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Posted Date: 27 January 2025

doi: 10.20944/preprints202501.2026.v1

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Article

Comparison of the Transformation Ability of the Major Saponins in *Panax notoginseng* by *Penicillium fimorum* Enzyme and Commercial β -Glucosidase

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Abstract: Ginsenosides with less sugar groups which were called minor ginsenosides might have greater pharmacological activity and a better adsorptive ability, but their content in nature is extremely low. In this study, a strain of *Penicillium fimorum* with strong saponin transformation ability was isolated from fresh *Gastrodia elata*. A comparative biotransformation experiment of the major saponins from *Panax notoginseng* root were conducted using crude enzyme from *P. fimorum* and commercial β -glucosidase to product minor ginsenosides. Specifically, the crude enzyme from *P. fimorum* was able to transform the major saponins from *P. notoginseng* root into 13 minor saponins within 72 hours, while commercial β -glucosidase was able to transform the same major saponins into 15 minor saponins within 72 hours. The most significant difference between their two enzymes is the ability to transform Rb₁. To the best of our knowledge, the biotransformation ability of crude enzyme from *P. fimorum* is reported for the first time. These two enzymes have the potential to improve the economic value of *P. notoginseng* root and expand the methods for preparing minor saponins by transforming the major saponins in the total saponins of *P. notoginseng* root.

Keywords: *Panax notoginseng* root; biotransformation; *Penicillium fimorum*; minor ginsenosides

1. Introduction

The genus of *Panax* belongs to the Araliaceae family, which has 17 species of the *P. genus*, including *P. ginseng*, *P. quinquefolius*, and *P. notoginseng*, etc [1]. Most members of this genus have medicinal properties and more than 150 ginsenosides have been identified and classified according to their structures, which can be divided into damarane, oktylon and oleanolic acid types [2,3]. *P. notoginseng* (Burk.) F. H. Chen is a traditional and valuable Chinese herbal medicine, mainly grown in Guangxi and Yunnan provinces in Southwest China. In *P. notoginseng*, dalmarane-type tetracyclic triterpene saponins can be further divided into propanaxadiol-type saponins (PPD, such as Rb₁, Rb₂, Rb₃, Rd and Rc) and propanaxtriol-type saponins (PPT, such as Re and Rg₁) according to the presence of a hydroxyl group attached to C-6. The kinds and contents of saponins in different parts of *P. notoginseng* are also different, and *P. notoginseng* root (PNR) mainly contains PPD-type ginsenosides (Rb₁ and Rd) and PPT-type ginsenosides (Re, Rg₁ and R₁) [4]. The major saponins in PNR mainly showed great polarity because of the different types and amounts of sugar moieties linked to the C-3, C-6 and C-20 positions of the dammarane triterpenoid backbone [5]. After oral administration, the major saponins need to be hydrolyzed by digestive enzymes and intestinal microorganisms before they can become more active and easily absorbed minor ginsenosides, but the efficiency of these conversions is very low [6].

Most studies have shown that the function of ginsenosides is closely related to the position, type and quantity of glycosidic bonds [7]. The main ginsenosides contain more sugar groups, which leads to their low pharmacological activity and is not easy to be absorbed by human body. Compared with the main ginsenosides, the minor ginsenosides have better pharmacological activities such as anti-hypertension, anti-aging, etc [8]. These minor saponins are produced mainly by deglycosylation, isomerization and dehydration [9,10]. The main ginsenosides can be hydrolyzed into minor ginsenosides by physical, chemical and biotransformation. Compared with other methods, biotransformation has the advantages of mild conversion conditions, product stability and pollution-free [11].

In order to prepare minor ginsenosides, many microorganisms capable of transforming ginsenosides have been discovered and applied. In particular, the relatively safe strain of *Aspergillus niger* is well known. *A. niger* JGL8 isolated from *Gynostemma pentaphyllum* can transform *Gynostemma pentaphyllum* saponins to ginsenoside F₂ through GypV → Rd → F₂ [12]. Ginsenoside Rb₁ can be transformed by extracellular enzymes produced by *A. niger* WU-16. The transformation pathway of Rb₁ is Rb₁ → F₂, CK [13]. *A. Niger* XD101 and its crude enzymes screened from the planting soil of *P. notoginseng* also have the ability to hydrolyze ginsenoside Rb₁, and its transformation pathway is: Rb₁ → Rd → F₂ → CK [14]. The results of all these studies indicate the feasibility of using microorganisms to carry out the transformation of major saponins for the preparation of minor saponins. Moreover, previous studies has shown that there are four main enzymes used in the enzymatic production of minor ginsenosides, these four enzymes hydrolyze glycosidic bonds at different positions on major ginsenosides [15–20].

In this study, a strain of the plant endophytic fungus *P. fimorum* with excellent saponin transformation capacity was isolated from fresh *Gastrodia elata*, and its extracellular crude enzyme was extracted to transform several major saponins in PNR for 72 hours. The enzyme was found to be capable of transforming four major saponins (Rb₁, Rg₁, R₁, and Re) in PNR into 13 minor saponins, of which ginsenoside CK had the highest yield at 11.88%, followed by ginsenoside Rd with a yield of 13.03%. To the best of our knowledge, this biotransformation characteristic of *P. fimorum* is reported here for the first time in fungi. In addition, commercial β-glucosidase was purchased for comparison, and it was found to transform the four major saponins in PNR into 15 minor saponins, of which ginsenoside Rd had the highest yield at 53.87%, with the yields of the remaining products being similar to those obtained from the transformation by the extracellular crude enzyme of *P. fimorum*.

By analyzing two comparative experiments, we found that the extracellular crude enzyme of *P. fimorum* and commercial β-glucosidase exhibited the following transformation capacities: deglycosylation, epimerization, and dehydration. The main difference is that the extracellular crude enzyme of *P. fimorum* specifically hydrolyzes glucose at the C-3 position of ginsenoside Rb₁, efficiently producing ginsenoside CK, whereas β-glucosidase specifically hydrolyzes glucose at the C-20 position of ginsenoside Rb₁, producing ginsenosides Rd, 20(S/R)-Rg₃, Rk₁, and Rg₅. Analysis via TLC and HPLC, we have hypothesized that the biotransformation pathway of *P. fimorum* extracellular crude enzyme and commercial β-glucosidase for transforming four major saponins in PNR, along with the yields of the substrates and products involved. This study is expected to facilitate the conversion of total saponins from PNR into minor saponins, thereby greatly enhancing the economic and functional value of *P. notoginseng*.

2. Materials and Methods

2.1. Materials

Authentic standards of ginsenosides Rb₁, Rd, F₂, CK, 20(S/R)-Rg₃, Rk₁, Rg₅, 20(S/R)-Rh₁, Rh₄, Rk₃, Re, 20(S/R)-Rg₂, F₄, Rg₆ and notoginsenosides R₁, 20(S/R)-R₂ were purchased from the Sichuan Victory. The commercial β-glucosidase was purchased from the Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China). The solvents methanol and acetonitrile for HPLC were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The HSGF₂₅₄ silica gel TLC plate was purchased from Yantai

Jiangyou Silicone Development Co., Ltd. (Shangdong, China). A Welchrom C₁₈ column (4.6 × 250 mm, 5 µm) was sourced from Yuexu Technology Co., Ltd. (Sichuan, China). An Agilent 1260 high-performance liquid chromatography instrument was purchased from Agilent (Grand Island, NY, USA).

2.2. Medium

PDA medium: potato extract powder 6 g/L, glucose 25 g/L, and agar 25 g/L. PDB medium: potato extract powder 6 g/L and glucose 25 g/L.

2.3. Isolation and Identification of Plant Endophytic Fungi

A single colony, S62, was isolated from fresh *Gastrodia elata* using the isolation and purification method of plant endophytic fungi [21,22]. The amplification and sequencing of the ITS rDNA gene was performed by Kunming Branch of Tsingke Biotechnology Co., Ltd. The ITS rDNA gene was submitted to the National Centre for Biotechnology Information (NCBI) to obtain the NCBI-recorded RSA registry number OR958833. Phylogenetic tree construction by neighbour-joining (NJ) method using MEGA 11.0 software. And the morphologies of conidiophores and ascospores were observed by optical microscopy. A voucher specimen (No. Li20221001) was deposited at the Faculty of Life Science and Technology, Kunming University of Science and Technology.

2.4. Screening of Fungi for Its Ability to Biotransform Saponins

Microbial fermentation of the fungus was carried out using PDB medium. After 5 days of fermentation, a solution of total saponins from PNR was added to the fermentation broth to continue microbial transformation for an additional 18 days. The total saponins solution of PNR was dissolved in 75% ethanol and filtered through a 0.22 µm filter before being added to the fermentation broth, achieving a final concentration of 4 mg/mL in the broth. After 18 days of fermentation, the fermentation broth was extracted with an equal volume (1:1) of aqueous saturated n-BuOH. The upper phase was dried by evaporation and then analyzed by TLC to assess its saponin conversion capacity [23,24].

2.5. Preparation of Crude Enzyme from Microorganism

The extracellular crude enzymes of the strain were extracted using supersaturated ammonium sulfate [4,25]. The fermentation broth from the 18-day fermentation was extracted with aqueous saturated n-BuOH. Ammonium sulfate was then added to the lower phase of the fermentation broth to form a saturated solution, followed by the extraction of the crude enzyme.

2.6. Determination of Optimal pH and Optimal Temperature

Optimization of the optimal conditions for the enzyme reaction was carried out using ginsenoside Rb₁. A 0.5 mL aliquot of 1 mg/mL ginsenoside Rb₁ and 1 mL of 4 mg/mL enzyme solution were mixed well, and the reaction was then carried out. The buffer solution was prepared using phosphate and citrate with a pH range of 3–8. The reaction temperature was varied between 30–70 °C.

2.7. Biotransformation of Monomer Saponins by Enzymes

Enzymatic biotransformation of four monomeric saponins (Rb₁, Rg₁, Re, and R_i) from PNR was carried out for 72 hours under optimal reaction conditions. At the end of the reaction, the solution was extracted with 2 times volume of aqueous saturated n-BuOH. The upper solution was then evaporated to dryness and analyzed by TLC and HPLC.

2.8. Monitoring of Substrate Conversion and Product Yields

To monitor changes in the substrate and products during biotransformation by the enzyme, samples from different time points were collected. Enzyme-substrate conversion reactions were performed at 3, 6, 12, 24, 48, and 72 hours, respectively. At the end of the reaction, the solution was extracted with 2 times volume of aqueous saturated n-BuOH. The upper solution was then evaporated to dryness and analyzed by TLC and HPLC.

$$\text{Substrate conversion (\%)} = \frac{C_s (\text{mg}) - C_{fs} (\text{mg})}{C_s (\text{mg})} \quad (1)$$

$$\text{Yield of product (\%)} = \frac{C_p (\text{mg})}{C_s (\text{mg})} \quad (2)$$

where C_s is the initial concentration of the substrate, C_{fs} is the final concentration of the substrate, C_p is the final concentration of the product ginsenoside.

2.9. Analytical Methods of TLC and HPLC

Experimental samples were extracted using water saturated n-butanol, and the upper solution was dried up and used for subsequent experiments.

For the TLC analysis, $\text{CHCl}_3\text{-CH}_3\text{COOH-CH}_3\text{OH-H}_2\text{O}$ (6.75:0.25:2.5:0.5, v/v/v) was used as a developing solvent, and 10% (v/v) H_2SO_4 -ethanol was used as a chromogenic solvent.

For the HPLC analysis, the samples were analyzed by HPLC, using a Agilent 1260 system (NY, USA) connected to a Welchrom C_{18} chromatography column (4.6 × 250 mm, 5 μm). The mobile phase entailed a mixture of water (A) and acetonitrile (B). The gradient elution program was set as follows: 0–15 min (20% B), 15–20 min (20–30% B), 20–45 min (30–36% B), 45–52 min (36–38% B), 52–55 min (38–40% B), 55–63 min (40–42% B), 63–69 min (42–44% B), 69–75 min (44–51% B), 75–85 min (51–53% B), 85–90 min (53–60% B), 90–91 min (60–63% B), 91–100 min (63–66% B), 100–105 min (66–100% B). The system maintained a flow rate of 1.0 mL/min. Absorbance measurements were taken at 203 nm with an injection volume of 30 μL and the column temperature set at 25 °C.

3. Results

3.1. Screening and Characterisation of Strain for the Transformation of Saponins

The plant endophytic fungus S62 was isolated from fresh *Gastrodia elata*. The morphology of the colonies after 5 days of culture on PDA medium is shown in Figure 1A and B. The surface appeared white, fluffy, or flocculent, while the back side was light yellow. Under optical microscopy, asymmetrical broom-like branches and subglobose conidia were observed.

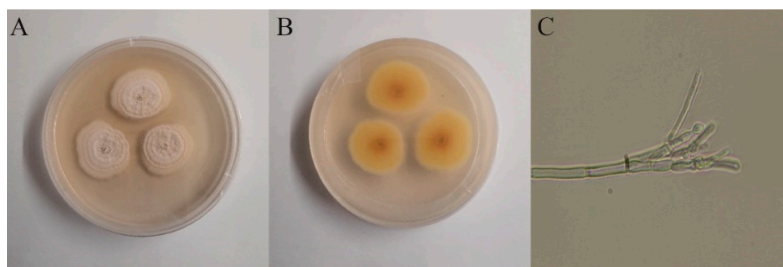


Figure 1. Morphology identification of strain-S62. (A) Front view of morphology of strain-S62. (B) Back view of morphology of strain-S62. (C) Mycelium spore morphology at 10×40 times microscope of strain-S62.

Biotransformation of total saponins from PNR was carried out for 18 days using strain S62 in PDB medium. TLC analysis of the results shown in Figure 2 reveals new spots with a larger R_f value above the original substrate, indicating that strain S62 has better saponin conversion ability.

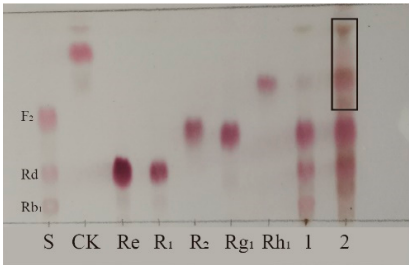


Figure 2. TLC analysis of the transformation products of total saponins from *P. notoginseng* root by *P. fimorum* for 18 days. S: Standards; 1: Substrate; 2: Transformation products after 18 days of biotransformation.

Based on ITS rDNA gene sequencing and comparison with the GenBank database, strain S62 was found to belong to the genus *Penicillium* and exhibited remarkable similarity to *Penicillium fimorum*, as shown in Figure 3.

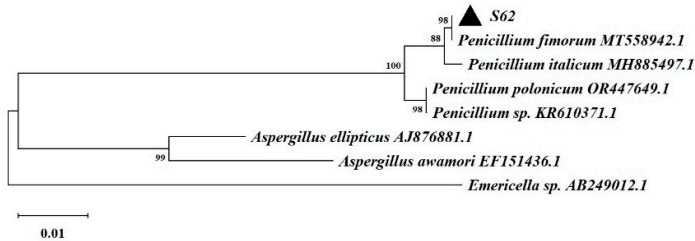


Figure 3. The neighbor-joining tree based on ITS rDNA gene sequences of strain-S62.

3.2. Results of Optimal pH and Temperature for Enzyme Transformation of Monomeric Saponin

Ginsenoside Rb₁, the major saponin in PNR, the content of Rb₁ is the highest in common ginsenosides, it could be used to determine the optimal conditions for biotransformation [26]. Figure 4 shows the optimal conditions for the conversion by *P. fimorum* crude enzyme, with an optimal pH of 5 and an optimal temperature of 60 °C. Similarly, the optimal reaction conditions for commercial β-glucosidase are also pH 5 and 60 °C, as shown in Figure 5. These results indicate that both enzymes exhibit optimal activity under weakly acidic conditions, which is similar to the enzyme reaction conditions reported for other strains with saponin conversion ability [27,28]. Both enzymes also exhibit good temperature tolerance, with activity remaining effective up to 60 °C. Additionally, a control experiment was conducted to examine the effects of pH and temperature on the reaction conditions. It was found that acidic hydrolysis of ginsenoside Rb₁ occurred at both pH 3 and pH 4, while high-temperature pyrolysis was observed at 70 °C, as shown in Figure S1.

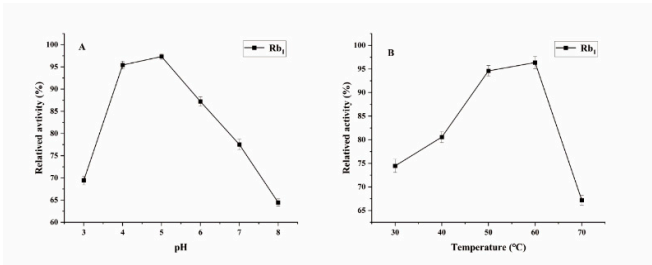


Figure 4. The optimum reaction temperature and pH for saponins transformed by the crude enzyme from *P. fimorum*. (A) pH effect on the transformation of Rb₁. (B) Temperature effect on the transformation of Rb₁.

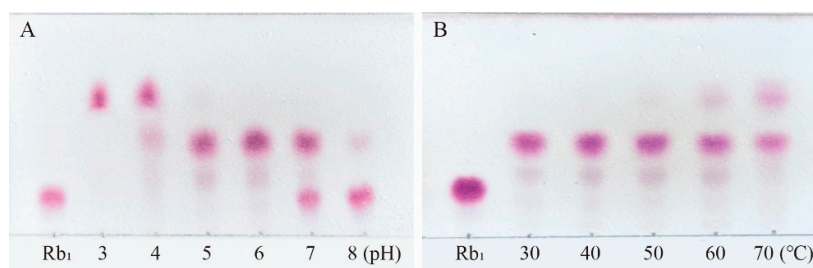


Figure 5. The optimum reaction temperature and pH for saponins transformed by β -glucosidase. (A) pH effect on the transformation of Rb₁. (B) Temperature effect on the transformation of Rb₁.

3.3. Transformation Ability of Enzymes on Monomer Ginsenosides Rb₁, Rg₁, Re and Notoginsenoside R₁

Among the total saponins of PNR, Rb₁ is PPD-type saponin, Rg₁ is PPT-type saponin, and R₁ is also PPT-type saponin which is a kind of saponin mainly contained in PNR [29]. Therefore, the enzyme's ability to transform the total saponins of PNR can be indirectly inferred from its conversion of these three saponins. Figure 6A shows the TLC analysis of the crude enzyme transformation of these three types of saponins by *P. fimorum*, where ginsenoside Rb₁ was almost completely transformed to ginsenoside CK, while ginsenoside Rg₁ and notoginseng R₁ were transformed to other minor saponins. Figure 6B presents the TLC results of the transformation of these three saponins by commercial β -glucosidase, where ginsenoside Rb₁ was predominantly transformed into ginsenosides Rd and Rg₃, while commercial β -glucosidase also transformed ginsenoside Rg₁ and notoginseng R₁, similar to the crude enzyme of *P. fimorum*.

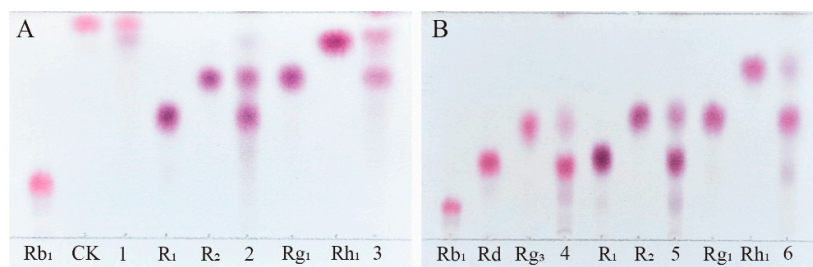


Figure 6. TLC analysis of the transformation products of different types of saponins (Rb₁, R₁ and Rg₁) by crude enzyme from *P. fimorum* and β -glucosidase, respectively. (A) TLC analysis of the transformation products by the crude enzyme from *P. fimorum*. (B) TLC analysis of the transformation products by β -glucosidase. 1, 2, 3: The transformation products of Rb₁, R₁ and Rg₁ by crude enzyme from *P. fimorum*, respectively. 4, 5, 6: The transformation products of Rb₁, R₁ and Rg₁ by β -glucosidase, respectively.

To further evaluate the enzyme's ability to transform the total saponins of PNR, we qualitatively analyzed the products of enzyme-mediated conversion of ginsenosides Rb₁, Rg₁, Re and notoginseng R₁ over 72 hours by HPLC. The 72 hours transformation products of *P. fimorum* crude enzyme transforming ginsenoside Rb₁ were found to have F₂ and CK and an unknown product (Figure 7A). The transformation products of ginsenoside Rg₁ included 20(S/R)-Rh₁, Rk₃, and Rh₄ (Figure 7B). The transformation of notoginseng R₁ yielded 20(S/R)-R₂ (Figure 7C), while the transformation of ginsenoside Re produced 20(S/R)-Rg₂, Rg₆, and F₄ (Figure 7D).

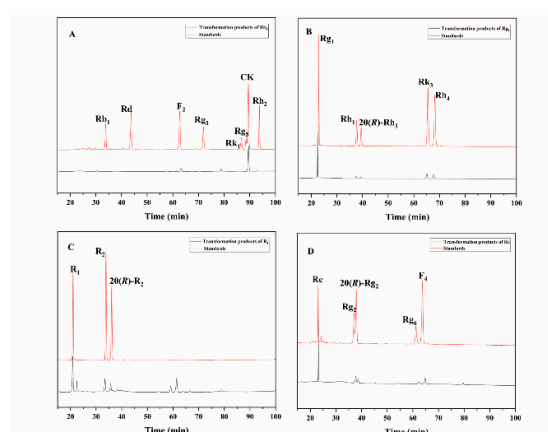


Figure 7. HPLC analysis of the transformation products of different types of saponins for 72 hours by crude enzyme from *P. fimorum*. (A) The transformation products of Rb₁. (B) The transformation products of Rg₁. (C) The transformation products of R₁. (D) The transformation products of Re.

The 72 hours conversion products of commercial β -glucosidase transforming ginsenoside Rb₁ had Rd, 20(S/R)-Rg₃, Rk₁ and Rg₅ (Figure 8A); the 72 hours conversion products of transforming ginsenoside Rg₁ had 20(S/R)-Rh₁, Rk₃ and Rh₄ (Figure 8B); and the 72 hours conversion products of transforming notoginseng R₁ had 20(S/R)-R₂ (Figure 8C); The 72 hours conversion products of transforming ginsenoside Re had 20(S/R)-Rg₂, Rg₆ and F₄ (Figure 8D). The HPLC results of these two enzymes were in general consistent with the results of TLC analyses.

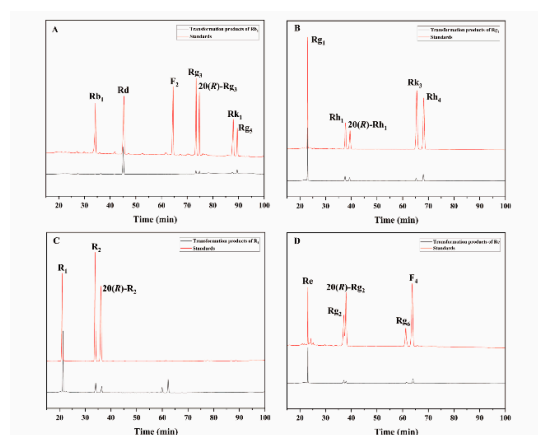


Figure 8. HPLC analysis of the transformation products of different types of saponins for 72 hours by β -glucosidase. (A) The transformation products of Rb₁. (B) The transformation products of Rg₁. (C) The transformation products of R₁. (D) The transformation products of Re.

3.4. Propose Possible Biotransformation Pathways of Major Ginsenosides Rg₁, Re, Rb₁ and Notoginsenoside R₁ of PNR

Based on qualitative and quantitative analyses by TLC and HPLC, as well as dynamic monitoring of the conversion reactions of several major saponins using linear regression equations (Table S1), we have proposed a pathway analysis for the conversion of the four major saponins in *P. notoginseng* by the crude enzyme from *P. fimorum* and commercial β -glucosidase. We also calculated the substrate conversion rates and product yields. Figure 9 presents the results of the dynamically monitored TLC analysis of the conversion of four major saponins by the *P. fimorum* crude enzyme, while Figure 10 shows the corresponding results for β -glucosidase. Table 1 summarizes the substrate conversion rates, and Figure 11 illustrates the proposed transformation pathway along with the yield analysis for each product.

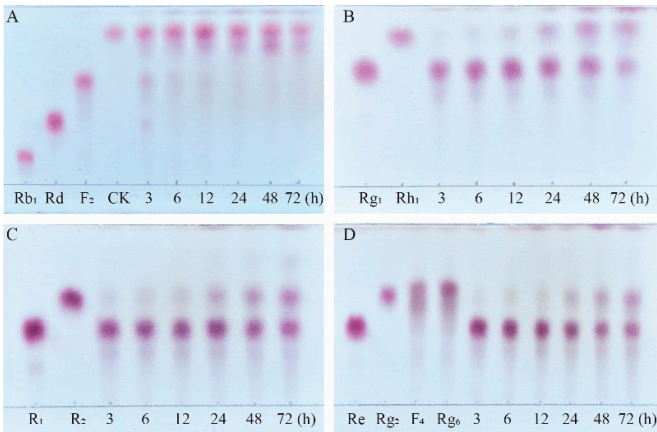


Figure 9. TLC analysis of the time-course variation in different types of saponins and their transformation products during the biotransformation process by the crude enzyme from *P. fimorum*. (A) Transformation products analysis of Rb₁ dynamic monitoring. (B) Transformation products analysis of Rg₁ dynamic monitoring. (C) Transformation products analysis of R₁ dynamic monitoring. (D) Transformation products analysis of Re dynamic monitoring.

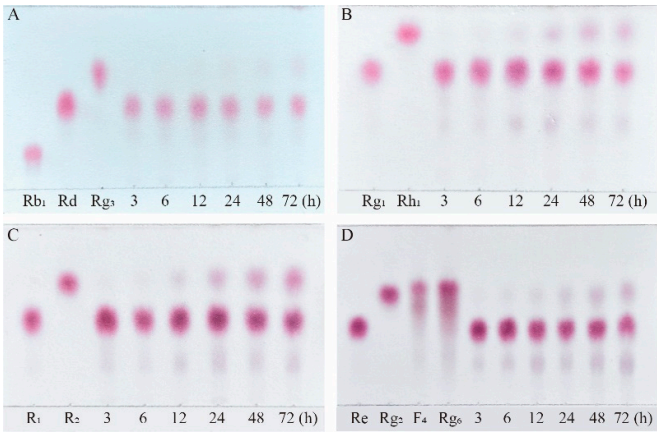


Figure 10. TLC analysis of the time-course variation in different types of saponins and their transformation products during the biotransformation process by β -glucosidase. (A) Transformation products analysis of Rb₁ dynamic monitoring. (B) Transformation products analysis of Rg₁ dynamic monitoring. (C) Transformation products analysis of R₁ dynamic monitoring. (D) Transformation products analysis of Re dynamic monitoring.

Table 1. The substrate conversion of four main saponins from the roots of *P. notoginseng* during biotransformation.

The types of enzymes	Substrates	Substrate conversion (%)
Crude enzyme from <i>P. fimorum</i>	Rb ₁	100
	Rg ₁	47.66
	R ₁	54.23
	Re	31.68
β -glucosidase	Rb ₁	100
	Rg ₁	41.94
	R ₁	41.58
	Re	41.72

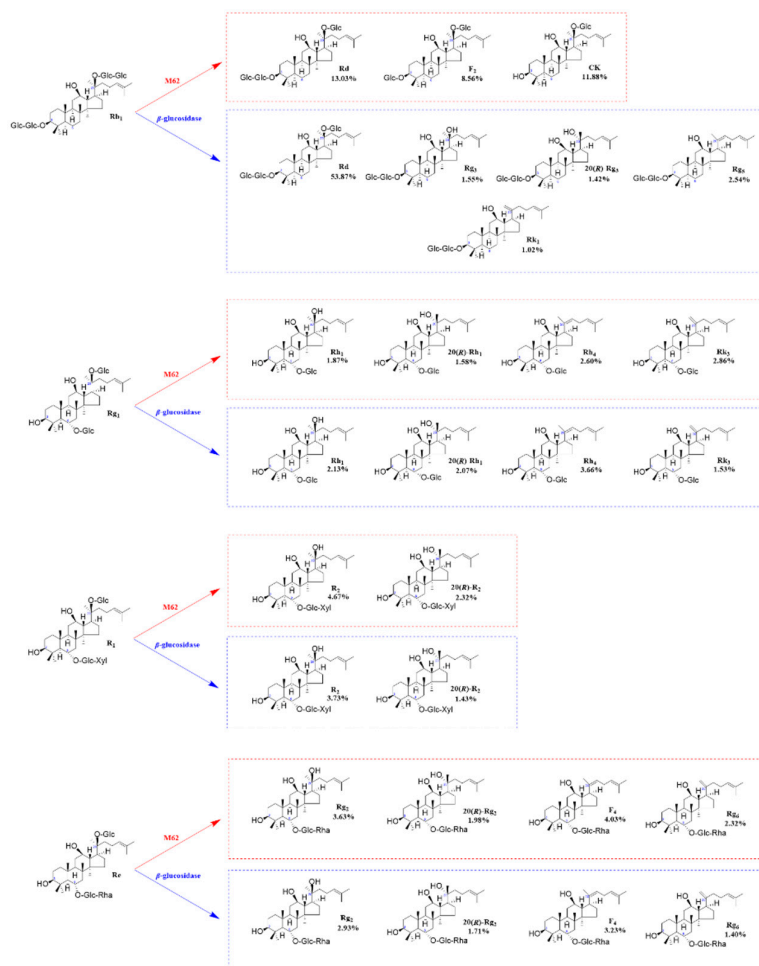


Figure 11. Structures and productivity of minor ginsenosides from the biotransformation of ginsenosides Rb₁, Rg₁, Re and notoginseng R₁ by crude enzyme from *P. fimorum* and β -glucosidase, respectively. Red dotted box: Products and yields of minor ginsenosides from biotransformation by crude enzyme from *P. fimorum*; Blue dotted box: Products and yields of minor ginsenosides from biotransformation by β -glucosidase.

3.5. Dynamic Change of Substrate Conversion of Major Ginsenosides and the Yield in the Transformation Products

The transformation analysis of several major saponins and dynamic monitoring of product generation were performed. As shown in Figure 12A, for the transformation of ginsenoside Rb₁ by *P. fimorum* crude enzyme, the substrate conversion rate reached its highest value of 100% at 6 hours. For the transformation of ginsenoside Rg₁, the highest transformation rate was 47.66% at 72 hours; for notoginseng R₁, the highest transformation rate was 54.23% at 72 hours; and for ginsenoside Re, the highest transformation rate was 31.68% at 72 hours.

As shown in Figure 12B, for the transformation of ginsenoside Rb₁ by commercial β -glucosidase, the substrate transformation rate reached its highest value of 100% at 3 hours. For the transformation of ginsenoside Rg₁, the highest transformation rate was 41.94% at 72 hours; for notoginseng R₁, the highest transformation rate was 41.58% at 72 hours; and for ginsenoside Re, the highest transformation rate was 41.72% at 72 hours.

Figure 13 shows the dynamic analysis results of the transformation product yields of four major saponins in *P. notoginseng* transformed by *P. fimorum* crude enzyme, while Figure 14 presents the corresponding results for the transformation by commercial β -glucosidase.

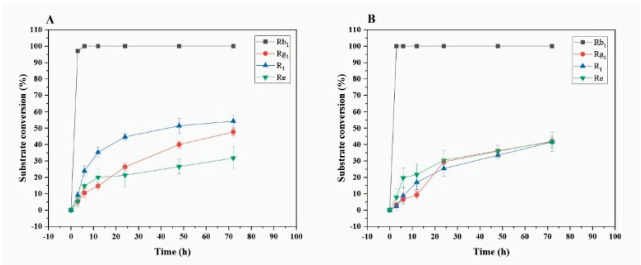


Figure 12. Dynamic changes in the substrate conversion of ginsenosides Rb₁, Rg₁, Re and notoginsenoside R₁ at different reaction time by crude enzyme from *P. fimorum* (A) and β -glucosidase (B), respectively.

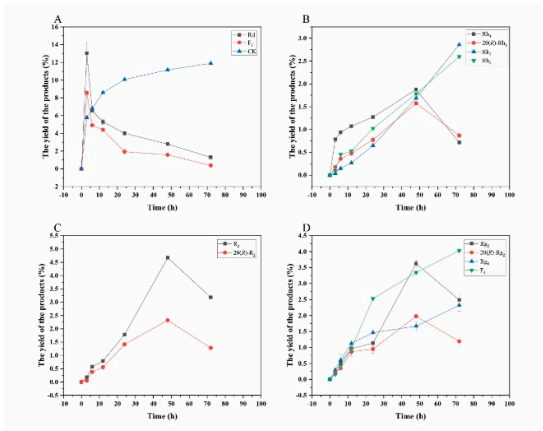


Figure 13. Dynamic changes in the yield of the transformation products of ginsenosides Rb₁ (A), Rg₁ (B), Re (D) and notoginsenoside R₁ (C) at different reaction time by crude enzyme from *P. fimorum*.

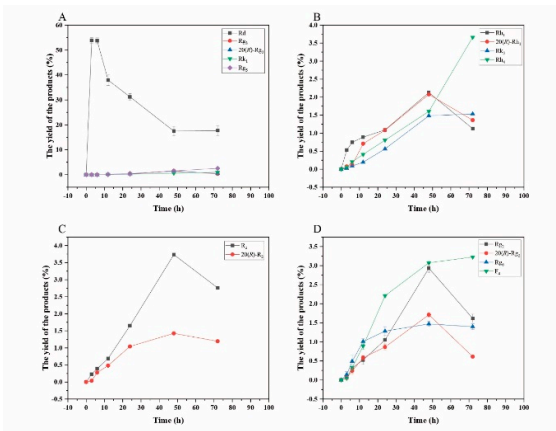


Figure 14. Dynamic changes in the yield of the transformation products of ginsenosides Rb₁ (A), Rg₁ (B), Re (D) and notoginsenoside R₁ (C) at different reaction time by β -glucosidase.

4. Discussion

In recent years, microbial transformation or biotransformation using enzymes have become a popular method for preparing minor ginsenosides, and many studies used these methods to transform the main ginsenosides for the preparation of minor ginsenosides [30–33].

In this study, *P. fimorum* was isolated from fresh *Gastrodia elata* and demonstrated the ability to transform the total saponins of PNR. The four major saponins (Rb₁, Rg₁, Re and R₁) from PNR were transformed using extracellular crude enzymes extracted from *P. fimorum*. The transformation products were thoroughly analyzed by TLC and HPLC. The transforming abilities of this fungus

were found to include deglycosylation, epimerization, and dehydration, which, to the best of our knowledge, have not been previously reported for *P. fimorum*. The crude enzyme effectively hydrolyzed glucose attached at the C-3 and C-20 positions of the major saponins, forming 20(*S/R*)-epimers at C-20 via isomerization, as well as double-bonded isomers at C-20 through dehydration.

According to most of the studies, it is known that β -glucosidase plays an important role in the conversion process against ginsenosides [27,28,30–34]. In this study, biotransformation experiments were conducted using commercial β -glucosidase to compare its transformation ability with that of *P. fimorum* crude enzyme. The results revealed significant differences in their transformation of ginsenoside Rb₁. The *P. fimorum* crude enzyme first hydrolyzed the glucose at the C-20 position of ginsenoside Rb₁ to form ginsenoside Rd, and then sequentially hydrolyzed the glucose at the C-3 position of ginsenoside Rd, ultimately efficiently transforming it into ginsenoside CK. In contrast, β -glucosidase also formed ginsenoside Rd by hydrolyzing glucose at C-20 of ginsenoside Rb₁, but then continued to hydrolyze it into 20(*S/R*)-Rg₃, which further underwent dehydration to produce double-bonded isomers Rg₅ and Rk₁ at C-20. For the other three major saponins (Rg₁, Re and R₁) the transformation abilities of commercial β -glucosidase and *P. fimorum* crude enzyme were nearly identical, as both enzymes efficiently transformed the major saponins into minor saponins.

Based on the analysis of transformation products by HPLC, several uncharacterized products were observed during the transformation of major saponins by *P. fimorum* crude enzyme. For example, for the transformation products F₂ and CK of ginsenoside Rb₁ there is an unknown product in the middle of them, while the transformation product 20(*S/R*)-R₂ from notoginseng R₁ was followed by several unknown products. Similarly, the transformation product F₄ from ginsenoside Re was followed by an unknown product. These uncharacterized products may be new saponin derivatives, as their retention times closely resemble those of known minor saponins. If monomeric saponins can be efficiently transformed by *P. fimorum* crude enzyme in large quantities through fermentation and subsequently purified via column chromatography, there is potential to obtain novel saponin derivatives.

For the HPLC analysis of the conversion products of commercial β -glucosidase, we found that two unknown products were also present in the conversion products of notoginseng R₁. By analysing the transformation products of other major saponins, we can conclude the regularity that ginsenosides Rb₁, Rg₁ and Re can be transformed by β -glucosidase to form 20(*S/R*)-epimers, C-20(21) and C-20(22) double-bond isomers. Therefore, we hypothesise that notoginseng R₁ can not only be transformed to 20(*S/R*)-R₂ only, but it can be further transformed to the notoginsenoside T₅ with a C-20(21) double bond but also to 3 β , 12 β -Dihydroxydammarane-(*E*)-20(22), 24-diene-6-O- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranoside with a C-20(22) double bond [4].

In addition, in this study, the transformation of various major saponins by *P. fimorum* crude enzyme and commercial β -glucosidase were monitored dynamically in terms of the conversion of the substrates and the yield of the conversion products. The results of the dynamic monitoring can thus be used for the qualitative production of a particular conversion product with a higher yield. In summary, this study investigated the transformation of major saponins from PNR using two enzymes, which is expected to improve the utilisation of PNR and increase the methods of producing minor saponins.

5. Conclusions

In this experiment, biotransformation of the major saponins in PNR was carried out using the extracellular crude enzyme of the plant endophytic fungus *P. fimorum* and commercial β -glucosidase. The extracellular crude enzyme, extracted in our laboratory, could be further purified to enhance transformation efficiency. Nevertheless, the biotransformation experiments demonstrated that the crude enzyme efficiently transformed ginsenoside Rb₁ with a 100% substrate conversion rate, producing ginsenoside CK. While commercial β -glucosidase also achieved 100% conversion of ginsenoside Rb₁, it generated multiple products, including Rd, 20(*S/R*)-Rg₃, Rk₁, and Rg₅, which followed a different transformation pathway compared to *P. fimorum* crude enzyme. For the total

saponins in PNR, including notoginseng R₁, ginsenoside Rg₁, and Re, both *P. fimorum* extracellular crude enzyme and commercial β -glucosidase exhibited similar transformation abilities, transforming the major saponins into various minor saponins. This study also proposed a transformation pathway and analyzed the transformation rates and product yields through dynamic monitoring. Overall, the study introduces two methods for transforming major saponins in *P. notoginseng* into minor saponins, thereby improving the utilization of PNR and expanding the production pathways of minor saponins. These findings may be applicable to other parts of *P. notoginseng* and lay the groundwork for the development of genetically engineered strains and enzyme immobilization technologies for large-scale production of minor saponins.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: The control experiment of the condition optimization process; Table S1: Linear regression equation of various saponins.

Author Contributions: Conceptualization, F.L.; methodology, F.L. and R.Z.; software, J.Y. and D.L.; validation, X.C., Y.Y. and X.Y.; formal analysis, F.L., R.Z. and X.Y.; investigation, F.L.; resources, X.C., Y.Y. and X.Y.; data curation, F.L.; writing—original draft preparation, F.L.; writing—review and editing, F.L., R.Z. and X.Y.; visualization, F.L.; supervision, X.Y.; project administration, F.L. and X.Y.; funding acquisition, X.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the National Natural Science Foundation of China, grant number 32460114.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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