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Full Length Research Paper

Biotransformation of major ginsenosides into compound K by a new *Penicillium dipodomyicola* strain isolated from the soil of wild ginseng

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A new strain, GH9, having ß-glucosidase activity was isolated from the soil of wild ginseng using Esculin-R2A agar. It shows the strongest activities to convert ginsenoside Rb1 to minor ginsenosides compound K. The transformation products were identified by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), and strain GH9 was found to transform major ginsenoside to minor ginsenoside compound K as the key sole product. The optimal biotransformation conditions of GH9 with C-K were obtained as follows: media, yeast broth (YB); transforming temperature, 40°C; pH of the medium, 4 - 6; transforming time, 7 days. At these optimum conditions, the maximum yield was 86.1%. Strain GH9 was identified as a *Penicillium dipodomyicola* species based on the internal transcribed spacers (ITS) ITS1-5.8S-ITS2 rRNA gene sequences constructed phylogenetic trees.

Key words: Biotransformation, *Panax ginseng*, ginsenoside Rb1, ginsenoside compound K, minor ginsenoside.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C. A. Meyer (Araliaceae), is a very popular medicinal plant. It has been used as traditional natural medicine in China, Korea and Japan for thousands of years. Its chemical properties and pharmaceutical functions have been intensively examined in investigations throughout the world. Ginsenosides are regarded as the principal components responsible for numerous pharmacological properties. Until now, more than 40 ginsenosides have been isolated and characterized from ginseng roots, with the major ginsenosides Rb1, Rb2, Rc, Rg1 and Re constituting more than 90% of the total ginsenosides (Park, 2004).

In recent decades, many studies have focused on the pharmacological activities of the minor ginsenosides, as their activities were found to be superior to those of the major ginsenosides. The minor ginsenoside compound K has significant biological actions, such as antigenotoxic activity, anti-allergic effect and the prevention of tumor invasion and metastasis (Hasegawa, 2004; Lee et al., 1999). In addition, Compound K shows potential hepatoprotective and anti-inflammatory activities (Choi et al., 2007; Lee et al., 2005). Various transformation methods, including mild acid hydrolysis, base treatment, enzymatic conversion, and microbial transformation (Chen et al., 2008; Cheng et al., 2006; Chi and Ji, 2005; Han et al., 2007) have been reported. Amongst these, biotransformation has the advantages over other methods because of its conditions, high specificity, low cost, and environmental compatibility.

In this study, we isolated the ß-glucosidase-producing microorganisms from the soil of wild ginseng field using Esculin-R2A agar, and investigated the activity involved in transforming major ginsenosides to minor ginsenosides

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compound K (C-K).

MATERIALS AND METHODS

Standard ginsenosides including 20(S)-Rb1, 20(S)-Rd, 20(S)-Rg1, F2, F1 and C-K were obtained from the Chengdu Mansite Pharmaceutical Co., Ltd., China. R2A agar was purchased from Difco. Silica gel-60 used for thin layer chromatography (TLC) was purchased from Merck KGaA Darmstadt, Germany. All chemicals and solvents were of analytical or high performance liquid chromatography (HPLC) grade.

Screening of microorganisms producing ß-glucosidase

Esculin-R2A agar was used to isolate ß-glucosidase-producing microorganisms. Esculin-R2A agar contains (per 1 L): esculin 1 g and ferric citrate 0.5 g with 15.2 g R2A agar, and is autoclaved at 121°C for 15 min. The microorganisms producing ß-glucosidase and those hydrolyzing esculin appeared as colonies surrounded by a reddish-brown to dark brown zone. The microorganisms were isolated from the soil of wild ginseng field (Changbai Mountain, China) by direct plating onto Esculin-R2A agar. Single colonies from these plates were purified by transferring onto new plates. Pure cultures were checked for shape, color and size of colonies.

Biotransformation of ginsenosides

The biotransformation procedure was carried out in 100 ml Erlenmeyer flasks containing 40 ml biotransformation medium and 0.4 mgL^{-1} Rb1 or Rg1 as the carbon source in a shaking incubator (150 rpm) at 30°C. The biotransformation medium consisted of 0.5 g ammonium chloride (NH_4Cl), 1.0 g dipotassium phosphate (K_2HPO_4), 0.5 g potassium dihydrogen phosphate (KH_2PO_4), 0.25 g magnesium sulfate ($MgSO_4$) and 1.0 g yeast extract per liter. All medium was sterilized at 121°C for 15 min and the initial pH was 7.0. The reaction mixture was extracted with *n*-butanol saturated with water (H_2O) and analyzed by TLC and HPLC.

Analytical methods

TLC analysis was carried out using a Silica gel 60 plates and a solvent system of $CHCl_3$-CH_3OH-H_2O (10:5:1 v/v/v) as the developing solvent. The spots on the TLC plates were detected by spraying 10% (v/v) H_2SO_4 (in ethanol) followed by heating at 110°C for 10 min. The reaction mixture was extracted with *n*-butanol saturated with H_2O, evaporated *in vacuo*, and the residue was dissolved in methanol (CH_3OH) and applied to the HPLC analysis. HPLC used a C_{18} column (250×4.6 mm, ID 5 µM) with H_2O (solvent A) and acetonitrile (solvent B) at A/B ratios of 75/25, 68/32, 45/55, 40/60, 0/100, 0/100, 75/25 and 75/25, with run times of 0, 10, 15, 20, 25, 27, 40 and 50 min, respectively at a flow rate of 1 ml min^{-1}. Detection wavelength was 203 nm.

The bioconversion rates of ginsenoside C-K and F1 were calculated as follows:

$$\text{Bioconversion rate of ginsenoside C-K (\%)} = \frac{\text{Weight of C-K / MW of C-K}}{\text{Weight of Rb1 / MW of Rb1}}$$

$$\text{Bioconversion rate of ginsenoside F1 (\%)} = \frac{\text{Weight of F1 / MW of F1}}{\text{Weight of Rg1 / MW of Rg1}}$$

Where, MW is the molecular weight; MW of Rb1 is 1108, MW of C-K is 622, MW of Rg1 is 801 and MW of F1 is 639. All the experiments were conducted in triplicate and the results were expressed as mean values ± SD.

Molecular methods

The ITS rDNA gene sequences of the strain GH9 was sequenced by the Shanghai Majorbio Bio-Pharm Technology Co. Ltd., China. The ITS rDNA gene sequences of the related taxa were obtained from GenBank. The phylogenetic tree was constructed using the neighbor-joining method through the MEGA 4.1 program. A bootstrap analysis with 1000 replicates was also conducted to obtain confidence levels for the branches. The closest type strains were included in the phylogenetic trees.

Optimization of fermentation conditions

Fermentation conditions for strain GH9 (pH value, temperature, and type of liquid culture media) were optimized to enhance the efficiency of biological transformation.

Determination of optimal liquid medium

The solution of strain GH9 was inoculated into nutrient broth (NB), R2A, de Man, Rogosa and Sharpe (MRS), yeast malt (YM), Luria-Bertani (LB), H_2O and yeast broth (YB) (prepared in the laboratory) media and cultivated in a 30°C shaking incubator. After seven days of incubation, they were extracted using water saturated butyl alcohol and stored. The liquid supernatants were later subjected to TLC analyses to determine the optimum medium.

Determination of the optimal starting pH value

After cultivation of the basic medium for 7 days at 3°C and 160 rpm in a 100-ml volume containing 10% inoculated bacteria, the effects of variety of pH values (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0) on the bacterial conversion rate were measured to determine the optimal pH value.

Determination of the optimal fermentation temperature

After cultivation of the basic medium for 7 days at 30°C and 160 rpm in a 1000-ml volume containing 10% inoculated bacteria, the effects of a range of temperatures (20, 30, 40, 50, and 60°C) on the bacterial conversion rate were measured, and the optimal fermentation temperature was determined.

Microbial conversion of ginseng monomer saponins

The solution of sterilized Rb1, Rb2, Rc, Rd, and Rg1 4 mg/ml were

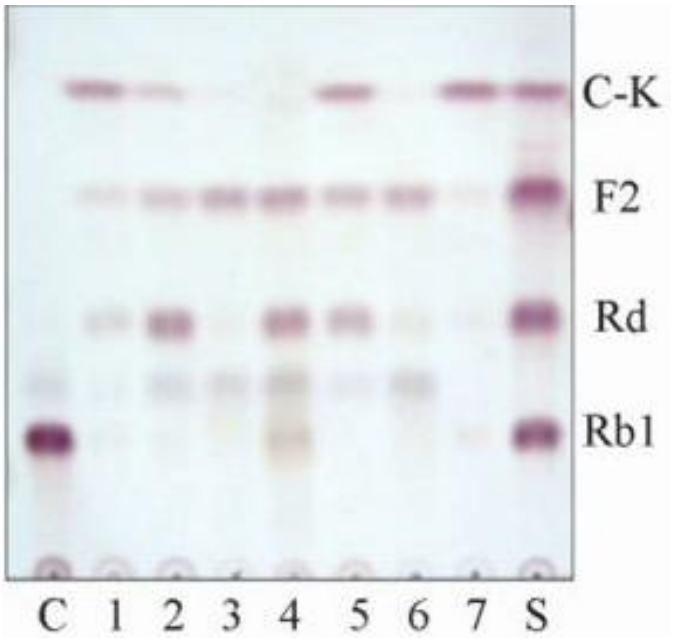

Figure 1. TLC analysis of conversion products of ginsenoside Rb1 by GH9 in different media. Lane 1, H_2O; lane 2, NB; lane 3, YM; lane 4, MRS; lane 5, R2A; lane 6, LB; lane 7, YB; lane C, control; lane S, saponin standards.

added to culture liquids that supported vigorous bacterial growth. During incubation in a 160 rpm shaking incubator under constant temperature for 7 days, samples were removed at 24-h intervals for extraction with water-saturated butyl alcohol, and the liquid supernatants were then used for TLC and HPLC analyses.

Microbial conversion of total ginsenoside leaching agent

Extraction of total ginsenoside

The powder of ginseng roots (1 g) were weighed and added to 50 ml 70% ethanol and water solution extraction for three times. After filtration, the filtrates were combined and then pooled for concentration under reduced pressure to dryness. It was then dissolved in liquid medium with total ginsenoside leaching agent.

Microbial conversion

After sterilization, leaching agent total ginsenoside was added to the nutrient solutions for 7-day fermentation in a 160 rpm shaking incubator at constant temperature, after which they were extracted with water-saturated butyl alcohol. The liquid supernatant was then used for HPLC analyses.

RESULTS

Initial screening of ß-glucosidase-producing microorganisms

Twenty-two microorganisms were initially screened using the Esculin-R2A agar, for the production of ß-glucosidase. The microorganisms producing ß-glucosidase and hydrolyzing esculin appeared as colonies surrounded by a reddish-brown to dark brown zone, which showed ß-glucosidase activity. The black colonies were picked and transferred to the fresh Esculin-R2A agar. The morphological characteristics of pure cultures were checked for size, shape and color of colonies.

Determination of an optimal liquid medium

In this experiment, liquid medium was the main nutrition matrix for GH9, thus effecting its growth and conversion. In YB medium, the substrate and intermediate products of GH9 were all hydrolyzed, resulting in the highest levels of C-K and the greatest level of conversion as shown in Figure 1.

Determination of an optimal initial pH value

The pH values of the home-made medium (YB) were adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. Under these conditions, conversion experiments were conducted with ginsenoside Rb1 as the substrate. Results indicated that GH9 produced the most C-K and achieved the best conversion under pH values from 4.0 to 6.0, a partially acidic environment, as shown in Figure 2.

Determination of an optimal temperature for fermentation

With the pH value fixed at 4.0 - 6.0 for YB medium with ginsenoside Rb1 as the substrate, microbial conversion experiments were conducted at temperatures of 20, 30, 40, 50 and 60°C, and 40°C was obtained as the best fermentation temperature for GH9 as indicated in Figure 3. The experimental results showed that the optimal liquid medium for strain GH9 should contain 1.0 g yeast extract powder, 0.5 NH_4Cl g, 0.25 g $MgSO_4$, 1.0 g K_2HPO_4, 0.5 g KH_2PO_4 and 1 L distilled water, with an initial pH value between 4.0 and 6.0 and an incubation temperature of 40°C.

Biological conversion of ginseng monomer saponins by GH9

After sterilization, 4 mg/ml ginseng monomer saponin solution was added to liquid media. During the 7 days fermentation in a 160 rpm shaking incubator at 40°C, samples were removed every 24 h for extraction with water-saturated butyl alcohol. The liquid supernatants were then used for TLC and HPLC analyses.

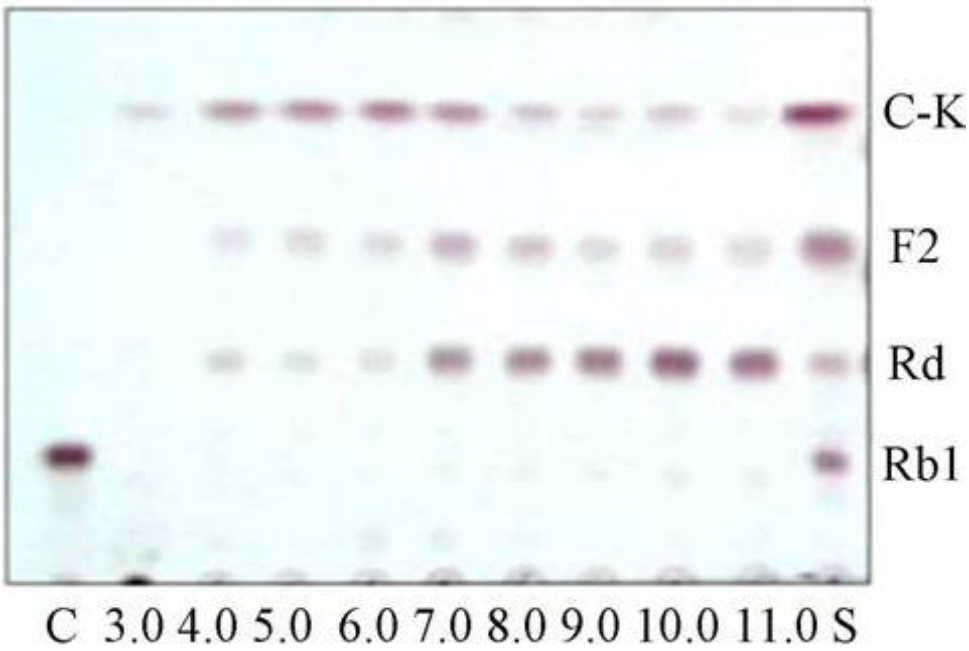

Figure 2. TLC results of the conversion products of ginsenoside Rb1 by GH9 at pH values from 3.0 to 11.0. Lane C, Control; lane S, saponin standard.

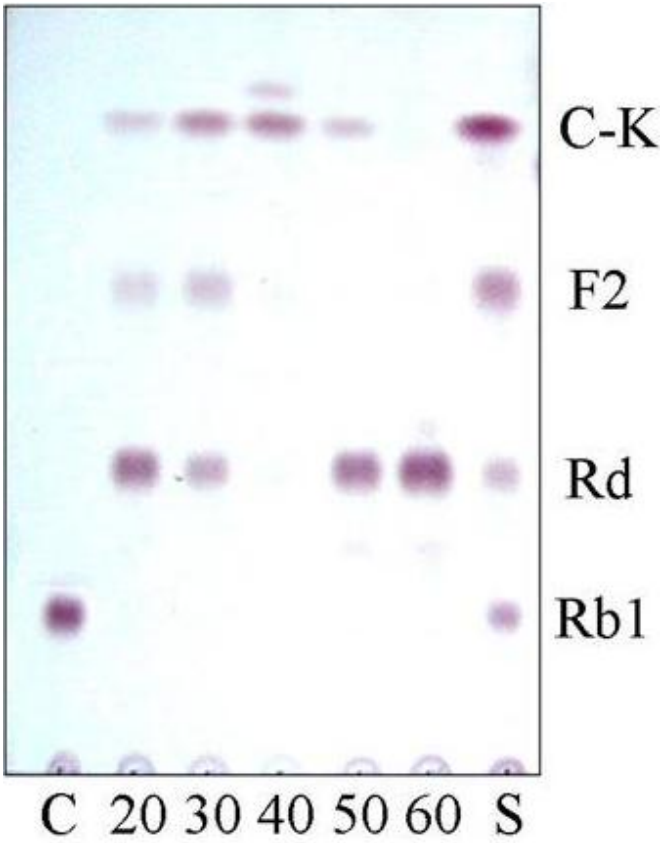

Figure 3. TLC analysis of the conversion products of ginsenoside Rb1 by GH9 under different temperatures. Lane C, Control; lane S, saponin standard.

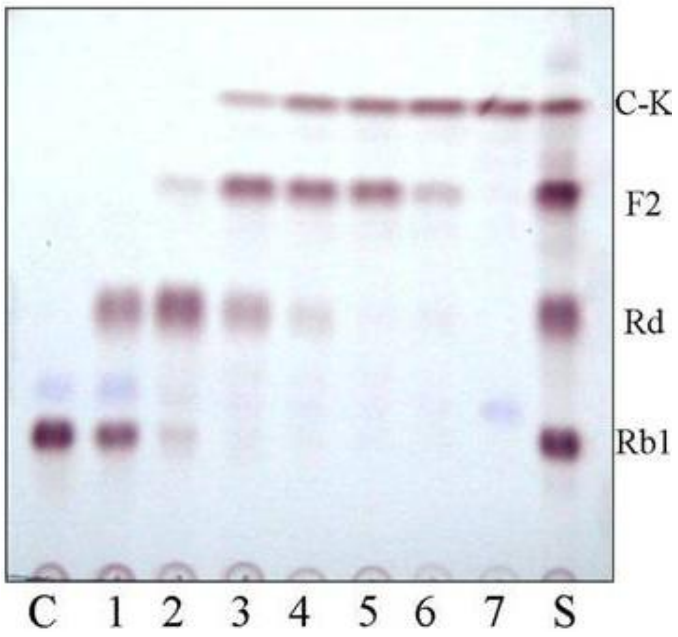

Figure 4. TLC analysis of GH9 conversion of ginsenoside Rb1. Lane C, Control; lane S, saponin standards.

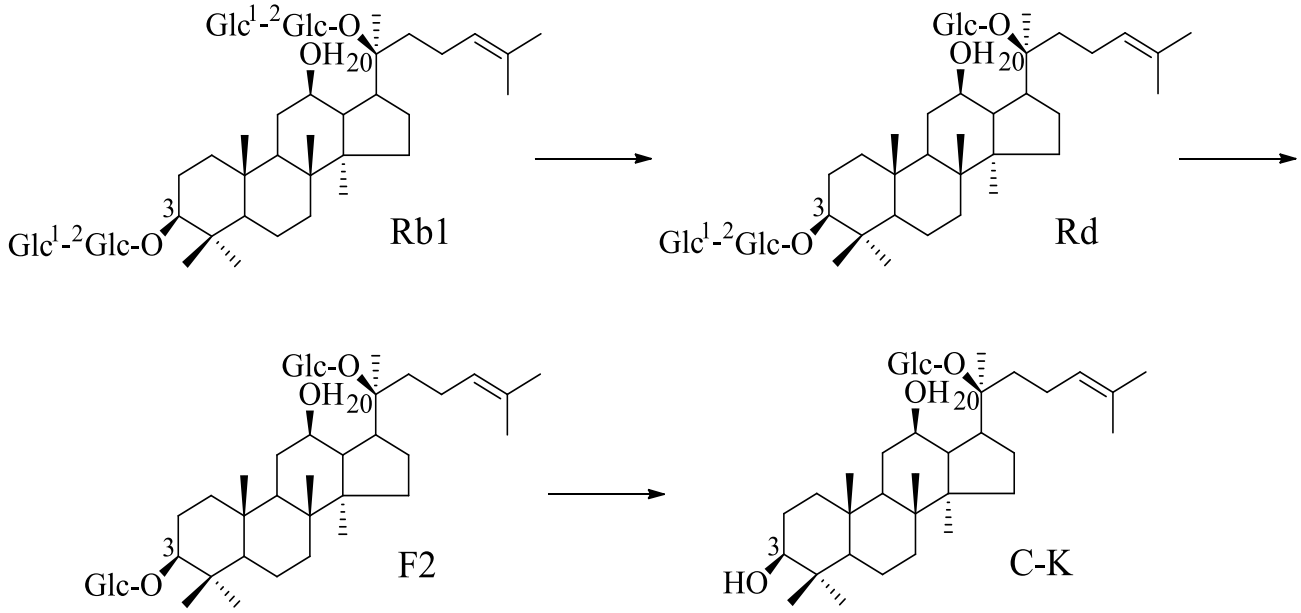

Figure 5. The mechanism by which GH9 converts Rb1 into C-K.

Biological conversion of ginsenoside Rb1 by GH9

We conducted thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses to measure the biological conversion of ginsenoside Rb1 by GH9. TLC analysis indicated that ginsenoside Rb1 had been partially converted into Rd by fermentation at day one, and after two days of fermentation, the conversion of Rb1 to Rd was nearly complete and a small quantity of F2 was also detected as shown in Figure 4. Then, after 3 days of fermentation, C-K was observed. Moreover, its concentration increased gradually over time. The mechanism of GH9 conversion of ginsenoside Rb1 proceeded as follows: Rb1→Rd→F2→C-K (Figure 5). By

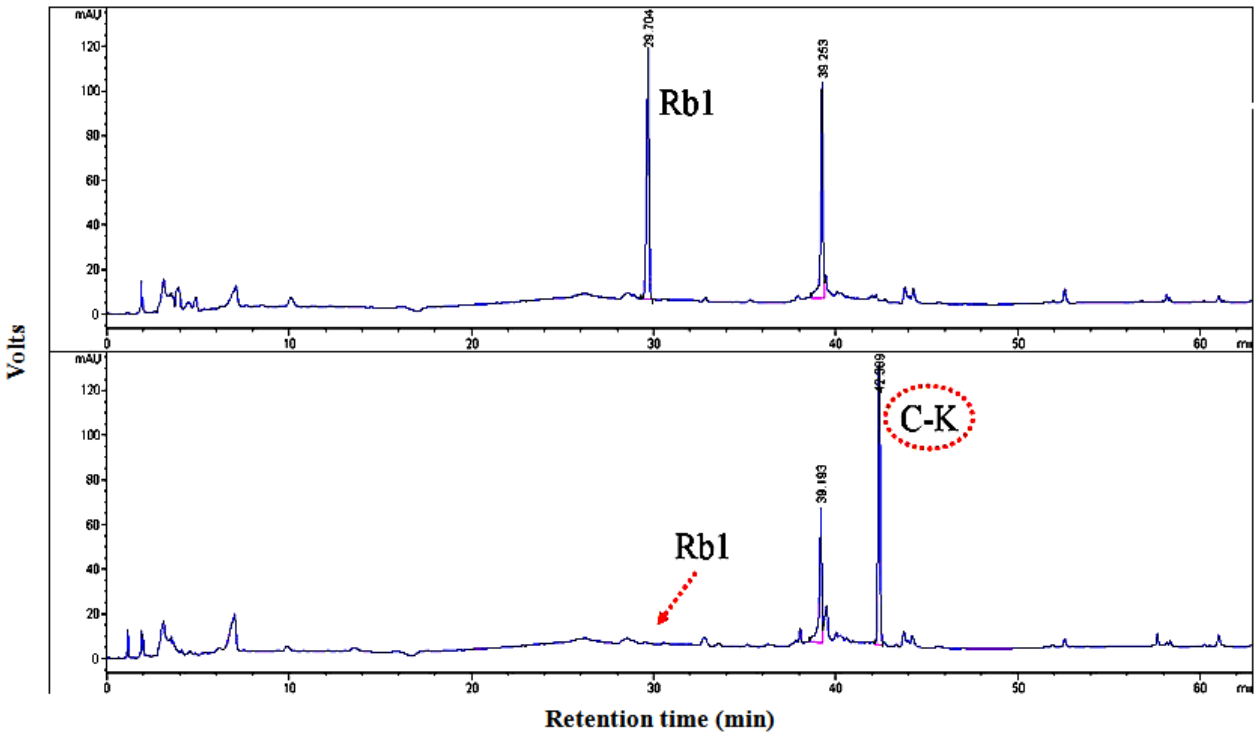

Figure 6. HPLC analysis of GH9 conversion of ginsenoside Rb1.

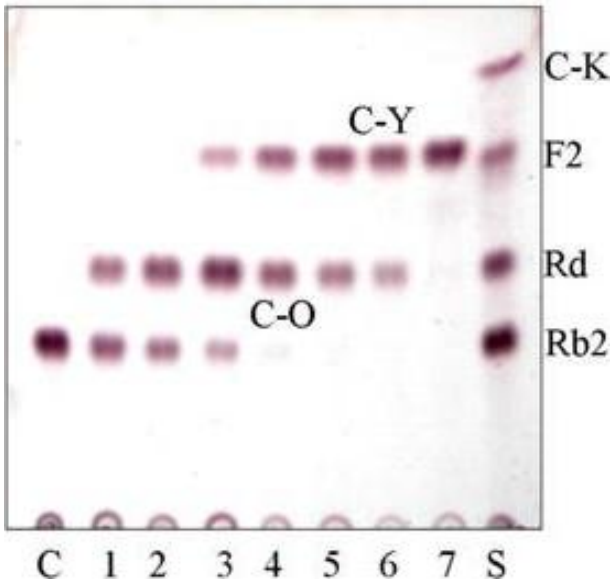

Figure 7. TLC analysis of GH9 conversion of ginsenoside Rb2. Lane C, Control; lane S, saponin standard.

the seventh day, the HPLC Rb1 peak had completely disappeared and a C-K peak was present (Figure 6). The conversion rate of Rb1 to C-K by GH9 was 86.1%, and it was calculated as follows:

C-K conversion rate (%) = Converted quantity of Rb1 / Total Rb1 before conversion.

GH9 conversion of ginsenoside Rb2

Production of compound O (C-O) began at day one of fermentation, and compound Y (C-Y) production appeared at day three of fermentation (Figure 7). Over time, C-Y increased in concentration. The mechanism by which GH9 converts Rb2 is as follows: Rb2→C-O→C-Y

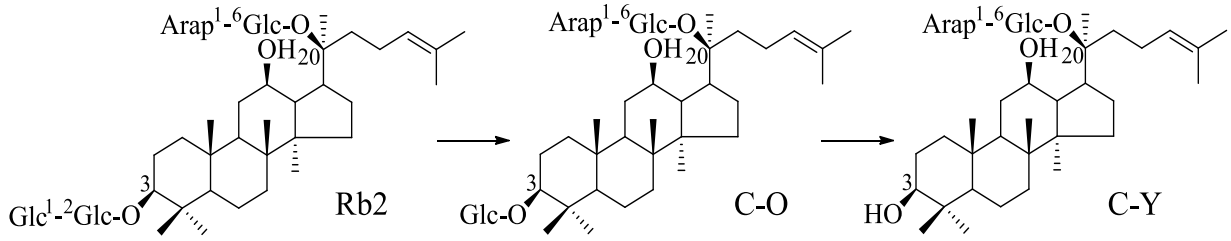

Figure 8. The mechanism by which GH9 converts Rb2 into C-Y.

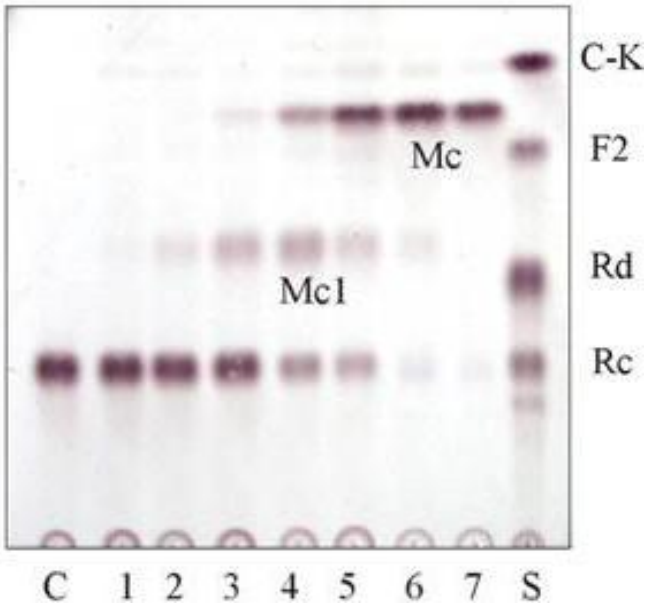

Figure 9. TLC analysis of GH9 conversion of ginsenoside Rc. Lane C, control; lane S: standard.

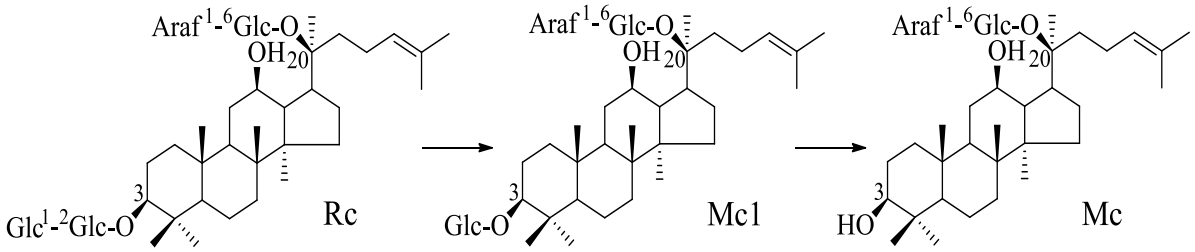

Figure 10. The mechanism by which GH9 converts Rc into Mc.

(Figure 8).

GH9 conversion of ginsenoside Rc

The fermentation began yielding Mc1 on fermentation at day one, while Mc was found after three days as shown in Figure 9. Subsequently, during fermentation Mc1 levels were low, while Mc levels increased. The mechanism by which GH9 converts Rc is as follows: Rc→Mc1→Mc shows (Figure 10).

GH9 conversion of ginsenoside Rd

As shown in Figure 11, ginsenoside F2 was produced on

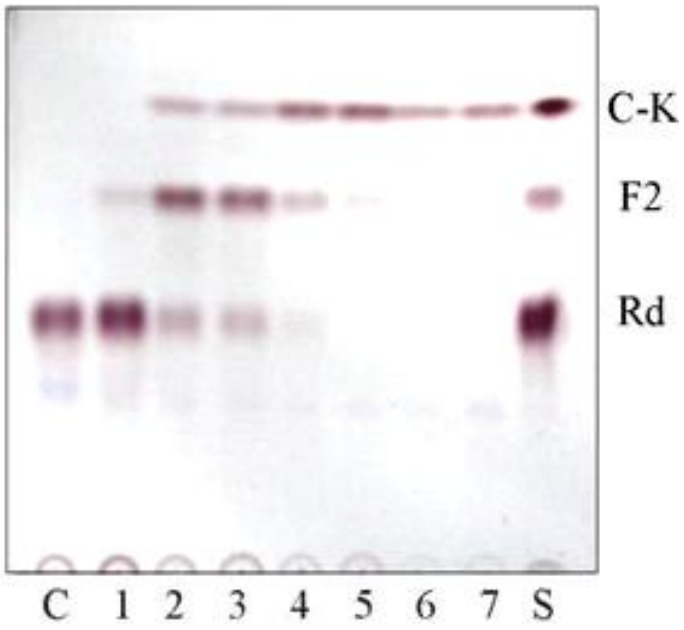

Figure 11. TLC analysis of GH9 conversion of ginsenoside Rd. Lane C: control; lane S: saponin standards.

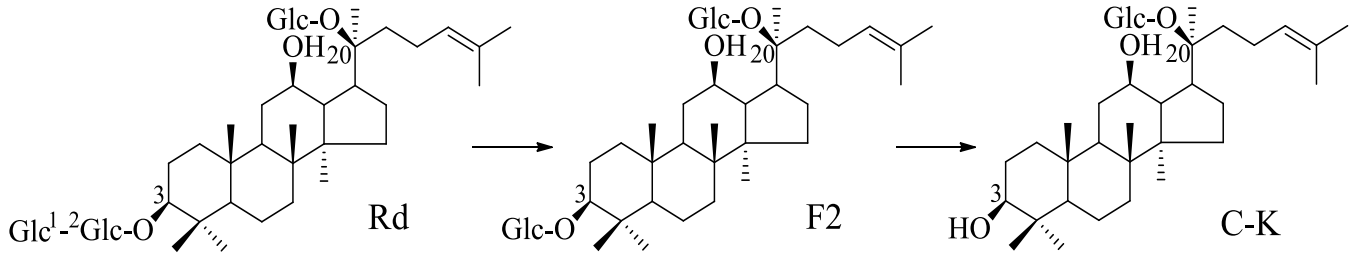

Figure 12. The mechanism by which GH9 converts Rd into C-K.

day one and ginsenoside C-K began to accumulate on day three. Over time, the concentration of Rd decreased and that of ginsenoside C-K increased. The mechanism by which GH9 converts Rd is as follows: Rd→F2→C-K (Figure 12). By the seventh day, the HPLC peak for Rd had totally disappeared and a significant ginsenoside compound K peak was found as shown in Figure 13. The conversion rate of Rd into compound K by GH9 was 52.6%, as calculated using the following formula: C-K conversion rate (%) = Converted quantity of Rd / The total Rd before conversion.

Microbial conversion of total ginsenoside leaching agent

The HPLC analysis of the fermented solution after 7 days of the microbial conversion of total ginsenoside leaching agent is shown in Figure 14; Rb1 had been converted almost completely, the level of Rc decreased, and peaks corresponding to Rd and C-K were present.

Morphologic features

The colony morphology is shown in Figure 15A; on Czapek's medium, the 7-day colony had a diameter of 35 mm, a grey-green color with white margins, and a powdery texture. The cell morphology is shown in Figure 15B; mycelia had asymmetric, broom-shaped transeptae, the conidia were round, and the cell walls were smooth.

Phylogenetic study

Internal transcribed spacers ITS1-5.8S-ITS2 rRNA gene sequences of the strain GH9 were aligned with those of the type strains found to have the closest taxonomic relationships. The phylogenetic tree is shown in Figure 16; the ITS rRNA gene sequences of the related taxa were

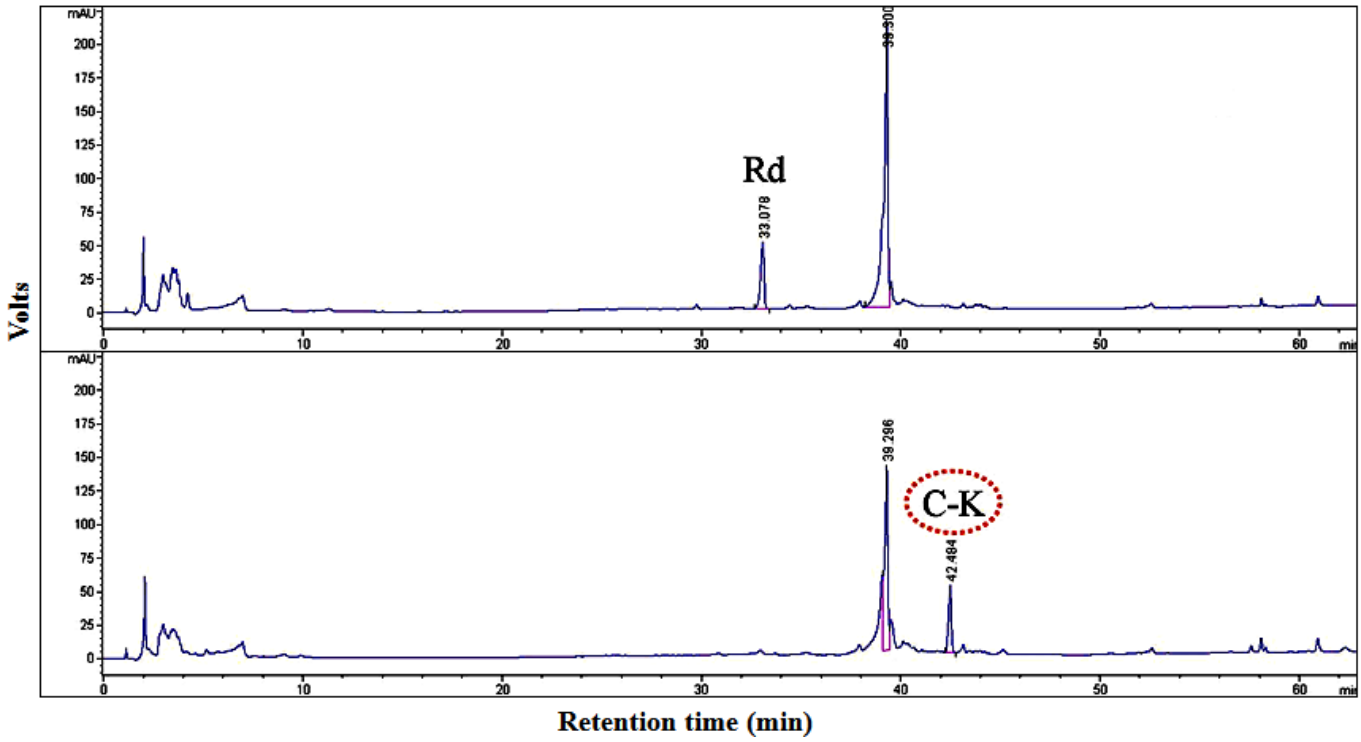

Figure 13. HPLC analysis of GH9 conversion of ginsenoside Rd.

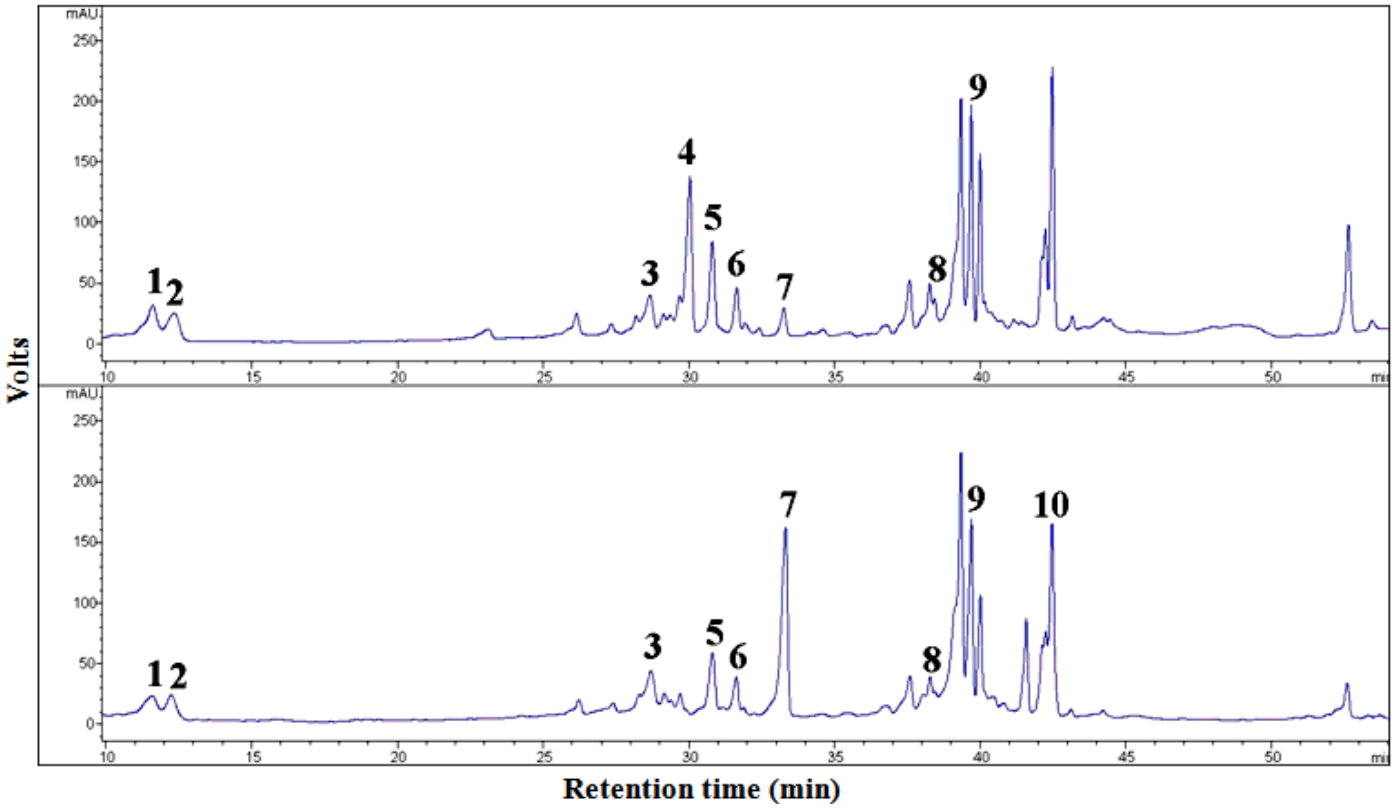

Figure 14. HPLC analysis results of GH9 conversion of total ginsenoside leaching agent. 1, Rg1; 2, Re; 3, Rh1; 4, Rb1; 5, Rc; 6, Rb2; 7, Rd; 8, F2; 9, Rg3; 10, C-K.

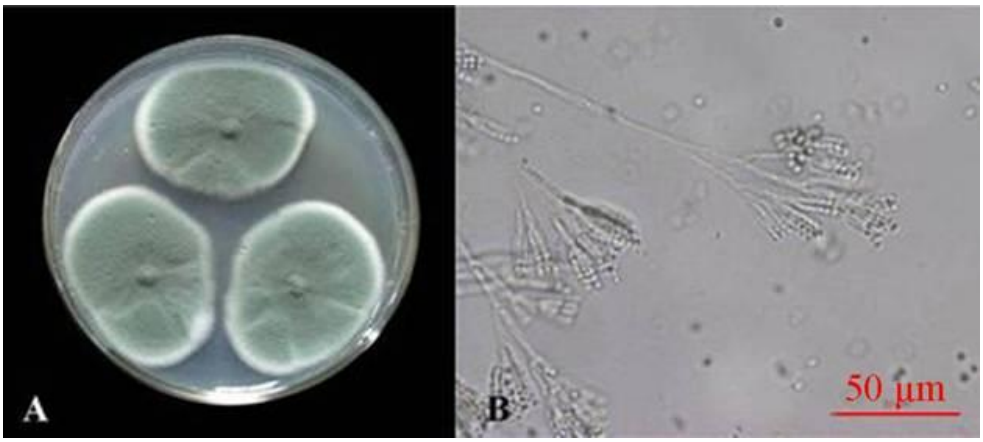

Figure 15. Morphological features of strain GH9.

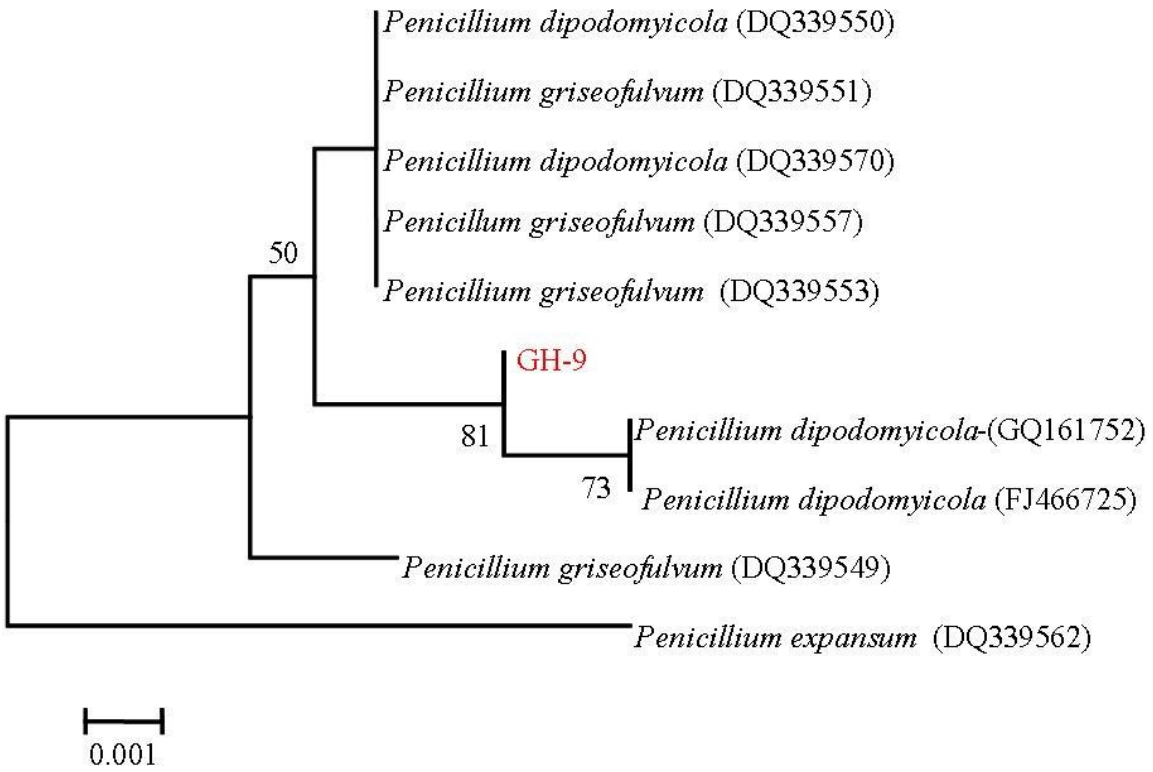

Figure 16. A phylogenetic tree based on the ITS rRNA gene sequences, showing the phylogenetic relationships of the strain GH9.

obtained from Genbank. Strain GH9 was calculated to belong to the *Penicillium*, with high similarity to *Penicillium dipodomyicola.* Therefore, the strain GH9 was classified as a *P. dipodomyicola*. As determined by taxonomic evaluation, the strain showing less than 1% difference in its internal transcribed spacers (ITS) rRNA gene sequence with the corresponding type stains were assumed to belong to the same species as the type stains.

DISCUSSION

Ginsenoside C-K is a promising natural product that could be used for the treatment of numerous human diseases. It is the metabolites of ginsenosides Rb1, Rb2, Rc and Rd by intestinal microflora of humans, rats, microorganisms, and enzymes. Unfortunately, the methods currently available for the commercial production of ginsenoside C-K is difficult, thus limiting the

availability and development of these compounds. There are reports on microbial sources able to convert the major ginsenoside Rb1 or ginsenoside Rg1 to minor ginsenosides usually through the pathway of Rb1→Rd→F2→C-K by microorganisms. Other pathways for C-K production such as Rb1→gypenoside X VII→gypenoside LXXV→C-K have been reported (Cheng et al., 2007; Lee et al., 2006). However, most of them lack specificity.

In this study, we have successfully isolated, for the first time, *P. dipodomyicola* species that showed potent transforming capacity of C-K. Furthermore, the conditions of transforming C-K by *P. dipodomyicola* species were optimized, which permitted us to obtain high levels of such bioactive compounds in the transformed products. These results may have practical importance in developing antitumor compounds. It should be mentioned that the bioconversion rate of *P*. *dipodomyicola* species depends on its media, PH and temperature. Compound K is the metabolites of protopanaxadiol ginsenosides Rb1, Rb2, Rc, and Rd, which are the main components of total ginsenoside. This is the first report on the optimization of culture conditions for the production of ginsenoside C-K by self-made media YB. Moreover, the degree of conversion was significantly higher than previous reports (Noh et al., 2009).

When biotransformation was performed using ginsenoside Rb1, Rb2, Rc and Rd to investigate transformation pathways into C-K, we found that the ginsenosides were converted into bioactive compound via three different transformation pathways, Rb1 or Rd→Rd→F2→C-K, Rb2→C-O→C-Y and Rc→Mc1→Mc. Ginsenosides Rb1, Rb2, Rd, F2, and C-K were identified by HPLC using standards, whereas compound C-O, C-Y, Mc1, and Mc are new compounds needed to be confirmed by liquid chromatography - mass spectrometry (LC-MS) based on their molecular weight. In the first pathway, hydrolysis of the glucose and arabinose units at C20 in Rb1 led to the formation of Rd, which was then hydrolyzed by the glucosyl-(1→2)-glucose unit at C3. In the second and third pathways, Rb2 and Rc were converted into C-Y and Mc, respectively. The strain GH9 was found to display high selectivity in cleaving the glucosidic linkage at the C-3 position of ginsenoside Rb1 and Rc without attacking the glucosidic linkage at the C-20 position. It has been reported that various microorganisms use the transformation pathway Rb1 or Rb2→Rd→F2→C-K, and ginsenoside Rc→ Compound Mc→C-K, due to its broad substrate specificity.

In conclusion, this is the first report of the high bioconversion rate of bioactive C-K by a new selected strain *P. dipodomyicola* species. In the course of optimization of culture conditions for the production of ginsenoside C-K by self-made media YB, the pH and temperature for maximum production were 4 - 6 and 90°C, respectively with transforming efficiency of 86.1%. Thus, it appears that *P. dipodomyicola* species may be a useful tool in preparing the bioactive products C-K.

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