d, and analyzed by HPLC showed that amount of ginsenoside Rb₁ significantly decreased, while that of ginsenoside Rd was significantly increased. The study confirmed that the β -glucosidase produced by *Paenibacillus* sp. MBT213 can hydrolyze the major ginsenoside Rb₁ and convert to Rd during fermentation of the ginseng. The β -glucosidase activity of this novel *Paenibacillus* sp. MBT213 strain may be utilized in development of variety of health foods, dairy foods and pharmaceutical products.

Keywords ginsenoside, β -glucosidase, β -galactosidase, *Paenibacillus* MBT213, raw milk

Introduction

Paenibacillus strains, a non-pathogenic and Gram-positive endospore-forming bacillus, are one of the most industrially significant facultative anaerobic bacteria. They are isolated from a variety of sources including soil, rhizosphere, water, diseased insect larvae and pasteurized food, cow manure, and raw and heat-treated milk (Daane *et al.*, 2002; Scheldeman *et al.*, 2004; Ziarno and Zareba, 2010). *Paenibacillus* strain can enter raw milk during milking, from the bedding materials, feed, or teat surfaces, during the transfer of milk to the tank car, and post-pasteur-

ization recontamination in the dairy plant (Ziarno and Zareba, 2010). *Paenibacillus* strains display physiologically diverse characteristics, one distinctive characteristic of members of the genus is their ability to degrade a variety of macromolecules, amongst others proteins (Ash *et al.*, 1993), polysaccharides (Kanzawa *et al.*, 1995) and polycyclic aromatic hydrocarbons (Daane *et al.*, 2002). Some *Paenibacillus* strains also produce antibacterial (Martin *et al.*, 2003; Piuri *et al.*, 1998) or antifungal compounds (Beatty and Jensen, 2002; Chung *et al.*, 2000).

The genus name of ginseng “Panax” is derived from the Greek *pan* (all) *akos* (cure), meaning “cure-all”. Although no single herb can be considered as a panacea, ginseng may come close to this effect. Ginseng is the most famous Asian medicinal plant, which has been used for therapeutic purpose for thousands of years (Li, 2004). Ginseng (the root of *Panax ginseng* C. A. Meyer, Araliaceae) has long been used as a medicinal purpose in Korea, China and Japan, since it exhibits anti-diabetic, anti-inflammatory (Shin *et al.*, 2006), and anti-allergic activities (Attele *et al.*, 1999). Active constituents found in most ginseng species include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids (Attele *et al.*, 1999).

Ginsenoside Rd is the main hydrolyzed product of ginsenoside Rb₁, Rb₂, and Rc, which are contained more than 80% in ginseng roots (Son *et al.*, 2008). Also ginsenoside Rd is the major precursor of ginsenoside Rg₃, Rh₂, F₂ and Compound-K, which have high biological activities (Cheng *et al.*, 2008). Among ginsenosides, minor ginsenoside Rd has been reportedly to have important biological functions including immunosuppressive (Wang *et al.*, 2012), anti-inflammatory (Yang *et al.*, 2012), immunological adjuvant (Han and Rhew, 2013), anticancer activity for gastric and breast cancer cell (Kim, 2013) and promotive effect on proliferation of neural stem cells (Palaniyandi *et al.*, 2015).

Various transformation methods have been used to produce ginsenosides including mild acid hydrolysis (Han *et al.*, 1982), enzymatic conversion (Ko *et al.*, 2003), and microbial conversion (Bae *et al.*, 2002). Of these methods, enzymatic conversion is the most desirable one for production of particular ginsenosides (Kim *et al.*, 2005). β -glucosidase produced by various microorganisms such as *Lactobacillus plantarum* CRNB22 (Renchinkhand *et al.*, 2015), *Enterococcus faecalis* CRNB-A3 (Renchinkhand *et al.*, 2016) and *Microbacterium* sp. (Cheng *et al.*, 2008) has been used for the production of biotransformed ginsenosides.

We hypothesize that the β -glucosidase activity of the *Paenibacillus* sp. could be used to hydrolyze the ginsenoside Rb₁. Therefore, objectives of this study were to: (1) isolate and characterize *Paenibacillus* sp. MBT213 possessing β -glucosidase activity from raw milk, and (2) examine the enzymatic capacity on the hydrolysis of a major ginsenoside (Rb₁) in the Korean ginseng.

Materials and Methods

Ginsenoside standards and Ginseng

Ginsenoside standards Rb₁, Rg₃, Rd and Compound-K were kindly obtained from the Department of Pharmacology Science, College of Pharmacology, Chungnam National University (Korea) and Ginseng was purchased from Seungshin BST (Korea).

Isolation of β -glucosidase activity from fermented medicinal plants

Lactic acid producing spore forming strains were isolated from raw milk. Samples were serially diluted 10 times with 0.1 M phosphate buffered saline (0.85% NaCl, pH 7.2) and were plated on a BCP agar (Bromo Cresol Purple agar, Eiken Chemical Co. Ltd., Japan) at 35°C for 24 h. Strains produced lactic acid, and the color of the media was changed from purple to yellow. Morphologically different colonies with yellow color were selected, identified and individually plated, which were subsequently tested for their β -glucosidase production with esculin iron agar (Sigma-Aldrich, Chemie GmbH, Germany) at 35°C for 24 h. The esculin positive colonies were selected for dark brown zones after incubation for 24 h at 37 and 20°C (Kim *et al.* 2005).

Enzyme activity patterns

Enzyme activity patterns of the selected strains were examined using the API ZYM enzyme system (version 4.0 of API LAB plus, BioMérieux, France) according to the manufacturer's instructions.

Conversion of ginsenoside Rb₁ by selected strain MBT213

Esculin positive colonies were inoculated in Trypticase Soy (pH 7.0) (BBL, USA) and supernatants were collected by centrifugations at 7,000×g for 15 min at 4°C. Supernatants of selected cultures with β -glucosidase activity were tested for the hydrolysis of the major ginsenoside Rb₁. The reaction mixture in the ratio 1:1 (v/v) of supernatant (pH

7.0) and each of 0.2 mg/mL of protopanaxadiol ginsenoside Rb₁, Rb₂ and protopanaxatriol ginsenoside Re (5 mM of sodium phosphate buffer, pH 7.0), respectively, were filtered through a 0.2 µm membrane filter (Toyo Roshi Kaisha Ltd, Japan). All reaction mixtures were incubated at 35°C for 7 d. The effect of pH on the conversion of ginsenoside by strain MBT213 was determined by examining the pH range of 4.0-9.0. These reaction mixtures were used to hydrolyze ginsenoside Rb₁ at 30°C for 7 d. The effect of temperature on the conversion of ginsenoside by strain MBT213 was determined by hydrolyzing ginsenoside Rb₁ in pH 7.0 at 30, 35 and 40°C for 7 d.

Thin layer chromatography (TLC) analysis

After incubation for 7 d, the reaction mixture was vigorously mixed with super butanol at the ratio 1:1 (v/v) and upper layer was collected for TLC analysis. Ginsenoside Rb₁ hydrolysis was monitored with TLC performed on Silica gel 60 F₂₅₄ plates (Merck, Germany). A solvent mixture of chloroform: methanol: water (65:35:10, v/v/v, lower phase) was used as the developing solvent. The resultant reaction spots were detected by spraying 10% sulfuric acid in methanol and heating under a lamp flame until the spots were clearly visible.

High performance liquid chromatography (HPLC) analysis of ginsenoside hydrolysates

Ginsenoside hydrolysates obtained from the β-glucosidase activity of strains MBT213 were analyzed using a reversed-phase HPLC system Shimadzu LC-6AD (Shimadzu Co., Ltd., Japan) and an ACE-5-C₁₈ column (4.6×250 mm) equilibrated with solvent A (H₂O). The ginsenoside hydrolysates were eluted with a linear gradient of solvent B (CH₃CN) using an elution gradient system [0-35 min (20% B), 35-85 min (40% B), 85-105 min (50% B), 105-135 min (65% B), 135-145 min (85% B), 145-155 min (100% B), 155-160 min (100% B), 160-163 min (20% B), 163-165 min (20% B), respectively]. Runs were conducted at 40°C using the same HPLC system at a flow rate of 1.0 mL/min, and the absorbance of the elute samples was read at 203 nm using a UV detector 1260 Infinity (Agilent Technologies Inc., Japan). All samples were filtered through 0.5 µm Acrodisc Syringe Filters (Gelman Laboratory, USA).

16S rDNA sequencing of the strain MBT213

Selected strains were cultured in TS broth for 24 h, and 1 mL of culture was harvested via centrifugation. Geno-

mic DNA was isolated using a Genomic DNA prep Kit (Solgent Co., Ltd., Korea). The primers used were 27F: 5'-AGAGTTTGATCACTGGC-TCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3'. PCR was performed using the 2×PCR pre Mix (EF-taq) in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA). PCR conditions were as follows: an initial denaturation step for 3 min at 95°C, 30 cycles of denaturation at 94°C for 20 s, annealing at 56°C for 40 s, extension at 72°C for 40 s, and a final elongation step at 72°C for 3 min. PCR sequences were compared with those in the NCBI database using BLAST. Phylogenetic trees of selected bacteria, including MBT213, were drawn using Bioedit (Tom Hall Ibis Biosciences, An Abbott Co., USA) and the MEGA 5 program (Arizona State University, USA).

Fermentation of ginseng root by the strain MBT213

Ginseng roots were sliced using mixer for 5 min and sterilized at 75°C for 10 min. A 20% ginseng root solution was incubated with 3% strain MBT213 at 35°C for 14 d. After 3, 7 and 14 d, fermented ginseng root solutions were freeze-dried and resolved in 50% methanol for protein precipitation. Centrifugation was performed at 7000×g for 20 min and the supernatant was collected for freezing-drying. The supernatant was resolved in methanol (95%) and filtered using the 0.5 µm Acrodisc Syringe Filters (Gelman Laboratory, USA). Filtrates were used to TLC and HPLC analysis in order to determine conversion ginsenosides. Determination of viable cell counts was performed by plating in triplicate using a TS Agar (BBL, USA) and incubating at 37°C for 48 h after serial dilution with sterile 0.85% saline.

Statistical analysis

Statistical analyses were performed using the SPSS version 22 statistical package (SPSS Inc., USA). Analysis of variance and paired *t*-tests were applied to the data to determine significant difference, and a level of *p*<0.05 was considered statistically significant.

Results and Discussion

Isolation and screening of *Paenibacillus* strain exhibiting β-glucosidase activity

Esculin is chromogenic substrate for detection of β-glucosidase. Many researchers have used esculin as quick screening method for isolation of microorganisms exerting β-glucosidase activity (Gabriel *et al.*, 2010). Using the

Table 1. Isolation of strain with β -glucosidase activity from 14 samples at 35°C for 24 hr in esculin agar

Sample number	Colonies with yellow color in BCP agar	Esculin positive	Selected for DNA analysis
1	-	-	-
2	MBT213	MBT213	MBT213
3	-	-	-
4	41S, 42S, 43S, 44L, 45L, 46L, 47L	-	-
5	51S	-	-
6	61L, 63L, 64L, 65L, 66L, 67L, 68L, 70L	61L, 63L, 64L, 65L, 66L, 67L, 68L, 70L	63L, 65L
7	71L, 72bS, 72S, 72'S, 73sS, 73bS	71L, 72bS, 72S, 72'S, 73sS, 73bS	71L, 72S, 73S
8	84S, 85L	-	-
9	96L, 97L	-	-
10	101L, 102L, 103L, 104L	103L	103L
11	-	-	-
12	122, 123, 124L, 125L	122, 123	123
13	-	-	-
14	141, 142	-	-
Total	37 colonies	18 colonies	8 colonies

esculin screening method in our study, a number of lactic acid producing bacteria were isolated from 14 types of Korean traditional fermented medicinal plants products. Among these bacterial strains, 37 selected strains were examined for β -glucosidase production capacities in the esculin agar for at 35°C for 24 h. It was found that MBT 213 was the most strong esculin positive strain. The results also showed that 18 strains, MBT213, 61L, 63L, 64L, 65L, 66L, 67L, 68L, 70L, 71L, 72bS, 72S, 73S, 73sS, 73bS, 103L, 122 and 123 exhibited the esculin positive reaction (Table 1). The 8 strains of these 18 esculin positive strains, the MBT213, 63L, 65L, 71L, 72S, 73S, 103L, 123 were selected for genomic DNA analysis based on cell morphology. The strain MBT213 had 4 types of enzymatic activity such as α -galactosidase, β -galactosidase, α -glucosidase and β -glucosidase in carbohydrate hydrolysis (Table 2). Among these enzymes, β -glucosidase can hydrolyze glycosidic linkage in ginsenosides. β -Glucosidase crude enzyme of strain MBT213 hydrolyzed glucose-glucose (6-1) linkages at C-20 in ginsenoside Rb₁(molecular weight = 1109.29) and released one glucose molecule and ginsenoside Rd (molecular weight = 963.15) (Fig. 1). This would be the main conversion pathway of ginsenoside Rb₁ to ginsenoside Rd by β -glucosidase enzyme, as shown in Fig. 1 (Suzuki *et al.* 2010). Among carbohydrate hydrolyzing enzymes, β -glucosidase can hydrolyze glucose-glucose (6-1) linkages (70%), glucose-arabinose (pyr) linkages (6-1) (20%), glucose-arabinose (fur) (6-1) linkages (20%) of ginsenoside Rb₁ in ginseng, and β -galactosidase can hydrolyze glucose-glucose (2-1) linkages (100%), glucose-glucose (6-1) linkages (100%) of ginsenoside Rb₁ in

Table 2. Enzymatic activities of strain MBT213 using API ZYM kit

Enzymes	Strain MBT213
Control	0*
Alkaline phosphatase	1
Esterase (c4)	3
Esterase lipase (C8)	1
Lipase (c14)	0
Leucine arylamidase	2
Valine arylamidase	2
Cystine arylamidase	0
Trypsin	0
α -chymotrypsin	0
Acid phosphatase	1
Naphtol-AS-BO-phosphohydrolase	2
α -galactosidase	1
β -galactosidase	3
β -glucuronidase	0
α -glucosidase	1
β -glucosidase	3
N-acetyl- β -glucosamidase	0
α -mannosidase	0
α -fucosidase	0

*Quantity of hydrolyzed substrate. 0: 0 nmol, 1: 5 nmol, 2: 10 nmol, 3: 20 nmol, 4: 30 nmol, 5: \geq 40 nmol.

ginseng (Suzuki *et al.*, 2010). This report supports the high hydrolyzing capacity of ginsenosides Rb₁ by β -glucosidase in the strain MBT213 in our study. When the major ginsenoside Rb₁, Rb₂ and Re in the fermented ginseng samples were hydrolyzed by the strain MBT213, ginsenoside Rb₁ was converted to Rd in minor quantity, while no conversions were occurred for ginsenosides Rb₂ and Re (Fig. 2).

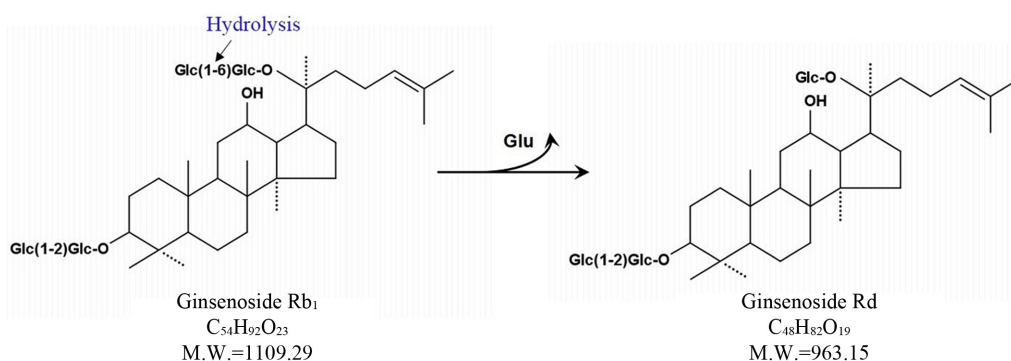


Fig. 1. Pathway on the conversion of ginsenoside Rb₁ to Rd. (Suzuki *et al.*, 2010)

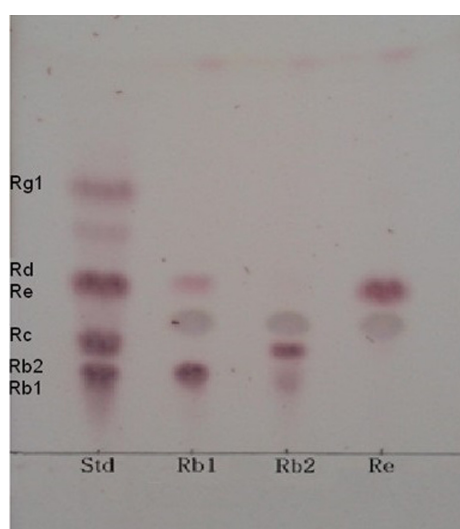


Fig. 2. TLC analysis of ginsenoside Rb₁, Rb₂ and Re by strain MBT213. TLC was performed on Silica gel 60 F₂₅₄ plates. A solvent mixture of chloroform: methanol: water (65:35:10, v/v/v, lower phase) was used as the developing solvent.

Optimum pH and temperature for crude enzyme and conversion of Rb₁

In conversion of ginsenoside Rb₁, the optimum pH and temperature for the crude enzyme extracted from strain MBT213 were found to be pH 7.0 (Fig. 3A) and 35°C (Fig. 3B), respectively. Under these conditions, the β -glucosidase enzyme obtained from MBT213 converted ginsenoside Rb₁ to ginsenoside Rd. After 14 d of hydrolysis, ginsenoside Rb₁ was fully converted to ginsenoside Rd, and the chromatographic analyses of the conversion of ginsenoside Rb₁ to ginsenoside Rd is as shown in HPLC (Fig. 4A) and TLC results (Fig. 4B). Under the above optimal conditions for 14 d, 3.794 \pm 0.061 mg/mL of ginsenoside Rb₁ was fully hydrolyzed by crude enzyme of strain MBT213, and was released 0.110 \pm 0.01 mg/mL ginseno-

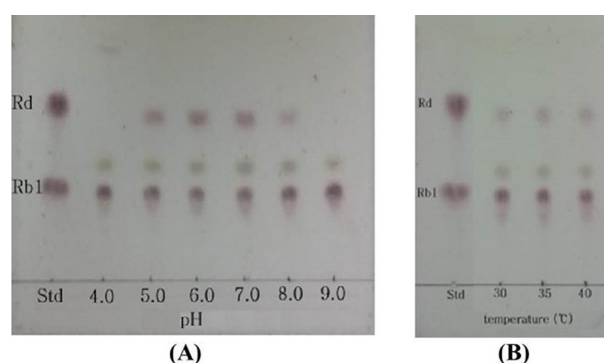


Fig. 3. The effect of pH and temperature on the conversion of ginsenoside by strain MBT213. (A) pH, (B) Temperature. TLC was performed on Silica gel 60 F₂₅₄ plates. A solvent mixture of chloroform: methanol: water (65:35:10, v/v/v, lower phase) was used as the developing solvent.

side Rd (Table 2). On the other hand, in a study of isolation of *Lactobacillus plantarum* CRNB22 and its screening of LAB exhibiting β -glucosidase activity from Kimchi (Renchinkhand *et al.*, 2015), observed that ginsenoside Rb₁ was hydrolyzed under optimum condition (pH 6.0 or 7.0 at 35°C) for 7 d. In addition, under these optimal conditions, the crude enzyme obtained from CRNB22 converted the ginsenoside Rb₁ to Rg₃ (epimer S/R) and Rg₅. After 7 d of hydrolysis, ginsenoside Rb₁ was decreased from 1.019 mg to 0.773 mg, and then converted to 0.135 mg Rg₃-S, 0.038 mg Rg₃-R, and 0.072 mg Rg₅. These reported data are in agreement with our observations in this study. Depending on types of enzymes and experimental conditions, different conversion compounds of ginsenoside Rb₁ have been generated, such as Rd, Rg₃, Compound-K and other compounds (Suzuki *et al.*, 2010).

16S rDNA sequencing of the strain MBT213

Sequencing of 16S rDNA was performed to determine

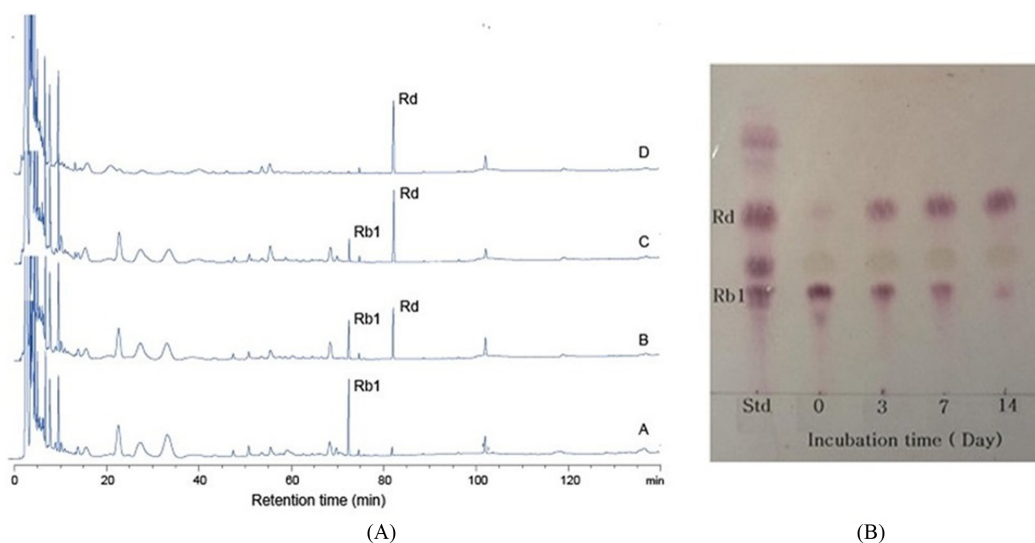


Fig. 4. Conversion of ginsenoside Rb₁ hydrolyzed by strain MBT213. (A) HPLC analysis of ginsenoside Rb₁ conversion at optimum temperature and optimum pH for 14 d hydrolysis by using ACE-5-C₁₈ column (4.6×250 mm) equilibrated with solvent A (H₂O). A-0 d, B-3 d, C-7 d, D-14 d; (B) TLC analysis of ginsenoside Rb₁ at optimum temperature and pH for 14 d hydrolysis.

the correct species of the isolated strain MBT213. PCR produced many sequences, which corresponded to the full length 16S rDNA sequence of the strain MBT213. The sequence consisted of 1450 bp, which were compared to those in the NCBI database using BLAST and this strain is homology in 95% (1374 bp within 1450 bp) to *Paenibacillus* sp. JDR2 and is homology in 95% (1374 bp within 1450 bp) to *Paenibacillus polyxa* E681. As shown in Fig. 5, the sequence was identical to *Paenibacillus* according to the phylogenetic tree. The isolated MBT213 strain was thus named *Paenibacillus* sp. MBT213.

Characteristics of fermented ginseng by *Paenibacillus* sp. MBT213

Table 3 shows the conversion of ginsenoside Rb₁ to ginsenoside Rd by *Paenibacillus* sp. MBT213 in 20% fer-

mented ginseng roots solution. In addition, it demonstrates the viable cell counts in the experimental fermented ginseng products. The initial cell count of *Paenibacillus* sp. MBT213 was 3.60±0.02 Log CFU/mL. Viable cell counts of *Paenibacillus* sp. MBT213 were increased to 8.07±0.021 Log CFU/mL for 7 d, and then after 14 d incubation, these cell counts were maintained at 7.16±0.016 Log CFU/mL level. Results of HPLC analysis of the fermented ginseng roots (Fig. 6) revealed that the peak of ginsenosides Rb₁ was decreased and ginsenoside Rd was significantly increased after 14 d. In addition, after 14 d fermentation, the amount of ginsenoside Rb₁ was decreased from 2.670±0.14 to 1.351±0.049 mg/mL during the fermentation, while amount of minor ginsenoside Rd was increased from 0 to 0.087±0.013 mg/mL (Table 3). The decrease in ginsenoside Rb₁ and increase in Rd in the fermented of

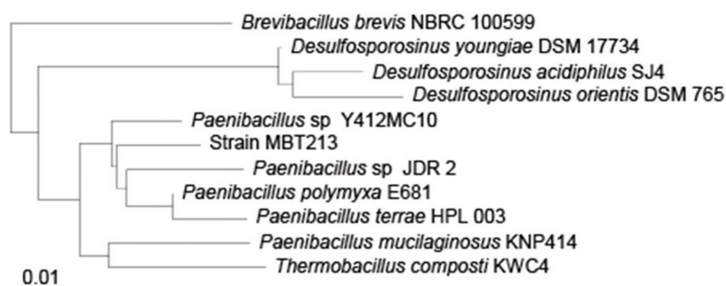


Fig. 5. Phylogenetic tree based on 16S rDNA sequences showing the position of strain MBT213, showing the phylogenetic relationships among strain MBT213 and related bacteria. Scale length is 0.01.

Table 3. Amounts of conversion of ginsenoside Rb₁ to Rd and viable cell counts

		Incubation time (d)			
		0	3	7	14
A	Rb ₁ (mg/mL)	3.794±0.061*	1.599±0.031	0.868±0.032	0
	Rd (mg/mL)	0	0.058±0.006	0.086±0.011	0.110±0.01
B	Rb ₁ (mg/mL)	2.670±0.14	1.821±0.071	1.521±0.179	1.351±0.049
	Rd (mg/mL)	0	0.017±0.005	0.069±0.011	0.087±0.013
C	<i>Paenibacillus</i> sp. MBT213 (Log CFU/mL)	3.60±0.02	7.48±0.021	8.07±0.021	7.16±0.016

*Means±SD (n=3).

A) Amounts of conversion of ginsenoside Rb₁ by supernatant of strain MBT213 during the 14 days; B) Amounts of conversion of ginsenoside Rb₁ and Rd in ginseng root by strain MBT213 during the 14 days; C) Viable cell counts of strain MBT213 in 20% ginseng root.

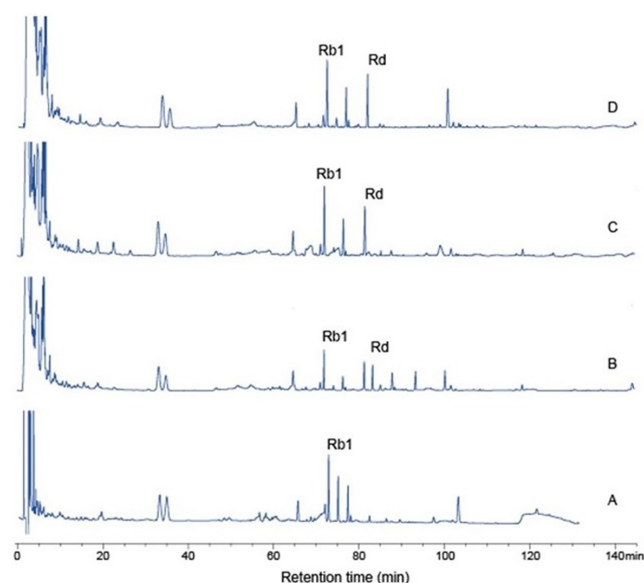


Fig. 6. HPLC analysis. Ginsenosides conversion in 20% ginseng by strain *Paenibacillus* sp. MBT213 for 3 and 14 d; A-0 d, B-3 d, C-7 d, D-14 d.

ginseng roots are apparently attributable to the conversion of ginsenoside Rb₁ to Rd, which was fermentation time dependent. Park *et al.* (2016) reported that 6 types of lactic acid bacteria in fermented ginseng multiplied to approximately 1×10^9 - 10^{11} CFU/mL on first day of fermentation, and after 5 d of fermentation, viable cell counts of LAB were dropped to 4×10^7 CFU/mL. They also found the continuous decreases in the amounts of ginsenoside Rb₁, Rb₂, Rc in PPD type, while increases in ginsenoside Rd during fermentation by the LAB. Consequently, viable cell counts of fermented ginseng roots were increased, since ginseng roots contain various substrates, such as polysaccharides, peptides, vitamins, minerals as growth factors of the microorganisms. These findings in bacterial growth and ginsenoside changes in the fermented ginseng roots in their study are in agreement with the results that were observed

in our study. This strain was deposited in the KACC database (<http://www.kacc.rda.go.kr>) under the registration number KACC-91831P and NCBI GenBank accession number is NCBINam.sqn A MF972642.

Conclusion

The study has confirmed that the β -glucosidase produced by *Paenibacillus* sp. MBT213 can hydrolyze the major ginsenoside Rb₁ and convert to Rd during fermentation of the ginseng. The β -glucosidase activity of this novel *Paenibacillus* sp. MBT213 strain may be utilized in development of variety of health foods and pharmaceutical products.

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