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Microbial Conversion of Protopanaxadiol-Type Ginsenosides by the Edible and Medicinal Mushroom *Schizophyllum commune*: A Green Biotransformation Strategy

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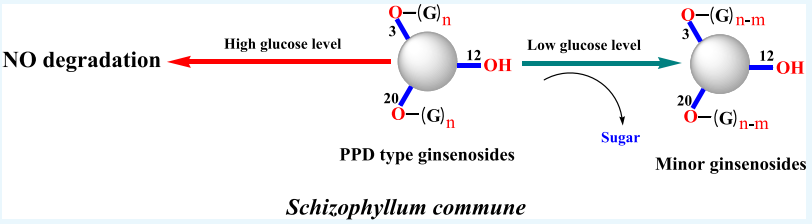
Supporting Information



ABSTRACT: Previous studies have shown that many kinds of microorganisms, including bacteria, yeasts, and filamentous fungi, can convert parent ginsenosides into minor ginsenosides. However, most microorganisms used for ginsenoside transformations may not be safe for food consumption and drug development. In this study, 24 edible and medicinal mushrooms were screened by high-performance liquid chromatography analyses for their ability to microbiologically transform protopanaxadiol (PPD)-type ginsenosides. We observed that the degradation of ginsenosides by *Schizophyllum commune* was inhibited by high concentrations of sugar in the culture medium. However, the inhibition was avoided by maintaining sugar concentration below 15 g L^{-1}. *S. commune* showed a strong ability to convert PPD-type ginsenosides (Rb_1, Rc, Rb_2, and Rd) into minor ginsenosides (F_2, C−O, C−Y, C−Mc_1, C−Mc, and C−K). The production and bioconversion rates of minor ginsenosides were significantly higher than those previously reported by food microorganisms. The fermentation process is efficient, nontoxic, eco-friendly, and economical, and the required biotransformation systems are readily available. This is the first report about the biotransformation of major ginsenosides into minor ginsenosides through fermentation by edible and medicinal mushrooms. Our results provide a green biodegradation strategy in transformation of ginsenosides using edible and medicinal mushrooms.

INTRODUCTION

Ginsenosides are the major active constituents in Asian ginseng, American ginseng, and Notoginseng and have been demonstrated to have a wide range of pharmacological properties, such as anti-inflammatory, antidiabetic, antifatigue, antioxidant, antiobesity, and antitumor activities.[1−4] They have been widely used in functional food, traditional medicine, and cosmetics industries with high economic value. Currently, more than 100 ginsenosides have been identified, isolated, and characterized.[4,5] According to the sugar moieties at the C-3, -6, and -20 position of sapogenins, major ginsenosides can be classified into protopanaxadiol (PPD)-type ginsenosides (e.g., Rb_1, Rc, Rb_2, and Rd) and propanaxatriol-type ginsenosides (e.g., Rg_1 and Re). They make up about 90% of the total ginsenosides in ginseng species.[5] Minor ginsenosides, present at low concentrations in ginseng, include Rg_3, Rh_1, Rh_2, F_2, compound K, compound Mc, and compound Y. These compounds have attracted great interest because they are readily absorbed into the bloodstream, and these minor ginsenosides have better pharmacological activities than the major ginsenosides.[6−9]

Microbial transformation is considered to be the most promising method for the preparation of minor ginsenosides. Previous studies have shown that many kinds of microorganisms can convert major ginsenosides into minor ginsenosides, including bacteria, yeasts, and filamentous fungi.[10,11] However, many microorganisms can produce toxic compounds such as biogenic amines, botulinum neurotoxins, aflatoxins, zearalenone, fumonisins, and ochratoxins. These toxins are of great concern due to their acute and long-term toxic, carcinogenic, nephrotoxic, mutagenic, teratogenic, hepatotoxic, and immunosuppressive effects.[12,13] Therefore, most micro-

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Table 1. Chromatographic Properties of Metabolites of Ginsenosides during Degradation by *Schizophyllum commune*[a]

peak no.	identification	retention time	$[M - H]^-$ (m/z)	MS/MS fragment ion (m/z)
1	Rb_1	36.807	1107	945$[M - H - Glc]^-$, 783$[M - H - 2Glc]^-$, 621$[M - H - 3Glc]^-$, 459$[M - H - 4Glc]^-$
2	Rd	41.241	945	783$[M - H - Glc]^-$, 621$[M - H - 2Glc]^-$, 459$[M - H - 3Glc]^-$
3	F_2	49.716	783	621$[M - H - Glc]^-$, 459$[M - H - 2Glc]^-$
4	compound K	62.569	621	459$[M - H - Glc]$
5	Rc	37.761	1077	945$[M - H - Araf]^-$, 915$[M - H - Glc]^-$, 783$[M - H - Araf - Glc]^-$, 621$[M - H - Araf - 2Glc]^-$, 459$[M - H - Araf - 3Glc]^-$
6	compound Mc_1	44.665	915	783$[M - H - Araf]^-$, 621$[M - H - Araf - Glc]^-$, 459$[M - H - Araf - 2Glc]^-$
7	compound Mc	55.890	753	621$[M - H - Araf]^-$, 459$[M - H - Araf - Glc]^-$
8	Rb_2	38.771	1077	945$[M - H - Arap]^-$, 915$[M - H - Glc]^-$, 783$[M - H - Arap - Glc]^-$, 621$[M - H - Arap - 2Glc]^-$, 459$[M - H - Arap - 3Glc]^-$
9	compound O	45.675	915	783$[M - H - Arap]^-$, 621$[M - H - Arap - Glc]^-$, 459$[M - H - Arap - 2Glc]^-$
10	compound Y	56.395	753	621$[M - H - Arap]^-$, 459$[M - H - Arap - Glc]^-$

[a]Glc: β-D-glucose, Arap: α-L-arabinose (pyranose), and Araf: α-L-arabinose (furanose).

organisms used for ginsenoside transformations are not safe for food consumption and drug development.

The human intestinal bacteria can transform ginsenosides into more active forms and can be used safely in a variety of foods.[14−17] However, human intestinal bacteria require an expensive medium and exhibit low yield and poor productivity. In addition, the fact that many filamentous fungi have been given generally regarded as safe (GRAS) status in many industrial applications could be questioned by mycotoxin production. For example, *Aspergillus niger* is one of the most important industrial filamentous fungal species used in biotechnology, where it is studied extensively for its ability to transform natural products. However, research data showed that *A. niger* has the potential to produce two groups of potentially carcinogenic mycotoxins.[18−21] Thus, it is a great challenge to establish green biotransformation methods to identify the appropriate microorganism for ginsenoside biotransformation.

Mushrooms are defined as macrofungi with distinctive and visible fruiting bodies that may grow above or below ground. There are at least 15 000 species of fungi that can be considered as mushrooms, and at least 2000 species are edible and medicinal.[22] Recently, edible and medicinal mushrooms have been widely consumed in many countries as food, nutraceuticals, and medicine. Their nutritional and health benefits come from their high protein, fiber, vitamin, and mineral contents, low-fat level, and bioactive secondary metabolites.[23,24] Compared with extensively studied bacteria, yeasts, and filamentous fungi, edible and medicinal mushrooms are safely used as edible microorganisms for food consumption and drug development. The required biotransformation systems are readily available, and the fermentation process is green, nontoxic, eco-friendly, and economical. In addition, edible and medicinal mushrooms play a very important role in many biotechnological processes, such as biodegradation of raw plant materials, bioremediation of soil, and industrial waters, as well as biopulping and biobleaching of paper pulp.[25,26] However, very little work has been carried out on the biotransformation of ginsenosides by edible and medicinal mushrooms.

The objective of this work was to study the role of edible and medicinal mushrooms in ginsenoside biotransformation. Special attention has been paid to their metabolic patterns and biotransformation pathways. The results highlight an important practical application of the green ginsenoside biotransformation strategy using edible and medicinal mushrooms.

RESULTS AND DISCUSSION

Edible and Medicinal Mushroom Screening. In this study, 24 edible and medicinal mushrooms were screened for the ability to convert ginsenosides. Among the cultures screened, *Schizophyllum commune* showed a strong ability to convert ginsenosides into a less polar metabolite. Therefore, this fungus was selected for ginsenoside biotransformation. *S. commune* is a popular edible and medicinal mushroom and has been demonstrated to have various pharmacological properties, such as antibacterial, immunomodulatory, and antitumor activities.[27−29] *S. commune* is known to produce a variety of hydrolytic enzymes, such as cellulase, xylanase, and β-glucosidase.[30,31] It has been studied for different industrial applications in areas like food, pharmacology, agro-industry, bioprocessing, and environmental technology. However, *S. commune* has rarely been investigated for the biotransformation of ginsenosides.

Identification of Metabolites. In recent years, high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESIMS) has been extensively used for the determination of ginsenoside structure. In this study, the metabolites of ginsenosides Rb_1, Rc, Rb_2, and Rd by *S. commune* were identified according to the standards and HPLC-ESIMS ion fragments. As shown in Figure S1, the $[M - H]^-$ ion at m/z = 945 was observed for compound 2, which confirmed its molecular mass as 946 Da. The major fragment ions at m/z 783$[M - H - Glc]^-$, 621$[M - H - 2Glc]^-$, and 459$[M - H - 3Glc]^-$ can be assigned to glycosidic cleavage fragments. The m/z = 459 ion corresponded to the characteristic ion of PPD-type aglycone. The MS of compounds 3 and 4 gave m/z of 783 and 621 as the deprotonated molecular ion $[M - H]^-$, which confirmed the molecular mass to be 784 and 622, respectively. The metabolites corresponding to compounds 2, 3, and 4 were identified as Rd, F_2, and C−K, respectively, by comparing the mass spectra and retention times with those of reference compounds. Similarly, the metabolites corresponding to compounds 6, 7, 9, and 10 were identified as C−Mc_1, C−Mc, C−O, and C−Y, respectively. Characteristic fragment ions of ginsenoside are summarized in Table 1.

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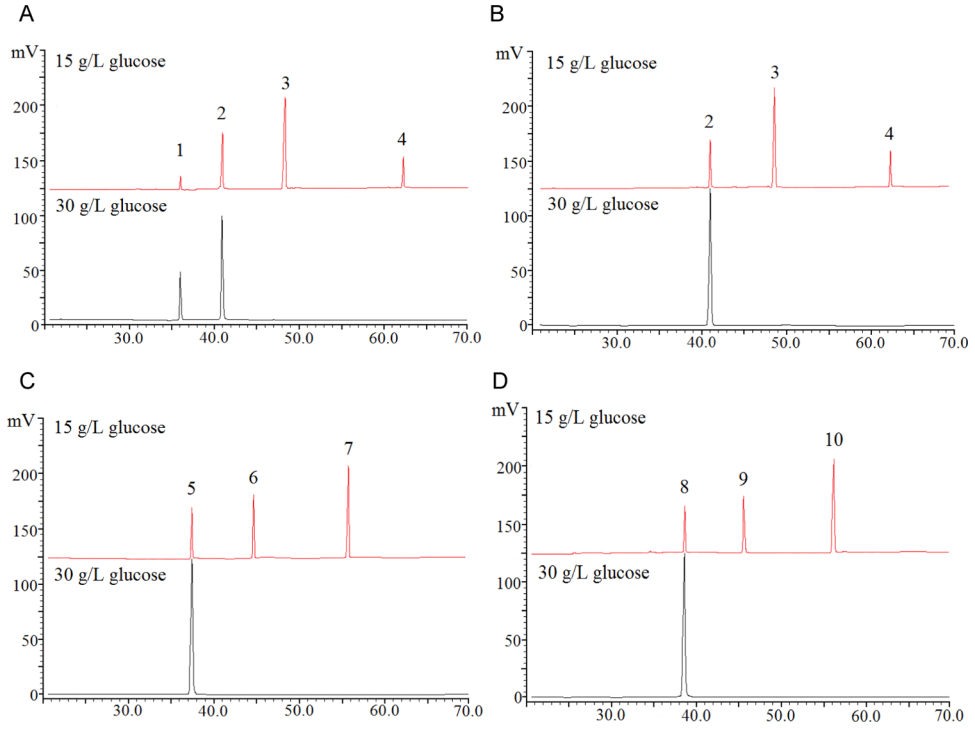

Figure 1. HPLC profile of the metabolites of ginsenosides (A) Rb_1, (B) Rd, (C) Rc, and (D) Rb_2 converted by *S. commune* with liquid medium containing 15 and 30 g L^{-1} glucose. Peak numbers: (1) Rb_1, (2) Rd, (3) F_2, (4) compound K, (5) Rc, (6) compound Mc_1, (7) compound Mc, (8) Rb_2, (9) compound O, and (10) compound Y. Peaks were identified using both authentic standards and LC-ESIMS.

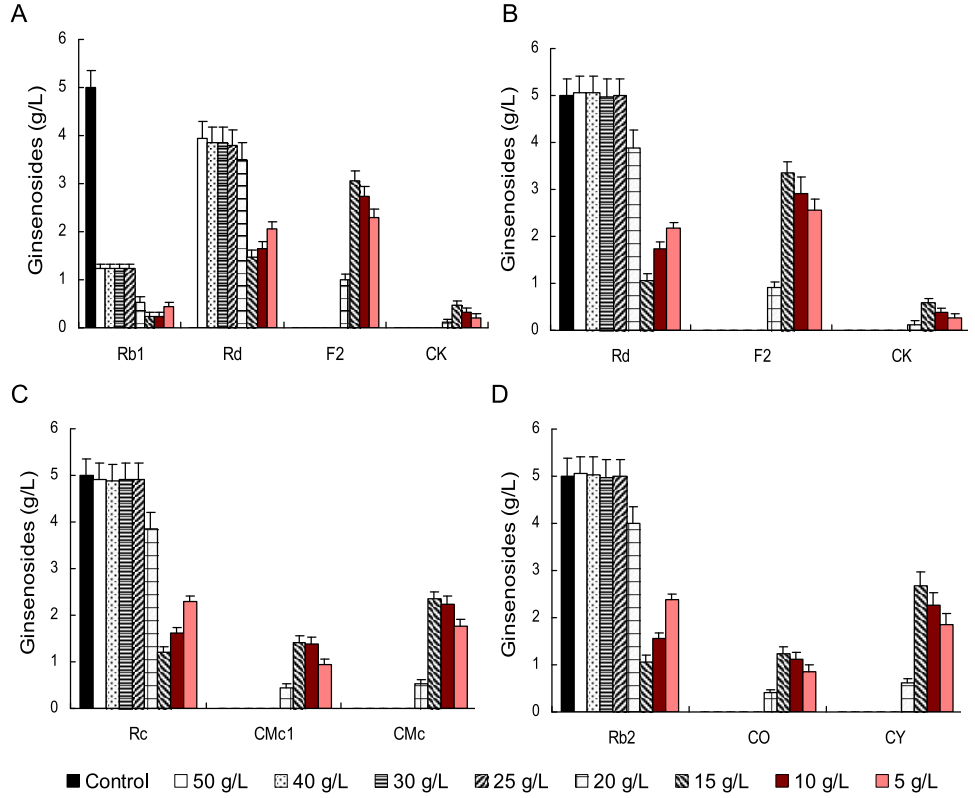

Figure 2. Effects of different glucose concentrations on biotransformation of ginsenosides (A) Rb_1, (B) Rd, (C) Rc, and (D) Rb_2 by *S. commune* with liquid medium containing 5, 10, 15, 20, 25, 30, 40, and 50 g L^{-1} glucose.

Effects of Glucose Concentration on Ginsenoside Biotransformation. To study the effect of the initial glucose concentration on PPD-type ginsenoside (Rb_1, Rb_2, Rc, and Rd) biotransformations, various concentrations of glucose (ranging from 5 to 50 g L^{-1}) were employed in the culture medium. As shown in Figures 1 and 2, when *S. commune* was

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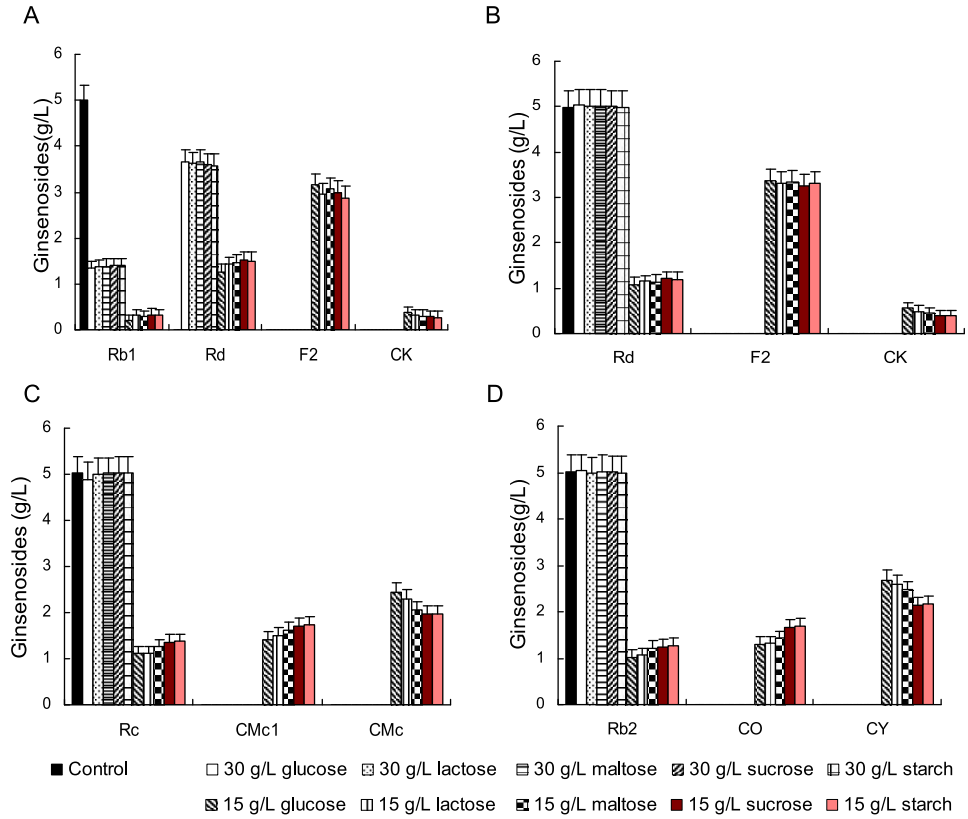

Figure 3. Effects of different carbon sources on biotransformation of ginsenosides (A) Rb_1, (B) Rd, (C) Rc, and (D) Rb_2 by *S. commune* with liquid medium containing 15 and 30 g L^{-1} sugar.

grown in fermentation medium containing 25−50 g L^{-1} glucose, after 4 days of fermentation, only ginsenoside Rb_1 was converted into Rd, whereas ginsenosides Rb_2, Rc, and Rd did not undergo any degradation. In contrast, ginsenosides Rb_1, Rb_2, Rc, and Rd were converted to minor ginsenosides when the glucose concentration was in the range of 5−15 g L^{-1}. These results indicate that the glucose level was the determining factor for the ginsenoside bioconversion by *S. commune*. The high glucose concentrations inhibited degradation of ginsenosides Rb_2, Rc, and Rd, whereas low glucose concentrations promoted the degradation of these ginsenosides.

Table S1 shows the mean biomass accumulation of *S. commune* grown in the fermentation medium containing 5, 15, and 30 g L^{-1} glucose or a combination of both glucose and ginsenoside Rb_1 (5 g L^{-1}). When *S. commune* was grown in fermentation medium containing 5 or 15 g L^{-1} glucose, the mycelial biomass in the presence of ginsenoside Rb_1 was higher than in the absence of ginsenoside Rb_1. When *S. commune* was grown in fermentation medium containing 30 g L^{-1} glucose, the mycelial biomass was not statistically different between cultures grown in the presence and absence of ginsenoside Rb_1 media. It was suggested that *S. commune* may use the sugars hydrolyzed from ginsenosides as a carbon source. Yousef and Bernards investigated and reported the metabolism of ginsenosides by the ginseng root pathogen *Pythium irregulare*.[32] The results showed that ginsenosides could significantly promote the in vitro growth of *P. irregulare*. The growth of *P. irregulare* in the presence of ginsenosides was more than 2-fold of that in the control group (no ginsenosides).

Effects of Different Carbon Sources on Ginsenoside Biotransformation. The effects of different carbon sources on the conversion of ginsenosides were also investigated. Each carbon source (glucose, lactose, maltose, sucrose, and starch) was added to the initial medium at a concentration of 15 or 30 g L^{-1}. Figure 3 shows that the effects of lactose, maltose, sucrose, and starch on the ginsenoside biotransformation were the same as the effects of glucose. The PPD-type ginsenosides (Rb_1, Rb_2, Rc, and Rd) could be degraded into minor ginsenosides by *S. commune* with liquid medium containing 15 g L^{-1} sugar. However, the degradation of ginsenosides Rb_2, Rc, and Rd did not occur using 30 g L^{-1} sugar as the sole carbon source. This phenomenon is similar to carbon catabolite repression (CCR). CCR is a key regulatory system found in most microorganisms that ensures preferential utilization of energy-efficient carbon sources. CCR has been sometimes called a glucose effect because the presence of glucose often prevents the use of other carbon sources. This is usually achieved through the inhibition of expression of genes encoding for enzymes involved in the catabolism of nonglucose carbon sources.[33] Therefore, it seemed that the degradation of ginsenosides by *S. commune* can be regulated by CCR.

Effects of Nitrogen Sources and Metal Ions on Ginsenoside Biotransformation. Nitrogen sources and metal ions are other two essential components for the growth and metabolism of microorganisms. Hence, we selected nitrogen sources and metal ions to investigate their influence on the biotransformation of ginsenosides. As shown in Figures S2 and S3, our results indicated that yeast powder was the most favorable for ginsenoside transformation in the fermentation medium with 15 g L^{-1} glucose as the carbon

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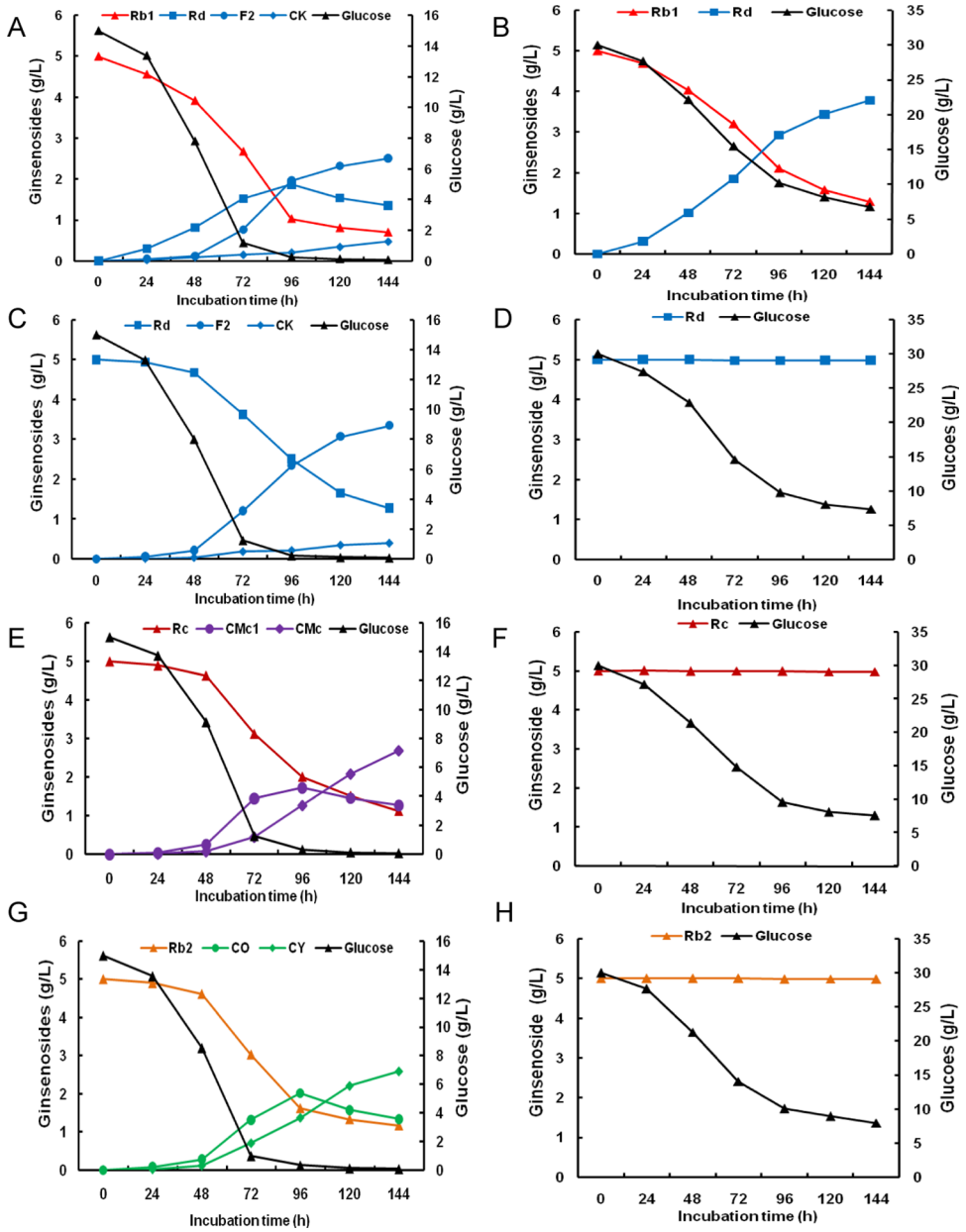

Figure 4. Transformation kinetic curve of ginsenosides Rb_1, Rb_2, Rc, and Rd and glucose utilization by *S. commune*. (A) Rb_1 transformation using 15 g L^{-1} glucose as a carbon source. (B) Rb_1 transformation using 30 g L^{-1} glucose as a carbon source. (C) Rd transformation using 15 g L^{-1} glucose as a carbon source. (D) Rd transformation using 30 g L^{-1} glucose as a carbon source. (E) Rc transformation using 15 g L^{-1} glucose as a carbon source. (F) Rc transformation using 30 g L^{-1} glucose as a carbon source. (G) Rb_2 transformation using 15 g L^{-1} glucose as a carbon source. (H) Rb_2 transformation using 30 g L^{-1} glucose as a carbon source.

source. Out of all of the examined metal ions, $MgSO_4$ and KH_2PO_4 most significantly increased ginsenoside bioconversion. However, neither the nitrogen source nor the metal ion had an effect on the degradation of ginsenosides Rb_2, Rc, and Rd when *S. commune* was cultured in fermentation medium containing 30 g L^{-1} glucose as the sole carbon source. These results indicate that the nitrogen sources and metal ions were not the determining factors for the ginsenoside bioconversion by *S. commune*.

Transformation Pathways of Ginsenosides and Glucose Utilization. The profiles of glucose utilization and ginsenoside transformation during the fermentation processes are shown in Figure 4. When *S. commune* was cultured in fermentation medium containing 15 g L^{-1} glucose, the glucose was quickly utilized and almost completely metabolized within 96 h of fermentation. Using ginsenoside Rb_1 as a substrate of *S. commune*, three transformation products, Rd, F_2, and C−K, were detected throughout the transformation period. The results are presented in Figure 4A. Ginsenoside Rb_1 was gradually transformed into Rd during 0−48 h, and ginsenosides F_2 and C−K were produced very weakly. From 48 to 96 h of incubation, the content of ginsenoside Rb_1 decreased rapidly. Ginsenoside Rd quickly approached its maximum production as long as the content of ginsenoside F_2 and C−K accumulated gradually. Then, the amount of Rd decreased continuously after 96 h, leading to a significant increase in the amount of F_2. These results indicate that F_2 is the main transformation product of ginsenoside Rb_1 and the bio-

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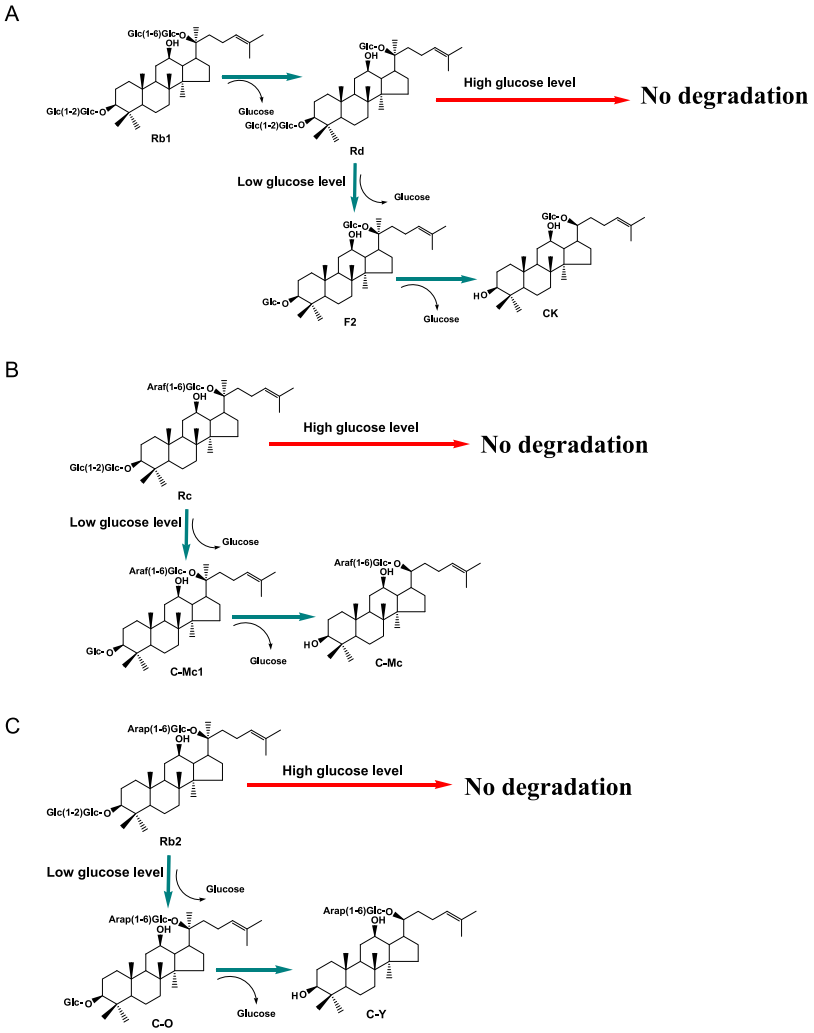

Figure 5. Biotransformation pathways of ginsenosides (A) Rb_1, (B) Rb_2, and (C) Rc by *S. commune*.

transformation pathway of Rb_1 by *S. commune* can be traced as follows: $Rb_1 \rightarrow Rd \rightarrow F_2 \rightarrow C{-}K$ (Figure 5A). However, the biotransformation pathway of Rb_1 by *S. commune* was different from that by human gut bacteria. The intestinal bacteria can hydrolyze the ginsenoside Rb_1 in two different pathways: $Rb_1 \rightarrow G{-}XVII \rightarrow G{-}LXXV \rightarrow C{-}K$ and $Rb_1 \rightarrow Rd \rightarrow F_2 \rightarrow C{-}K$.[14] When ginsenoside Rd was tested as substrate of *S. commune*, the transformation products, F_2 and C−K, were detected in the metabolites throughout the transformation period (Figure 4C). A similar result suggested that the transformation pathway of Rd was $Rd \rightarrow F_2 \rightarrow C{-}K$.

When ginsenoside Rc was used as a substrate, two major transformation products, $C{-}Mc_1$ and C−Mc, were detected. As shown in Figure 4E, $C{-}Mc_1$ and C−Mc were observed during 0−48 h and maintained at a low level, then increased gradually from 48 h until the end of the experimental period. The biotransformation pathway of Rc was $Rc \rightarrow C{-}Mc_1 \rightarrow C{-}Mc$ (Figure 5B). Ginsenoside C−Mc is the main transformation product of ginsenoside Rc. For ginsenoside Rb_2, two major transformation products, C−O and C−Y, were detected. As shown in Figure 4G, the level of Rb_2 decreased gradually throughout the transformation period. The amount of C−O and C−Y was relatively low during the first 48 h of incubation and then increased continuously from 48 h until the end of the experimental period. Our results showed that C−Y was the main transformation product of Rb_2 and the biotransformation pathway of Rb_2 can be traced as follows: $Rb_2 \rightarrow C{-}O \rightarrow C{-}Y$ (Figure 5C). The biotransformation pathways of Rb_2 and Rc by *S. commune* were also different from that by human gut bacteria. The intestinal bacteria can transform the ginsenosides Rb_2 and Rc with the pathway $Rb_2 \rightarrow C{-}O \rightarrow C{-}Y \rightarrow C{-}K$ and $Rc \rightarrow C{-}Mc_1 \rightarrow C{-}Mc \rightarrow C{-}K$.[15] However, *S. commune* is not able to hydrolyze the ginsenosides Rb_2 and Rc to C−K.

When *S. commune* was grown in fermentation medium containing 30 g L^{-1} glucose, the glucose was rapidly utilized within 96 h of incubation, then metabolized slowly from 96 h until the end of the experimental period. Under high levels of glucose, ginsenoside Rb_1 (Figure 4B) was gradually converted into Rd within 144 h of incubation, whereas the degradation of Rd (Figure 4D), Rc (Figure 4F), and Rb_2 (Figure 4H) was not observed. These results indicate that the hydrolysis of the terminal glucose residues at C-20 of Rb_1 was not affected by glucose levels, but the terminal and inner glucose residues at the C-3 of Rb_1, Rb_2, Rc, and Rd were inhibited by the high concentration of glucose. In addition, under the low glucose levels, the glucose and protopanaxadiol-type ginsenosides (Rb_1, Rb_2, Rc, and Rd) were simultaneously consumed by *S. commune*. However, the rate of glucose consumption was always higher than that of ginsenosides.

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CCR is a universal regulatory phenomenon in many microorganisms. However, there are exceptions. For some pathogenic bacteria, such as *Chlamydia trachomatis* and *Mycoplasma pneumoniae*, glucose is only a secondary carbon source, which is referred to as reverse CCR.[34,35] Another peculiarity is the co-fermentation of glucose and other carbon sources that occurs in *Corynebacterium glutamicum* and engineered *Escherichia coli*, although this co-fermentation is highly regulated.[36,37] In our work, the degradation of ginsenosides Rb_2, Rc, and Rd was inhibited by various carbon sources at high concentrations. The inhibition was avoided by maintaining sugar concentration below 15 g L^{-1}. In addition, the utilization of sugar and biotransformation of ginsenosides Rb_1 to Rd by *S. commune* occur simultaneously. The reason and mechanism behind these phenomena warrant further studies.

Optimization of Substrate Concentration and Cosolvent on Minor Ginsenoside Yield. The effect of different ginsenoside (Rb_1, Rc, Rb_2, and Rd) concentrations on the biotransformation for the production of minor ginsenosides was investigated under optimized culture conditions. The contents of minor ginsenosides in the media were detected by HPLC. As shown in Figure 6A, the bioconversion rate declined

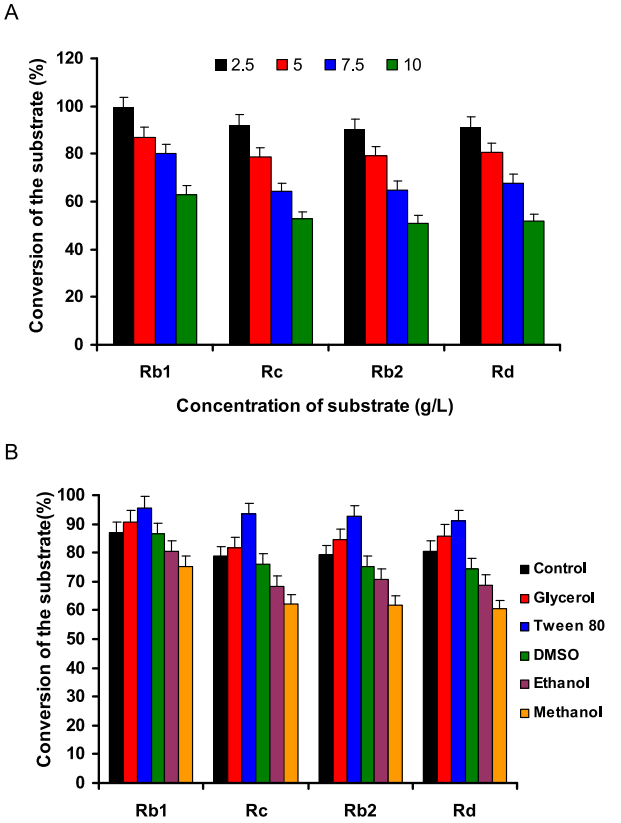

Figure 6. Effects of substrate concentration and cosolvent on ginsenoside degradation by *S. commune*. (A) Effect of different substrate concentrations. (B) Effect of various cosolvents.

gradually following a rise in the ginsenoside concentration from 2.5 to 5 g $L^{-1}$and decreased rapidly above this range. The results indicate that high ginsenoside concentration was not beneficial for the production of minor ginsenosides; it may be the poor aqueous solubility of ginsenoside that inhibited its utility by *S. commune*.

Usually, a cosolvent can improve the aqueous solubility of ginsenoside substrates and promote the permeability of cell membrane and increase the bioconversion efficiency.[38] Therefore, the effects of various cosolvents on the ginsenoside biotransformation were investigated in this study. As shown in Figure 6B, among the five different cosolvents, Tween 80 was found to be the optimal cosolvent to improve the ginsenoside solubility and enhance the production of minor ginsenosides. When the fermentation medium contained 5 g L^{-1} substrate and 0.1% (w/v) Tween 80, the bioconversion rates of ginsenosides Rb_1, Rc, Rb_2, and Rd were more than 90%. The maximum yields of ginsenosides F_2, C−Y, and C−Mc were raised to 75.2, 62.7, and 64.9%, respectively. The production and bioconversion rates were significantly higher than those previously reported by food microorganisms.[16,17,39]

Effects of Glucose Concentration on Ginsenoside Biotransformation by Various Edible and Medicinal Mushrooms. On the basis of the experimental results, we further investigated the effect of initial glucose concentration on the ginsenoside biotransformation by various edible and medicinal mushrooms. Our results showed that many edible and medicinal mushrooms, including *Ganoderma lucidum*, *Hericium erinaceus*, *Pleurotus ostreatus*, *Grifola frondosa*, and *Flammulina velutiper*, were only able to convert ginsenoside Rb_1 to Rd, which was not affected by sugar concentration. It has been shown that ginsenoside Rd possesses many pharmacological activities.[40−42] However, the concentration of ginsenoside Rd is extremely low in most natural ginseng plants. Thus, many studies have been aimed at biotransformation of major ginsenosides (Rb_1, Rb_2, and Rc) to pharmacologically active Rd using enzymes or microorganisms.[10] However, ginsenoside Rd is just an intermediate in the reaction and can further be transformed into other compounds. This leads to a low yield and bioconversion rate of ginsenoside Rd. Thus, edible and medicinal mushrooms are also an effective and green ginsenoside Rd producer.

CONCLUSIONS

In summary, this study investigated the role of edible and medicinal mushrooms in ginsenoside biotransformation. A total of 24 edible and medicinal mushrooms were selected and screened for their ability to microbiologically transform ginsenosides by HPLC analyses. Our results showed that the degradation of ginsenosides by *S. commune* was inhibited by high concentrations of sugar. However, the inhibition was avoided by maintaining sugar concentration below 15 g L^{-1}. *S. commune* showed a strong ability to convert ginsenosides into minor ginsenosides. We found that *S. commune* may use the sugars hydrolyzed from ginsenosides as a carbon source. Under the low glucose levels, the glucose and ginsenosides (Rb_1, Rb_2, Rc, and Rd) were simultaneously consumed by *S. commune*. The production and bioconversion rates were significantly higher than those previously reported by food microorganisms. In addition, many edible and medicinal mushrooms were only able to convert ginsenoside Rb_1 to Rd, which was not affected by the concentration of sugar. Thus, edible and medicinal mushrooms are also an effective and green ginsenoside Rd producer. This is the first report about the biotransformation of major ginsenosides into minor ginsenosides through fermentation by edible and medicinal mushrooms. The results indicate that edible and medicinal mushrooms would be potential food microorganisms for obtaining minor ginsenosides.

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MATERIALS AND METHODS

Materials. Ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rg_1, F_2, 20S-Rg_3, 20R-Rg_3, Rh_2, and C−K were obtained from Norman Bethune College of Medicine, Jilin University (Changchun, China). Ginsenosides C−O, C−Y, C−Mc, and C−Mc_1 were purchased from Dalian Green Bio Co Ltd (Dalian, China). Acetonitrile and methanol of HPLC grade were from Fisher (Fisher Scientific). Water was purified by a Milli-Q system (Millipore, Bedford, MA). All other chemicals used in this study were of analytical grade.

Microorganisms and Growth Conditions. A total of 24 mushroom species were obtained from the Engineering Research Center of Chinese Ministry of Education for Edible and Medicinal Fungi, Jilin Agricultural University (Jilin, China). They were stored at 4 °C on potato dextrose agar slants and subcultured once a month. The freshly inoculated slant was incubated at 25 °C for 7 days before use. The fermentation medium for the flask culture consisted of the following components: 15 g of glucose, 3 g of yeast powder, 1 g of KH_2PO_4, and 0.5 g of $MgSO_4{\cdot}7H_2O$ in 1 L of distilled water. Fermentations were carried out in 250 mL shake flasks on a rotary shaker (160 rpm) at 28 °C for 7 d. All media were sterilized at 121 °C for 30 min.

Screening of Microorganisms. Preliminary screening was performed in 100 mL Erlenmeyer flasks containing 30 mL of fermentation medium and 50 mg of protopanaxadiol-type ginsenosides. The flasks were placed on a rotary shaker operating at 160 rpm at 28 °C. After 7 days of incubation, the ginsenosides were extracted three times by sonification with aqueous saturated *n*-BuOH for 20 min. The *n*-BuOH extract was concentrated under reduced pressure to dryness, and the residue was dissolved in methanol and then analyzed by HPLC-UV.

Effects of Glucose Concentration. To study the effect of glucose concentration on ginsenoside transformation, the different concentrations of glucose (ranging from 5 to 50 g L^{-1}) in the initial culture medium were tested. The biotransformation medium was composed of 3 g L^{-1} yeast powder, 1 g L^{-1} KH_2PO_4, and 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$ and a certain initial concentration of glucose as investigated. Fermentation was carried out on a rotary shaker (160 rpm) at 28 °C. After 2 d of culture, 2 mL of the substrate solution was added to each shake flask, and then the initial concentration of ginsenoside in the culture medium was diluted to 5 g L^{-1}. Incubation was then allowed to proceed for 5 d. Finally, the ginsenosides were extracted by *n*-BuOH and analyzed by HPLC-UV.

Effects of Different Carbon Sources. Glucose, sucrose, maltose, lactose, and soluble starch were selected as alternative carbon sources in the fermentation medium to investigate influences on the ginsenoside biotransformation. The concentration of each carbohydrate as a sole carbon source in the culture medium was 15 or 30 g L^{-1} in these experiments. The biotransformation medium was composed of 3 g L^{-1} yeast powder, 1 g L^{-1} KH_2PO_4, and 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$ and a certain initial concentration of sugar (glucose, sucrose, maltose, lactose, or soluble starch) as investigated. The other fermentation conditions were the same as above.

Effects of Nitrogen Sources. The concentrations of 3 g L^{-1} yeast powder, soybean powder, peptone, corn steep powder, $NaNO_3$, and $(NH_4)_2SO_4$ were selected as alternative nitrogen sources in the fermentation medium to investigate influences on the ginsenoside biotransformation. The biotransformation medium was composed of 15 or 30 g L^{-1} glucose, 1 g L^{-1} KH_2PO_4, and 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$ and an alternative nitrogen source (yeast powder, soybean powder, peptone, corn steep powder, $NaNO_3$ or $(NH_4)_2SO_4$). The other fermentation conditions were the same as above.

Effects of Metal Ions. The concentrations of 1.5 g L^{-1} KH_2PO_4, NaH_2PO_4, $CaCl_2$, $MgSO_4{\cdot}7H_2O$, $FeSO_4$, $ZnSO_4$, and $CuSO_4$ were selected as alternative metal ions in the fermentation medium to investigate influences on the ginsenoside biotransformation. The biotransformation medium was composed of 15 or 30 g L^{-1} glucose, 3 g L^{-1} yeast powder, and an alternative metal ion (KH_2PO_4, NaH_2PO_4, $CaCl_2$, $MgSO_4{\cdot}7H_2O$, $FeSO_4$, $ZnSO_4$, or $CuSO_4$). The other fermentation conditions were the same as above.

Biotransformation Pathway and Glucose Utilization. Ginsenosides Rb_1, Rb_2, Rc, and Rd were dissolved in a 100 mL shake flask containing 30 mL fermentation medium. The initial concentration of each ginsenoside in the medium was diluted to 5 g L^{-1}. Incubation was then allowed to proceed for 0−144 h. The dynamic profiles of the ginsenoside biotransformation and glucose utilization were monitored during the biotransformation process.

Effects of Substrate Concentration and Cosolvent. The effect of initial ginsenoside concentration on the biotransformation for production of minor ginsenosides by *S. commune* was investigated with its levels of 2.5, 5, 7.5, and 10 g L^{-1}. Based on the above initial substrate concentration experiment result, the effect of five different cosolvents (glycerol, Tween 80, dimethyl sulfoxide, ethanol, and methanol) on the ginsenoside biotransformation was also investigated.

Analytical Methods. Ginsenoside analysis was carried out on an LC-20A liquid chromatograph (Shimadzu, Japan) equipped with two LC-20AT pumps and an SPD-20A UV/Vis detector. Samples were separated on a Cosmosil C18 reverse-phase silica column (5 μm, 250 × 4.6 mm^2) at 25 °C using acetonitrile (solvent A) and water (solvent B). A gradient elution program was performed according to the following profiles: 0−20 min, 22% (A), 78% (B); 20−25 min, 22−30% (A), 78−70% (B); 25−45 min, 30−46% (A), 70−54% (B); 45−55 min, 46−64% (A), 54−36% (B); 55−70 min, 64−66% (A), 36−34% (B). The injection volume was 20 μL. The flow rate was kept at 1.0 mL min^{-1}. The absorbance was measured at a wavelength of 203 nm. Glucose was determined by HPLC (LC-20AT; Shimadzu, Japan) equipped with a refractive index detector and a Cosmosil Sugar-D column (250 × 4.6 mm^2). The column was operated at 30 °C, and the mobile phase was acetonitrile/water (75:25 v/v) at 1 mL min^{-1}.

The ginsenoside degradation products were analyzed and identified by a Finnigan LCQ ion trap mass spectrometer (MAT; San Jose, CA) equipped with an electrospray ion source in negative-ion mode. The ESI parameters were the following: ion source voltage, 5000 V; capillary voltage, −40.0 V; source temperature, 150 °C; capillary temperature, 280 °C; and sheath gas and auxiliary gas flow rates, 65 and 11 arbitrary units, respectively. Full scan spectra were recorded in the m/z range of 200−2000. The HPLC conditions for the HPLC/ESIMS analysis were the same as above mentioned for the analysis method of ginsenosides.

Statistical Analysis. The statistical analysis was conducted based on an SPSS20.0 system (SPSS Inc., Chicago, IL). Data

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are expressed as the mean ± SEM. The ginsenoside contents of the two groups were analyzed by independent sample *t*-test. Differences between the groups were analyzed using analysis of variance ANOVA. A difference of $p < 0.05$ was considered to be statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01001.

Biomass accumulation of *S. commune*; LC/ESIMS analysis of metabolites; and effects of nitrogen sources and metal ions on biotransformation of ginsenosides (PDF)

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Notes

The authors declare no competing financial interest.

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