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# Bioconversion of Ginsenoside Rb1 to the Pharmaceutical Ginsenoside Compound K using *Aspergillus usamii* KCTC 6954

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β-Glucosidase from *Aspergillus usamii* KCTC 6954 was used to convert ginsenoside Rb1 to compound K, which has a high bio-functional activity. The enzymatic activities during culturing for 15 days were determined using ρ-nitrophenyl-β-glucopyranoside. The growth rate of the strain and the enzymatic activity were maximized after 6 days (IU; 175.93 μM $ml^{-1}$ $min^{-1}$). The activities were maximized at 60°C in pH 6.0. During culturing, Rb1 was converted to Rd after 9 d and then finally converted to compound K at 15 d. In the enzymatic reaction, Rb1 was converted to the ginsenoside Rd within 1 h of reaction time and compound K could be detected after 8 h. As a result, this study demonstrates that Rb1→Rd→F2→compound K is the main metabolic pathway catalyzed by β-glucosidase and that β-glucosidase is a feasible option for the development of specific bioconversion processes to obtain minor ginsenosides such as Rd and compound K.

**Keywords:** *Panax ginseng*, β-glucosidase, *Aspergillus usamii*, ginsenoside, bioconversion

## Introduction

Ginseng has been used as a traditional medicine in Asian countries for a long time. Ginsenoside, i.e., ginseng saponin, is responsible for the biological and pharmacological activities of ginseng species such as *Panax ginseng* Meyer, *Panax quinquefolius* L., *Panax notoginseng,* and *Panax japonicus*. Ginsenosides, glycosides with steroids or triterpenes as the aglycon, account for 2-10% in ginseng root [4].

Ginsenosides can be classified into three different groups according to the chemical structure of the aglycon: protopanaxadiol (PPD), protopanaxatriol (PPT), and oleanane saponin (OA). In addition, PPD, PPT, and OA are subdivided into Rb1, Rd, Re, Rc, and so on, depending on the type, number, and sites of attachment of their sugar moieties [23]. Currently, more than 60 types of ginsenosides have been reported in the roots of *Panax ginseng* Meyer [15]. However, six major ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1) comprise 90% (w/w) of the total ginsenosides in ginseng [12], and the rest is composed of minor ginsenosides. Despite their small numbers, minor ginsenosides including compound K have been known to have various physiological activities such as anticanceric [17, 26], antioxidative [13], antifungal [26], and antiinflammatory [18] effects as well as high bioavailability compared to major ginsenosides [20].

Minor ginsenosides can be produced by hydrolysis of the sugar moieties from the major ginsenosides. Deglycosylation methods such as heating [11], acid treatment [8], and enzymatic conversion [7] have been developed for produc-

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ing minor ginsenosides from major ginsenosides. However, chemical methods produce side reactions such as epimerization, hydration, and hydroxylation. Comparatively, enzymatic conversion is an efficient and environment-friendly method [25]. However, the production of minor ginsenosides by biological techniques has not been practically studied for industrial applications such as pharmaceutical or functional food materials.

*Aspergillus niger* is used in food fermentation industry, especially since their products have been recognized as 'generally recognized as safe' (GRAS) by the FDA. *Aspergillus usamii* belongs to the *A. niger* group. The strain has been used to make Asian traditional fermented food such as *doenjang meju* [9] because these strains have various hydrolyzing enzymes such as amylase and protease. Recently, many researchers have studied on production of extracellular enzymes such as glucanase and glucoamylase [21]. In particular, β-glucosidase from *A. niger* have been already studied recently [3], but that of *A. usamii* has not been studied yet. In study on industrial enzymatic treatments, there is a limit that the application of purified enzymes at a large scale is uneconomic due to its high cost for industrial production of minor ginsenosides.

Therefore, the objective of this study is to investigate the enzymatic conversion of ginsenosides Rb1 to compound K by using enzymes, particularly β-glucosidase, produced by using *Aspergillus usamii* KCTC 6954 as basic information for industrial production.

## Materials and Methods

### Microorganisms

*A. usamii* KCTC 6954 was purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). The fungus was grown on potato dextrose agar at 30°C for 4 days and the stock culture was maintained at 4°C. Broth medium was composed of glucose [10 g/l], peptone [5 g/l], malt extract [3 g/l], and yeast extract [3 g/l]), and the medium was inoculated with 5% (v/v) of 4-day-culture. The culture broth was incubated at 200 rpm at 30°C for 15 days. Glass beads were added into the culture to prevent lumping of the mycelia. During culturing, a broth was sampled at 3-day-intervals for 15 days for detection of β-glucosidase activity. In addition, the cell growth was determined at 600 nm with a spectrophotometer (Optizen 2120 UV plus, Mecasys Co. Ltd., Korea).

### Materials

Ginseng harvested in Young-dong, Korea was purchased from a local market in Seoul, Korea. Stems and leaves of the ginseng were collected for the extraction and then dried in an oven (OF12GW, Jeio-Tech Co., Seoul, Korea) at 60°C for 10 h until a moisture content of 4 to 5% was reached (w/w). The dried stems and leaves were ground to a particle size of less than 10 mm using a high speed mixer (Blender 7012S, Waring, Torrington, CT, USA) and were stored at 4°C until used.

Ginsenoside Rb1, Rd, F2, Rh2, and compound K were purchased from LKT Laboratories, Inc. (St. Paul, MI, USA). ρ-nitrophenyl-β-glucopyranoside (ρNPG), ρ-nitrophenol (ρNP), and β-glucosidase from almond were purchased from Sigma Aldrich (St. Louis, MO, USA). Peptone, yeast extract, and malt extract were purchased from BD Company (Miller, Becton Dickinson and Co., Sparks, MD, USA). Glucose was purchased from Merck (Darmstadt, Germany). All solvents used in chromatography were of HPLC grade, and other chemicals were of analytical reagent grade.

### Assay of β-glucosidase activity

Culture broth sampled was centrifuged at 14,000 × *g* for 10 min at 4°C, and the supernatant was used as crude enzyme. Enzyme activity of culture broth was evaluated by a colorimetric method using ρNPG as the substrate [19]. The reaction mixture, which contained 1 ml of 5 mM ρNPG and 100 μl of crude enzyme solution, was reacted at 50°C for 10 min. The reaction was terminated by adding 0.5 M sodium carbohydrate (1 ml), and the absorbance of the released ρ-nitrophenyl (ρNP) was determined at 400 nm and the amount was calculated by using a standard calibration curve.

### Effects of temperature and pH on enzyme activity

The temperatures were regulated at 30, 40, 50, 60, 70, 80, and 90°C to detect an optimal temperature [28]. The effect of pH on enzyme activity was tested at optimum temperature (60°C) with different buffers (50 mM). The pH value were controlled at the pH range of 3.0 to 9.0 by using citrate buffer (pH 3.0), acetate buffer (pH 4.0 and 5.0), sodium phosphate buffer (pH 6.0, 7.0, and 8.0), and Tris buffer (pH 9.0), respectively. Enzymatic activities on various temperatures and pH's were assayed by the methods mentioned above.

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### Bioconversion of the ginsenoside Rb1

Enzymatic conversion of the ginsenoside Rb1 was performed at 60°C for 48 h. The culture supernatant (100 μl) obtained from centrifugation at 14,000 × *g* for 10 min at 4°C was reacted with 0.1 mM ginsenoside Rb1 (100 μl) on a heating block [2]. The reaction mixture was extracted twice with 400 μl of water-saturated n-butanol. The water-saturated n-butanol fraction was lyophilized to obtain the crude saponin fraction. Crude saponin was dissolved in 50 μl of methanol, and then analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) [10].

### TLC and HPLC analysis for determination of ginsenosides

TLC was performed on Silica gel 60$F_{254}$ plates and the developing solvent was composed of chloroform, methanol, and water (65:35:10 v/v/v). The spots were detected by spraying them with 0.2% ρ-anisaldehyde and heating them under a lamp flame. Ginsenoside Rb1 and converted ginsenosides were compared with the ginsenoside standards (Rb1, Rd, F2, compound K, and Rh2) [6, 10].

HPLC was performed using an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) with a UV detection wavelength of 203 nm. The column was a reversed phase column (Eclipse XDB-C18; 4.6 × 160 mm, particle size; 5 μm, Agilent Techonlogies, USA), and the injection volume was 20 μl. The mobile phase utilized gradient conditions with solvents A ($CH_3CN$:$H_2O$ = 100:0) and B ($CH_3CN$:$H_2O$ = 14:86). The solvent A and B ratios were as follows: 20% A (0 min); 20% A (5 min); 30% A (10 min); 30% A (15 min); 60% A (20 min); 60% A (23 min); and 0% A (25 min), with a 1.2 ml/min flow rate [2, 10].

### Statistical analysis

All experiments were performed in triplicate. The data was analyzed by using SPSS 18 (Chicago, IL, USA). The mean values were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$).

## Results and Discussion

### Production of crude enzyme from *A. usamii* KCTC 6954

*Aspergillus* species are known to be useful source of β-glucosidase production [24], and *A. usamii* KCTC 6954 was the most efficient producer of β-glucosidase among the microorganisms investigated in our previous study (data not shown). Changes in the cell growth and β-glucosidase activity of *A. usamii* KCTC 6954 at 30°C were observed under aerobic conditions (Fig. 1). The growth of *A. usamii* KCTC 6954 sharply increased for 8 days, and then slowed for the subsequent 8 days. β-Glucosidase activity also sharply increased for 6 days (175.93 μM $ml^{-1}$ $min^{-1}$) at a middle of logarithmic phase. In this phase, it presumed that the cells capable of transforming the primary carbon source into biosynthetic precursor and Lu *et al*. presented the similar results of enzyme production in this study [16]. After 9 days of culturing, β-glucosidase activity was a little decreased, not significantly ($p < 0.05$), at this high level for the duration of the experiment.

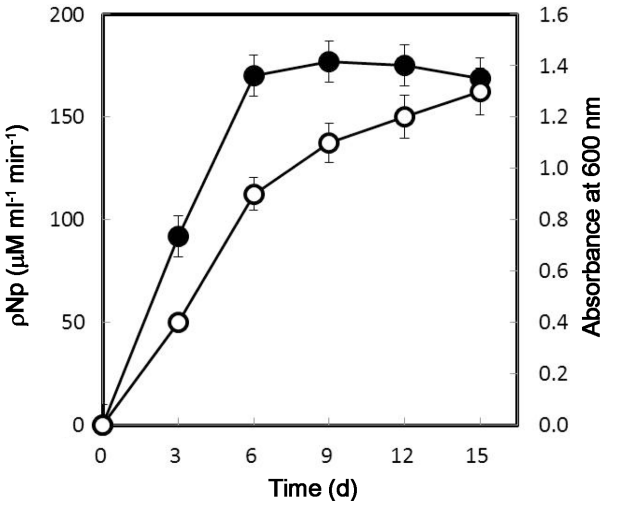


**Fig. 1. Cell growth and β-glucosidase activity of *A. usamii* KCTC 6954.** ●, β-glucosidase activity (μM $ml^{-1}$ $min^{-1}$); ○, growth curve. Enzymatic activities were assayed by the absorbance of the released ρ-nitrophenyl (ρNP) at 400 nm and the cell numbers were detected by the absorbance at 600 nm.

### Effect of pH and temperature on β-glucosidase activity

The effect of pH on β-glucosidase activity was surveyed at the pH range of 3.0 to 9.0. High levels of β-glucosidase activity were detected (151.10 μM $ml^{-1}$ $min^{-1}$) in the pH range. However, enzymatic activity was lost at pH 7.0 (4.11 μM $ml^{-1}$ $min^{-1}$, Fig. 2A). Our results showed that the optimal temperature for β-glucosidase activity was 60°C (179.92 μM $ml^{-1}$ $min^{-1}$). The enzyme activity was maintained at 70°C (171.87 μM $ml^{-1}$ $min^{-1}$), but it sharply decreased to 82.92 μM $ml^{-1}$ $min^{-1}$ at 80°C (Fig. 2B). These

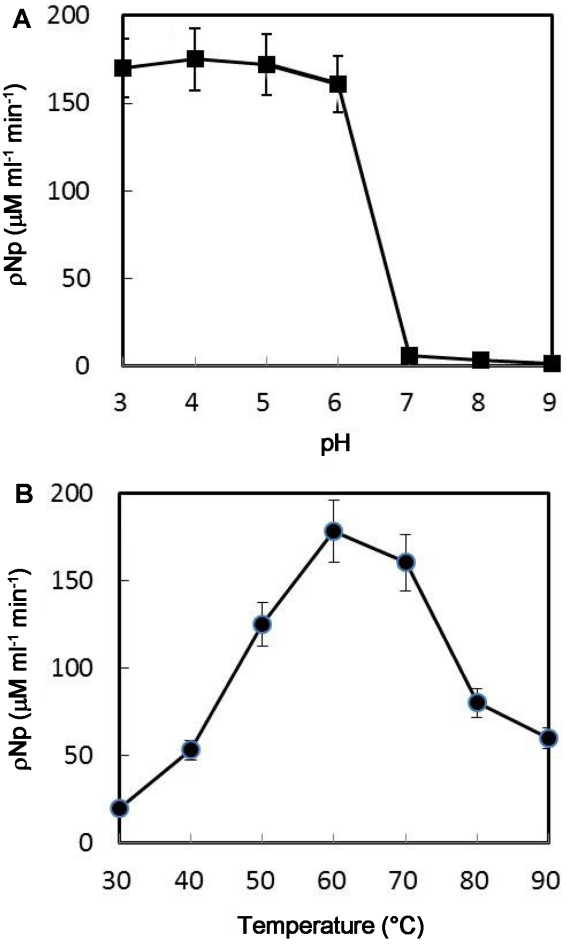


**Fig. 2. Effect of (A) pH and (B) temperature on β-glucosidase activity.** All data were calculated as relative activities. The pH conditions were prepared with citrate buffer (pH 3.0), acetate buffer (pH 4.0-5.0), sodium phosphate buffer (pH 6.0-8.0), and Tris buffer (pH 9.0). β-glucosidase activities were assayed after reaction for 10 min at each temperature.

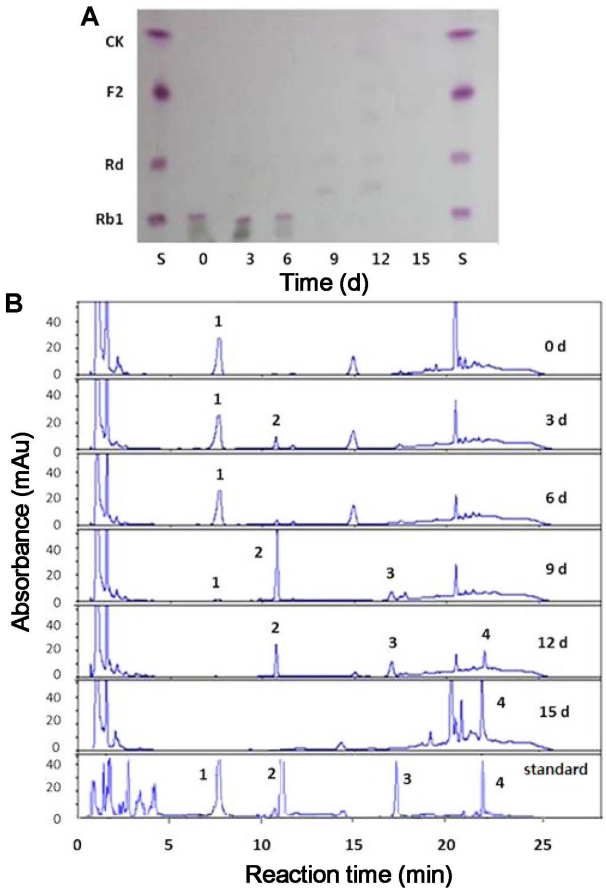


**Fig. 3. Analysis of conversion patterns of the ginsenoside Rb1 during culturing of *A. usamii* KCTC 6954 with (A) TLC and (B) HPLC analysis.** TLC was done by using silica gel $60F_{254}$ as a stationary phase and chloroform:methanol:water (=65:35:10 (v/v/v)) as a mobile phase (S: standard). In HPLC, Eclipse XDB-C18 column was used and a mobile phase was utilized with gradient conditions with $CH_3CN$:$H_2O$ (100:0) and $CH_3CN$:$H_2O$ (14:86). 1, Rb1; 2, Rd; 3, F2; 4, compound K (CK).

results are similar to those of the previous studies, which identified the optimal temperature of *A. niger* and *A. oryzae* to be 60°C, and that of *A. usamii* D5 to be 55°C [1, 10, 22].

### Detection of bioconversion of the ginsenoside Rb1 during culturing of *A. usamii* KCTC 6954

Comparison of the enzymatic conversion of the ginsenoside $Rb_1$ and the growth of *A. usamii* KCTC 6954 is shown in Fig. 3. The enzymatic transformation of the ginsenoside Rb1 was performed at 60°C for 48 h and detected by TLC (Fig. 3A) and HPLC (Fig. 3B). The enzymes of the 6-day-cultures were unable to convert the ginsenoside Rb1. However, the enzymes of the 9-day-culture converted almost all the ginsenoside Rb1 to Rd; their retention times (RT) were 7.667 min and 10.741 min, respectively. All the ginsenoside Rb1 was converted to Rd, F2 (RT, 17.019 min), and compound K (RT, 21.933 min) by the 12-day-culture. The enzymes of the 15-day-culture were completely converted the ginsenoside Rb1 to compound K. It has been known that *Aspergillus* sp. g48p strain from the traditional Chinese preparation Koji can hydrolyze PPD ginsenoside [27], and *A. niger* can convert the ginsenoside $R_f$ to PPT [14]. By these data, it was shown that *A. usamii* also can bio-convert major ginsenosides during culturing.

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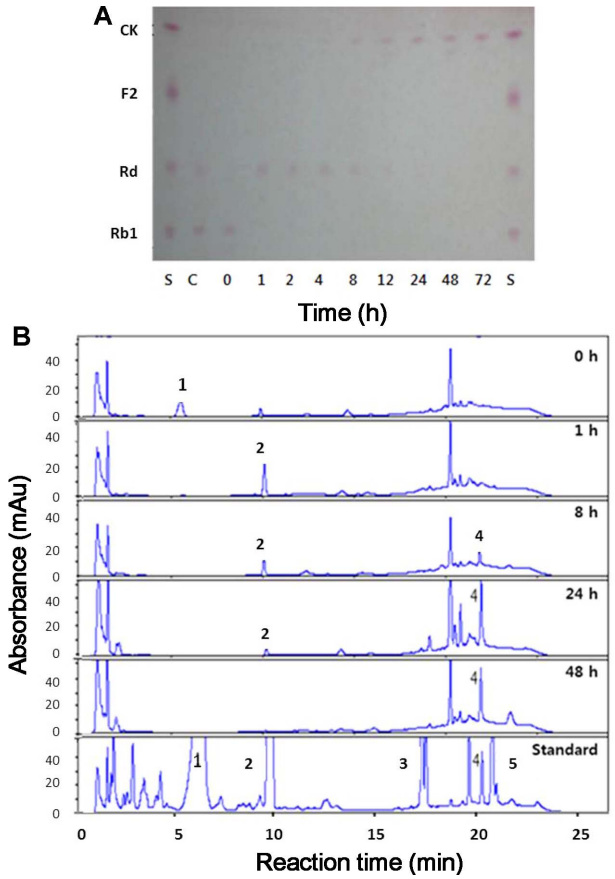


**Fig. 4. Conversion pattern of the ginsenoside Rb1 during enzymatic reaction time. (A) TLC analysis. S: standard; C: Rb1+ medium. (B) HPLC analysis.** TLC was done by using silica gel $60F_{254}$ as a stationary phase and chloroform:methanol:water (=65:35:10 (v/v/v)) as a mobile phase (S: standard). In HPLC, Eclipse XDB-C18 column was used and a mobile phase was utilized with gradient conditions with $CH_3CN$:$H_2O$ (100:0) and $CH_3CN$:$H_2O$ (14:86). 1, Rb1; 2, Rd; 3, F2; 4, compound K (CK); 5, Rh2.

### Reaction time of ginsenoside Rb1 conversion

The reaction time required for the enzymatic conversion of the ginsenoside Rb1 was investigated using the supernatant of the 15-day-culture. The reaction was performed at 60°C for 48 h, and the ginsenoside pattern was analyzed by TLC (Fig. 4A) and HPLC (Fig. 4B). In the absence of a reaction time, the ginsenoside Rb1 (RT, 5.551 min) and a small quantity of Rd (RT, 10.088 min) were detected. However, all the ginsenoside Rb1 was converted to Rd following the 1-h reaction. The compound K (RT, 21.933 min) was observed after a reaction of time of 8 h, and all the ginsenosides were converted to compound K after the 48-h reaction.

In conclusion, the results of HPLC demonstrated that ginsenoside Rb1→Rd→F2→compound K (Fig. 5) is the main metabolic pathway catalyzed by *A. usamii* KCTC 6954 enzymes indicating that this result is similar to the pathway in *Actinosynnema mirum* [5]. Specifically, β-glucosidase from *A. usamii* KCTC 6954 hydrolyzes two different glucosidic linkages of ginsenoside Rb1. That is, the former is to hydrolyze the β-1,6-glucosidic linkage of C-20 in ginsenoside Rb1 to produce ginsenoside Rd and the latter is to hydrolyze the β-1,2-glucosidic linkage of C-3 in ginsenoside Rd to produce ginsenoside F2. Furthermore, F2 can be deglucosylated to compound K by β-glucosidase. Importantly, it demonstrates that β-glucosidase from *A. usamii* KCTC 6954 is a feasible option for the development of specific bioconversion processes to obtain minor ginsenosides such as Rd, F2, and compound K which are more potent bio-functional materials for human health than major ginsenosides such as Rb1 are.

In our further research, β-glucosidase of *A. usamii* related with a bioconversion of Rb1 will be purified and its characterization and optimization for activity will be determined in more detail. These results suggest that *A. usamii* strains can be practically applied to develop new functional materials in a pharmaceutical as well as a functional food industry as basic information.

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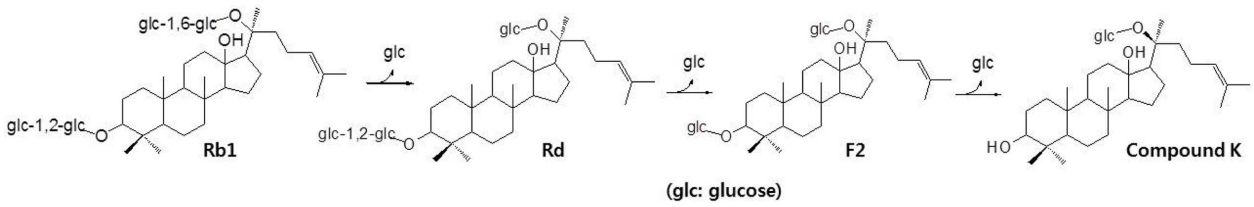


**Fig. 5. Pathway of bioconversion of Rb1 to compound K.**

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## 국문초록

**_Aspergillus usamii_ KCTC 6954에 의한 ginsenoside Rb1로 부터 의약용 소재인 compound K로의 생물학적 전환**

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본 연구는 인삼의 주요성분인 ginsenoside Rb1으로부터 보다 높은 생리기능성을 갖는 것으로 알려져 있는 compound K를 생산하기 위하여 *Aspergillus usamii* KCTC 6954에서 유래된 β-glucosidase를 사용하여 생물전환을 실시하였다. 15일 동안의 배양 중, 효소활성 측정은 ρ-nitrophenyl-β-glucopyranoside를 기질로 하여 분해 생성되는 ρ-nitrophenol (ρNP)을 비색계로 측정함으로써 실시되었다. 그 결과로서, 균주의 성장 속도는 접종 후 6일 후 최대로 나타났으며 이때의 β-glucosidas 활성도는 175.93 μM $ml^{-1}$ $min^{-1}$로 나타났다. 또한 효소 반응의 최적 조건은 pH 6.0 이내에서는 60°C인 것으로 나타났다. 배양 중 ginsenosides 분석 결과, 배양 9일 후에는 Rb1는 Rd로 전환되고 15 days 후에는 compound K로 순차적으로 전환되는 것으로 나타났다. 효소반응에 있어서는 Rb1는 1시간 이내에 ginsenoside Rd로 전환되었고 8시간 이후에 최종산물인 compound K가 측정되었다. 본 연구결과로부터 Rb1으로부터 주요 생물학적 전환 경로는 Rb1→Rd→F2→compound K로 나타났으며 이는 차후 Rd나 compound K와 같이 강한 생리기능성을 갖지만 자연에 미량 존재하는 물질의 대량생산에 응용될 수 있을 것으로 기대된다.