

Enzymatic Transformation of Ginsenoside Rb1 by *Lactobacillus pentosus* Strain 6105 from Kimchi

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Ginsenoside (ginseng saponin), the principal component of ginseng, is responsible for the pharmacological and biological activities of ginseng. We isolated lactic acid bacteria from Kimchi using esculin agar, to produce β -glucosidase. We focused on the bio-transformation of ginsenoside. Phylogenetic analysis was performed by comparing the 16S rRNA sequences. We identified the strain as *Lactobacillus* (strain 6105). In order to determine the optimal conditions for enzyme activity, the crude enzyme was incubated with 1 mM ginsenoside Rb1 to catalyse the reaction. A carbon substrate, such as cellobiose, lactose, and sucrose, resulted in the highest yields of β -glucosidase activity. Biotransformations of ginsenoside Rb1 were analyzed using TLC and HPLC. Our results confirmed that the microbial enzyme of strain 6105 significantly transformed ginsenoside as follows: Rb1 \rightarrow gypenoside XVII, Rd \rightarrow F2 into compound K. Our results indicate that this is the best possible way to obtain specific ginsenosides using microbial enzymes from 6105 culture.

Keywords: *Panax ginseng*, *Lactobacillus pentosus*, Transformation, Ginsenoside

INTRODUCTION

Panax ginseng Meyer is one of the most valuable Oriental medicinal plants. Ginseng saponin (ginsenoside) is the major pharmacological component in ginseng. More than 100 ginsenosides have been isolated from ginseng plant [1,2]. Among these, six major ginsenosides, such as Rb₁, Rb₂, Rc, Rd, Re, and Rg1, constitute more than 90% of the total ginsenosides in white and red ginseng [3]. In recent decades, many studies have been reported the successful transformation of these major forms of ginsenoside into the more active minor forms of ginsenosides, such as Rg3, Rh2, and compound K (anti-tumor, antidiabetic, antistress, and antioxidative) [4]. Minor saponins has high pharmacological activity, however the natural availability of these saponins are very less in ginseng.

Studies have been reported the success of converting major ginsenosides to the minor saponin varieties using methods such as heating [5,6], acid hydrolysis [7,8], and microbial enzymatic conversion [9-11]. Ginsenoside Rb1 has four glucose molecules with (1 \rightarrow 2)- β -glycosidic linkages at C-3 and (1 \rightarrow 6)- β -glycosidic linkages at C-20.

Kimchi is a traditional fermented food in Korea that accompanies many meals. The ingredients include Korean cabbage, vegetables, garlic, and other seasonings. During Kimchi fermentation, lactic acid bacteria (LAB) produce lactic acid by hydrolyzing carbohydrates (glucose and lactose). LAB is not only widely recognized, but is also well known for its physiological effects such as drug metabolism, antimutation, anticancer, gastric sec-

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retomotor activity, and radiation resistance [12-14].

In this study, we isolated β -glucosidase producing bacteria from Kimchi and tested the bio conversion ability of ginsenoside Rb1. The crude enzyme from strain 6105 was reacted with ginsenoside Rb1 to convert it into specific components. Finally, the results were confirmed with TLC and HPLC.

MATERIALS AND METHODS

Materials

Ginsenoside Rb1 was obtained from the Ginseng Genetic Resource Bank at Kyung Hee University, Korea. Standard ginsenosides including 20(*S*)-Rb1, 20(*S*)-Rd, 20(*S*)-Rg3, 20(*S*)-Rh2, and compound-K were obtained from KT&G in Daejeon, Korea. De Man, Rogosa and Sharpe (MRS) broth was purchased from Difco (Detroit, MI, USA). *p*-Nitrophenyl- β -D-glucopyranoside (*p*-NPG) and *p*-nitrophenol (*p*-NP) were purchased from Sigma Aldrich (St. Louis, MO, USA). The 60 F254 silica gel plate (Merck, Darmstadt, Germany) was used for TLC and silica gel 60 (Merck) was used for column chromatography. An HPLC (NS 3000i system, Futecs Co., Daejeon, Korea) was used with a UV detector and gradient pump.

Isolation of β -glucosidase producing bacteria from Kimchi

We collected many kinds of Kimchi from various regions in Korea, and β -glucosidase producing bacteria were isolated using a esculin agar medium. Single colonies from the plates were purified onto new plates. The isolated strains were incubated at 37°C for 24 h in MRS agar (Difco). We suggested that the bacteria could convert the major ginsenoside Rb1 to the minor ginsenoside Rg3.

Culture conditions

One micro liter of cells were grown in 100 mL Erlenmeyer flasks containing 50 mL of MSR broth and incubated at 30°C on a rotary shaker (160 rpm) until it reaches the absorbance 0.8 to 1.0. Later the cells were harvested and re-inoculated onto the MRS broth containing 1% (w/v) cellobiose, glucose, lactose, sucrose, hesperidin and soluble starch. Growth was monitored using spectrophotometer at O.D. 600.

Preparation of crude enzyme

Bacterial cells were removed by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant containing the extracellular β -glucosidases was used as the crude enzyme and was precipitated using acetone. The precipi-

tates were dissolved in 20 mM sodium phosphate buffer, pH 7.0. Later, their activity was checked using 1 mM ginsenoside Rb1.

Assay of ginsenoside Rb1 transformation activity

To determine the β -glucosidase activity, 50 μ L of crude enzyme were added to 50 μ L of 10 mM *p*-NPG suspended in 20 mM sodium phosphate buffer (pH 7.0). Then the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 100 μ L of 1 M Na₂CO₃, and the *p*-NP produced was measured at 405 nm by a UV spectrophotometer (Ultrospec 2100 Pro; Amersham Biosciences, Cambridge, UK). One unit of enzyme activity was defined as the amount released 1 nmol *p*-NP per min from *p*-NPG under the above reaction conditions.

Effect of temperature and pH on enzyme activity

The optimal temperature for enzyme activity was determined over a temperature ranges between 25°C to 70°C using the standard assay conditions. To determine the optimal pH the enzyme activity was measured over a pH range of 4.5 to 8.0. The buffers used for the assay are 20 mM acetate buffer (pH 4.5-5.5), 20 mM sodium phosphate buffer (pH 6-7.5), and 20 mM Tris-HCl buffer (pH 8.0).

Biotransformation

The reaction mixture, containing 200 μ L of 1 mM ginsenoside Rb1 and 200 μ L of bacterial suspension were cultured in MRS broth, was incubated for 72 h at 37°C. Then extracted twice with 200 μ L of *n*-butanol. The *n*-butanol fraction of the reaction mixture was evaporated *in vacuo*, and the residue was dissolved in CH₃OH.

Analysis of ginsenosides by thin-layer chromatography

TLC was performed using a silica gel 60 F254 plate. CHCl₃-CH₃OH-H₂O (65:35:10, v/v/v, lower phase) mixture was used as the developing solvent for TLC. The spots on the TLC plates were detected by spraying 10% H₂SO₄ followed by heating at 110°C for 5 min.

Analysis of ginsenosides by high pressure layer chromatography

HPLC was performed using a NS 3000 system (Futecs Co.), equipped with a UV detector and a gradient pump. The C18 column (YMC c18 pro, 250×4.6 mm, 5 μ m) was used for this analysis with 20 μ L sample volume. The mobile phase consisted of CH₃CN (A) with distilled H₂O (B). The solvent A/solvent B form the gradient in the ratios as follows: 15:85, 21:79, 58:42, 90:10, 90:10,

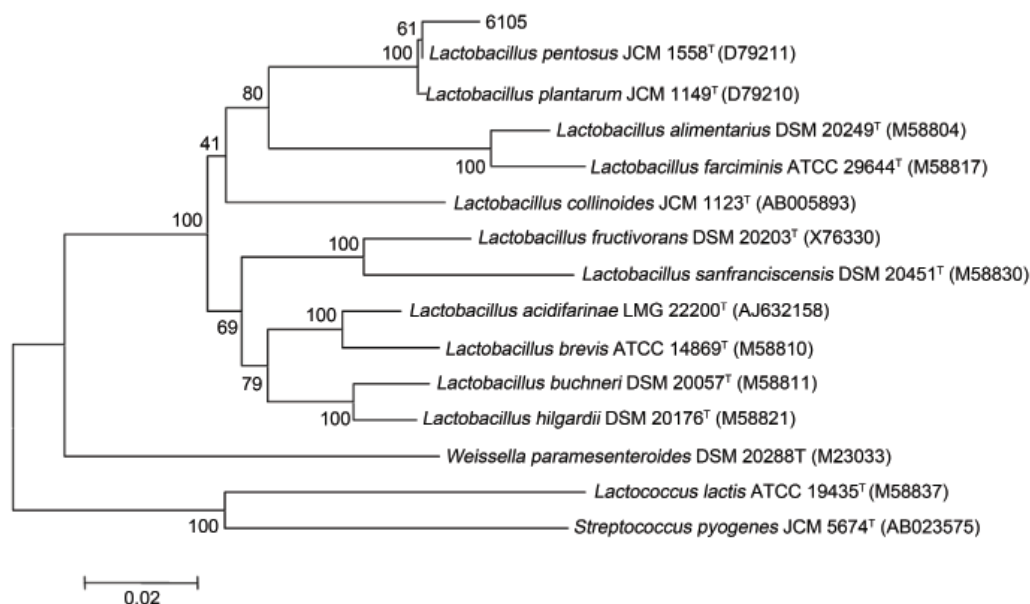


Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain 6105 and related species.

15:85, and 15:85, with run times of 0-5, 5-25, 25-70, 70-72, 72-82, 82-84, and 84-100 min, respectively, at a 1.6 mL/min flow rate. Detection was done at 203 nm.

Analysis of nuclear magnetic resonance spectrum

An 80 mL suspension of strain 6105 from the MRS broth was mixed with the same volume of 1 mM ginsenoside Rb1 and incubated at 30°C, in 160 rpm for 60 h. The reaction mixture was extracted with 150 mL of *n*-butanol twice and was evaporated *in vacuo* at 45°C. The residue was applied to the silica gel 60 column and eluted with CHCl₃-CH₃OH-H₂O (65:35:10, v/v, lower phase). Twenty milliliter of fraction was collected, separated and the metabolite transformed from ginsenoside Rb1 was dissolved in pyridine-*d*₅ and identified by ¹H-NMR and ¹³C-NMR using FT-NMR spectrometry (400 MHz; Varion Inova AS 400, Varion, Palo Alto, CA, USA)

RESULTS

Phylogenetic study

β -glucosidase producing bacteria was isolated from Kimchi using esculin-MRS agar. The 16S rRNA gene sequences of the strain 6105 were aligned with type strains found to have the closest taxonomic relationships. Strain 6105 was grouped with a *Lactobacillus* species, and the highest degrees of 16S rRNA gene sequence identities were to *L. plantarum* JCM 1149^T (D79210) (99.1%), *L. pentosus* JCM 1558^T (D79211) (99.2%) (Fig. 1).

Characterization of the crude enzyme of strain 6105

The effect of temperature and pH on the activity of β -glucosidase was shown in Fig. 2. The optimum temperature for maximal activity was 37°C. At 37°C ginsenoside Rb1 was converted into compound K, however, the crude enzyme activity was decreased over 40°C dramatically and not converted any compounds at 50°C to 70°C. Under optimal temperature conditions, the crude enzyme activity was high at the pH ranges from 6.0 to 8.0. Ginsenoside Rb1 was almost hydrolyzed on pH 6.0 to 7.5 and the crude enzyme shows maximum activity at the pH 7.0 which converts ginsenoside Rb1 into ginsenoside Rd, F2 and compound K.

The HPLC profile of the enzymatic transformation of ginsenoside Rb1 by crude enzyme of strain 6105 is shown in Fig. 3. The identities of the peaks were established by comparison of retention time values with those of standards. In the HPLC chromatogram, the peaks with retention time 37.23, 41.68, 56.01, and 61.56 min correspond to ginsenoside Rb1, gypenoside XVII, F2, and compound K.

Effect of carbon source supplements on β -glucosidase activity

The relative rates of hydrolysis of various substrates by crude enzyme are shown in Fig. 4. Strains were cultured on MRS broth and inoculated on MRS broth with 1% carbon source such as glucose, cellobiose, lactose, su-

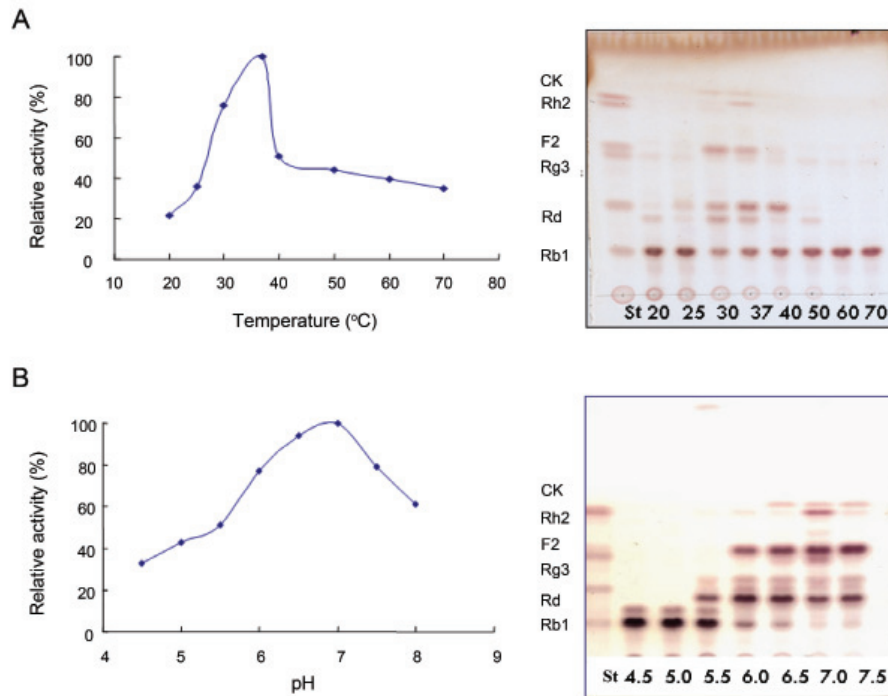


Fig. 2. Effect of temperature (A) and pH (B) on the transformation of ginsenoside Rb1 by crude enzyme strain 6105. CK, compound K; ST, standard.

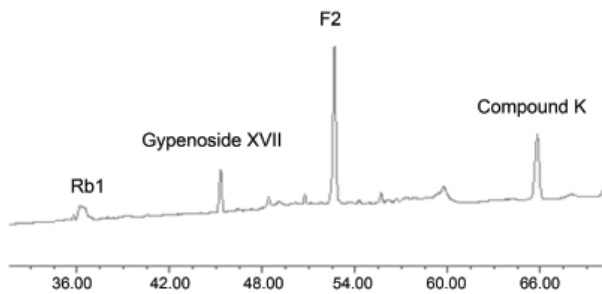


Fig. 3. HPLC assay of transformation of ginsenoside Rb1 to compound K by crude enzyme strain 6105.

Table 1. β -glucosidase produced by strain 6105 on various carbon sources

	β -glucosidase (U/mL)	Relative activity (%)
Control	0.78	100
Cellobiose	0.83	110.2
Glucose	0.82	108.16
Lactose	0.95	132.35
Sucrose	0.86	116.33
Hesperidin	0.59	65.31
Soluble starch	0.72	89.8

crose, hesperidin, and soluble starch to prepare crude enzyme (supernatant). The highest yields of β -glucosidase

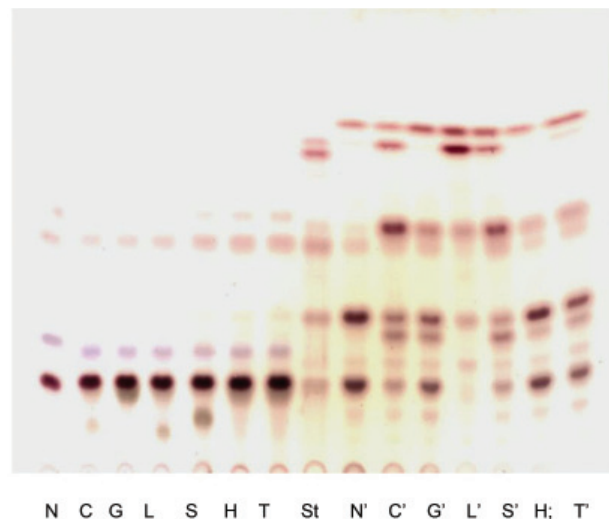


Fig. 4. Effects of carbon source supplementation on the transformation of ginsenoside Rb1 by crude enzyme of strain 6105. N, without elicitor; C, cellobiose; G, glucose; L, lactose; S, sucrose, H, hesperidin; T, soluble starch; St, standard; N-T, reaction with suspension culture; N'-T', reaction with crude enzyme precipitated by acetone.

activity (0.95 U/mL) were obtained in lactose culture broth (Table 1). We hypothesized that carbon sources cause an increase β -glucosidase activity. The ginsenoside Rb1 was converted in the following sequence: Rb1→Rd and gypenoside XVII→F2→compound K (Fig. 5) by the

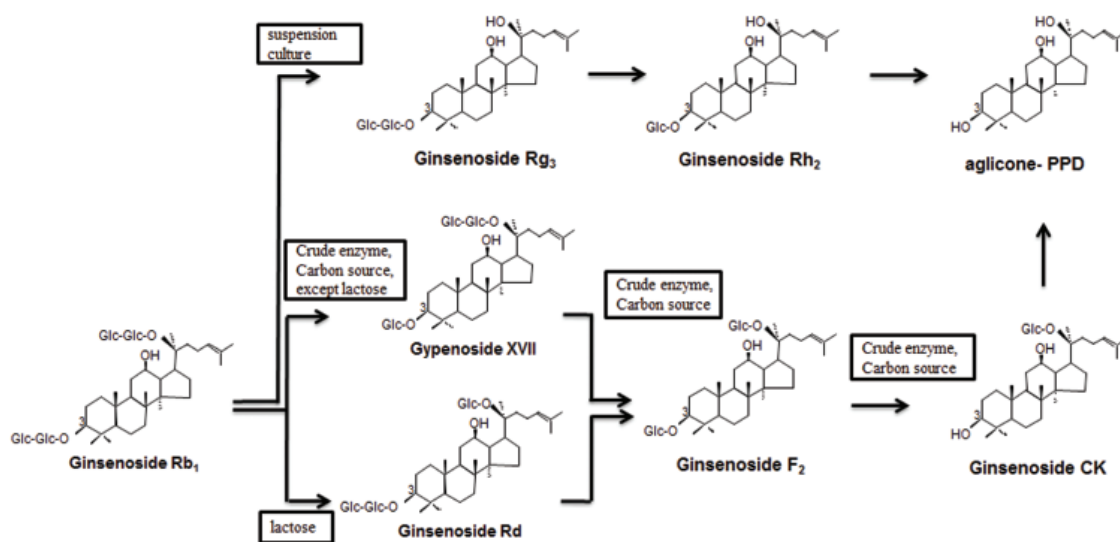


Fig. 5. The conversion pathway of ginsenoside Rb1 using crude enzyme strain 6105.

crude enzyme from strain 6105, consecutively hydrolyzing of ginsenoside Rb1.

Structural identification of metabolites

We identified the metabolites of ginsenoside Rb1 by $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectroscopy, because the R_f value and retention time of the metabolites were the same as the standard compound K in TLC and HPLC analysis.

Metabolite 1

In the $^1\text{H-NMR}$ spectrum of metabolite 1, the proton signals for the H-1 of the 3-O-inner-glucopyranosyl moiety, 3-O-outer-glucopyranosyl moiety, and 20-glucopyranosyl moiety appeared respectively, at δ 4.87 ppm (1H, d, $J=8.2$ Hz, H-3-glc-1H'), δ 5.05 ppm (1H, d, $J=7.8$ Hz, H-20-glc-1H'''), δ 5.08 ppm (1H, d, $J=7.6$ Hz, H-20-glc-1H'''), showing that the aglycon of metabolite 1 harbored three β -D-glucoses. In the $^{13}\text{C-NMR}$ (pyridine- d_5 , 100 MHz) spectrum of metabolite 1, the signals for C-2' of the 3-inner-glucose was shifted up field, from 83.5 ppm to 76.0 ppm, but the other signals were similar to those of ginsenoside Rb1. Compared with as previous reported[15], we indicated that the metabolite is 3-O- $[\beta$ -D-glucopyranosyl]-20-O- $[\beta$ -D-glucopyranosyl-(6,1)- β -D-gluco-pyranosyl]-20(S)-protopanaxadiol, identical to gypenoside XVII.

Metabolite 2

In the $^1\text{H-NMR}$ spectrum of metabolite 2, the proton signals for the H-1 of the 3-O-inner-glucopyranosyl moiety, 3-O-outer-glucopyranosyl moiety, and 20-glucopy-

ranosyl moiety appeared respectively, at δ 4.94 ppm (1H, d, $J=7.7$ Hz, H-20-glc-1'''), δ 4.87 ppm (1H, d, $J=8.2$ Hz, H-3-glc-1H'), δ 5.19 ppm (1H, d, $J=7.4$ Hz, H-3-glc-1''); Compared with as previous reported[16], metabolite 2 was identified as 3-O- $[\beta$ -D-glucopyranosyl]-20(S)-protopanaxadiol, identical to ginsenoside Rd.

Metabolite 3

In the $^1\text{H-NMR}$ spectrum of metabolite 3, the proton signals for the H-1 of the 3-O-inner-glucopyranosyl moiety and 20-glucopyranosyl moiety appeared respectively, at δ 5.20 ppm (1H, d, $J=7.4$ Hz, H-3-glc-1'), δ 4.94 ppm (1H, d, $J=7.4$ Hz, H-20-glc-1'''); for $^{13}\text{C-NMR}$ (pyridine- d_5 , 100 MHz) data see Table 2. According to the comparison of the $^{13}\text{C-NMR}$ spectrum of metabolite 3 with that of metabolite 1, the signal for the C-2 of the 3-inner-glucopyranoxyl moiety was shifted up field, from 83.2 ppm to 78.4 ppm, and other signals were similar to those of metabolite 2. Additionally, based on the fact that the anomeric carbon signals at δ 106.9 and δ 98.2 ppm showed to be similar to signals for C-1 of the 3-O-inner-glucopyranosyl moiety and 20-O-inner-glucopyranosyl moiety of metabolite 2, it was confirmed that two glucoses connected with the C-3 and C-20 sites of the aglycon moiety. Compared with as previous reported[17], metabolite 3 was identified as 3-O- $[\beta$ -D-glucopyranosyl]-20-O- $[\beta$ -D-glucopyranosyl]-20(S)-protopanaxadiol, identical to ginsenoside F2.

Metabolite 4

In the $^1\text{H-NMR}$ spectrum of metabolite 4, the anomeric-

ic proton signals appeared at δ 3.42 ppm (3H, dd, 11,8, $J=5.2$ Hz), δ 5.18 ppm (1H, H-20-glc-1', $J=8.0$ Hz), δ 5.25 ppm (24H, t, $J=6.4$ Hz). The ^{13}C -NMR analysis result of metabolite is shown in Table 2. The signals for, C-24, C-25, 20-glc-6' and 20-glc-1' of the metabolite appeared δ 126.01 ppm, δ 130.92 ppm, δ 63.11 ppm and δ 98.33 ppm, respectively, which are similar to those for the same carbon in compound K. Compared with as previous reported [18], we indicated that the metabolite is 20-O- $[\beta$ -D-glucopyranosyl]-20(S)-protopanaxadiol, identical to compound K.

DISCUSSION

This study focused on the isolation of LAB strain *Lactobacillus* sp. 6105 from Kimchi, finding β -glucosidase with the ability to convert ginsenoside Rb1. In previous studies we reported that the isolation of β -glucosidase producing bacteria using esculin [4,10] MRS medium. Strain 6105 converted ginsenoside Rb1 to Rg3 by live cell cultures (data not shown), however, it was shown to convert the ginsenoside Rb₁ into compound K using crude enzyme. Previously, Chi and Ji [19] and Chi et al. [20] reported the transformation of major ginsenoside using food microorganisms. But low β -glucosidase activity was noted, when they incubated for transformation ginsenoside with live cells, however, crude enzyme of their bacteria shows higher activity to convert a minor saponin like compound K.

We confirmed the hydrolysis of ginsenoside Rb1. We standardized the optimal conditions of the β -glucosidase activity, and the crude enzyme of strain 6105 had the highest activity at 37°C. According to other studies, the optimal temperature was lower than those reported such as 70°C for *Paecilomyces thermophila* derivative β -glucosidase [21]. An optimal temperature of 50°C was found for *Aspergillus oryzae* derivative β -glucosidase [22], for *Fusarium proliferatum* ECU2042 derivative β -glucosidase [23], and for *Candida peltata* derivative β -glucosidase [24]. The 45°C optimal temperature was found for *Rhizopus japonicus* derivative β -glucosidase [25] and for *Bacillus subtilis* natto derivative β -glucosidase [26]. Additionally, the crude enzyme of strain 6105 has optimum activity at pH 6.0 to 7.5. Comparing with others reported strain optimum pH such as *P. thermophila* [21], *A. oryzae* [22], *F. proliferatum* ECU2042 [23], *R. japonica* [25], *C. peltata* [24], *B. subtilis* natto [26], pH 6.2, 5.0, 5.0 to 6.0, 4.8 to 5.0, 5.0, 6.0, respectively.

In order to find out the maximum β -glucosidase activ-

ity, we added carbon sources such as glucose, cellobiose, lactose, sucrose, hesperidin, and soluble starch on MRS medium. Among them, the addition of lactose increased the β -glucosidase activity about 30 percent over control. On the MRS medium containing hesperidin and soluble starch, there was less activity. These results suggest that strain 6105 can increase β -glucosidase activity on the MRS medium including glucose, cellobiose, lactose, sucrose like *C. peltata* [24].

We found that compound K has a highly efficient function through this study. Compound K has many pharmacological activities including protection of the liver from injury [27], inhibition of the P450-mediated metabolism [28], and inhibition at the α (1G)-type of Ca^{2+} channel [29]. It also triggers apoptosis in activated hepatic stellate cells via caspase-3 activation [30] and potent cytotoxic activity against tumor cell lines [31]. However, minor saponins like ginsenoside F2 and compound K are found in small amounts in ginseng roots. Therefore, it is desirable to produce a greater amount of minor ginsenoside such as compound K. Our experiment demonstrates that strain 6105 is capable of producing the minor ginsenoside F2 and compound K using crude enzymes.

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