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Diversity and epimedium biotransformation potential of cultivable endophytic fungi associated with *Epimedium brevicornum Maxim* in the Qinling Mountains, China

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Abstract

Background The use of biocatalysis technology to manufacture rare natural products can solve the contradiction between the market demand for rare natural products in large health industry fields and the protection and sustainable development of wildlife resources. However, the currently available research on fungal endophytes, which are great potential resources for glycoside hydrolase biocatalysts, is still insufficient. In this study, endophytic fungi from *Epimedium brevicornum Maxim.* were isolated in the Qinling Mountains, identified and tested for their potential to biotransform epimedium extracts into minor epimedium flavonoids.

Results A total of 84 representative morphotype strains were isolated and identified via ITS rDNA sequence analyses and were grouped into 32 taxa. The Shannon–Wiener index (*H'*, 3.089) indicated that *E. brevicornum Maxim.* harboured abundant fungal resources. Ten strains showed strong β-glucosidase activity and exhibited the ability to biotransform major epimedium flavonoids into deglycosylated minor epimedium flavonoids, such as baohuoside I and icaritin, via various glycoside-hydrolysing pathways. Among these strains, strains 8509 and F8889, which were initially characterized as *Aspergillus ochraceus* and *A. protuberus*, have the potential for further development in the biotransformation of epimedium extracts into minor epimedium flavonoids because of their excellent biosafety, enzyme activity, and enzymatic characteristics. The enzyme activity of the crude enzyme obtained by freeze-drying from the F8509 fermentation broth supernatant reached 78.24 ± 2.48 U/g. Further research revealed that major glycosylated flavonoids from 100 g/L epimedium extracts were bio-transformed completely into minor deglycosylated flavonoids in 90 min after the addition of 1 g/L crude enzyme. In addition, the liquid phase separation conditions were optimized, and ethyl alcohol and water were ultimately used as the mobile phase for efficient separation of the conversion products at equal flow degrees.

Conclusions This study not only identified a series of candidates for the biotransformation of minor epimedium flavonoids but also provided an efficient purification method. More importantly, this study also demonstrated the important value of endophytes in the biotransformation of rare natural products.

Keywords Endophytic fungi, Biotransformation, *Epimedium brevicornum Maxim*, Diversity

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Background

Herba epimedii, a classical Chinese herbal medicine and an effective ingredient for diet therapy, has been widely used in China for thousands of years because of its remarkable cardiovascular protection effects and its ability to ameliorate osteoporosis and neurological defects [1]. Epimedium flavonoids are the most valued and major active components in *H. epimedii* [2]. The major epimedium flavonoids include epimedin A, B, C and icariin, which account for more than 52% of the total epimedium flavonoids [3], and the deglycosylated minor epimedium flavonoids, such as baohuoside I and icaritin, which are present in very low amounts (< 0.15%) in most natural ginseng plants. These flavonoids are generally more pharmaceutically active than major glycosylated epimedium flavonoids because of their higher bioavailability, and deglycosylated minor epimedium flavonoids, such as baohuoside I and icaritin, are present in very low amounts (< 0.15%) in most natural ginseng plants and are generally more pharmaceutically active than major glycosylated epimedium flavonoids because of their good capillary absorption properties in intestinal epithelial cells [4, 5]. Therefore, the conversion of major glycosylated epimedium flavonoids to baohuoside I and icaritin has attracted considerable attention and been widely studied [6–8].

The general methods for preparing baohuoside I and icaritin include direct acid hydrolysis and enzyme/microbial bioconversion [6, 9]. Direct acid hydrolysis usually has poor selectivity, leads to low yields, creates product that are difficult to purify, and easily causes environmental pollution [9]. The enzyme bioconversion method has the advantages of notable selectivity, mild reaction conditions, high efficiency, and environmental friendliness [10, 11]. Some reactions that are difficult to carry out via chemical methods can be completed via enzyme bioconversion, which is considered a highly effective method for the production of high-value epimedium flavonoids [12]. However, enzymes, as biological macromolecule catalysts, have limitations in large-scale industrial use, such as easily reduced activity and the high cost associated with enzyme expression and purification [13]. The use of immobilized enzyme technology may solve these technical defects. For example, the relative enzyme activity of immobilized glycosyltransferase was still maintained at 67.8% after the immobilized enzyme was reused six times, which was significantly greater than that of the free enzyme [14]. However, the catalytic efficiency, material transfer and enzymatic reaction conditions of the immobilized enzyme were still limited by the immobilization medium. Whole-cell catalysis, an intermediate approach between fermentation and in vitro enzyme catalysis, offers several advantages over the use of purified or immobilized enzymes. These include significantly reduced catalyst costs, increased stability due to residual cell wall compounds, and no need for external cofactor addition [15]. In recent years, whole cells have been used in some studies to biotransform and synthesize epimedium flavonoids. For example, recombinant *E. coli* expressing the α-L-rhamnosidase^{syn} AnRhaE from *A. nidulans* was used as a whole-cell biocatalyst for the biotransformation of epimedin C to icariin [16]. Lin et al. constructed a recombinant *E. coli* strain that simultaneously expresses α-L-rhamnosidase SPRHA2 and the β-glucosidase and was capable of transforming icariin into icaritin with high conversion efficiency (yield rate, 95.23%) [17].

Overall, these findings highlight the potential of whole-cell catalysis as a promising approach for the future industrial production of high-value epimedium flavonoids.

Endophytes generally inhabit healthy plant tissues without causing disease or injury to the host [18]. Owing to their special living environment and long-term coexistence with their hosts, endophytes can produce diverse novel metabolites and incorporate genetic information on bioactive substance biosynthesis from their host plants [19]. For example, Xu et al. isolated the endophyte *Penicillium* sp. F5 from *Polygonum cuspidatum* and demonstrated the biotransformation of resveratrol to pterostilbene [20]. In another study, 20 endophytic fungi were shown to be capable of mogroside V transformation, suggesting that plant endophytic fungi are valuable resources for the biocatalysis of natural compounds [21]. Therefore, endophytes are widely considered valuable resources of natural enzymes for the biotransformation of several types of natural compounds, such as flavonoids, alkaloids and saponins [22–24].

The Qinling Mountains (32°30′-34°45′ N, 104°30′-112°45′ E), which is located mainly in southern Shaanxi Province in Central China [25], constitute the most important natural climatic boundary between the subtropical and warm temperate zones of China and support astonishingly high biodiversity and original species resources [26]. Despite numerous reports on the survey of wild medicinal plant resources in the Qinling Mountains [27], the community and function of their endophytes have been investigated less often. In fact, during the long-term coevolution of endophytes and their host wild plants, endophytic fungi have developed rich genetic resources to adapt to and participate in the biotransformation of host phytochemicals [28]. *Epimedium brevicornum Maxim.*, which is a traditional medicinal plant in the Qinling Mountains, contains various epimedium flavonoids and is thus believed to be rich in the epimedium flavonoid glucosidase resource; it was therefore used to

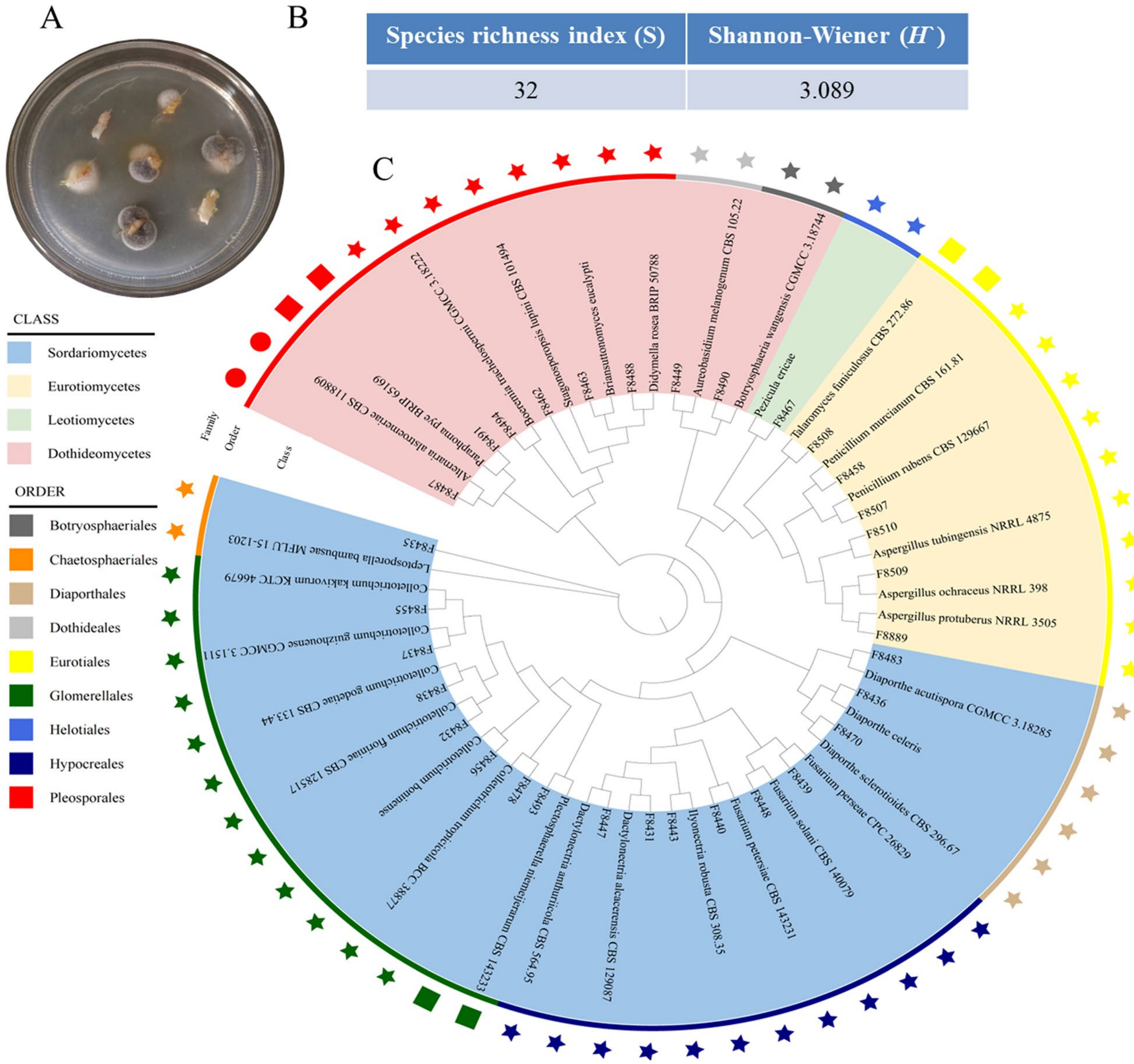


Fig. 1 Isolation and diversity analysis of the culturable endophytic fungi from *E. brevicornum Maxim*. **A** The isolates were subsequently grown on PDA plates. **B** Species richness and Shannon–Wiener diversity index analysis. **C** Phylogenetic tree of culturable endophytic fungi from *E. brevicornum Maxim.* in the Qinling Mountains, China

identify novel fungi with potent β-glucosidase activity for the production of minor epimedium flavonoids. In this study, endophytic fungi were first isolated from wild *Epimedium brevicornum Maxim.* from the Qinling Mountains. Epimedium extracts were used as substrates to examine the biotransformation potential of epimedium flavonoids. To the best of our knowledge, this is the first report on the diversity and biotransformation activities of the epimedium flavonoids of cultivable endophytic fungi associated with *E. brevicornum Maxim.* in the Qinling Mountains, China.

Results

Isolation, sequencing, identification, and diversity analyses of the endophytic fungi from *E. brevicornum Maxim*

In this study, a total of 84 fungal colonies were successfully isolated from *E. brevicornum Maxim.* on potato dextrose agar (PDA) media (Fig. 1A). As shown in Fig. 1B, the species richness index (S) and Shannon–Wiener index (H'), two important parameters for diversity analysis, were 32 and 3.089 for *E. brevicornum Maxim.*, respectively. All the isolates were grouped into 32 taxa by conducting searches with their ITS rDNA regions using

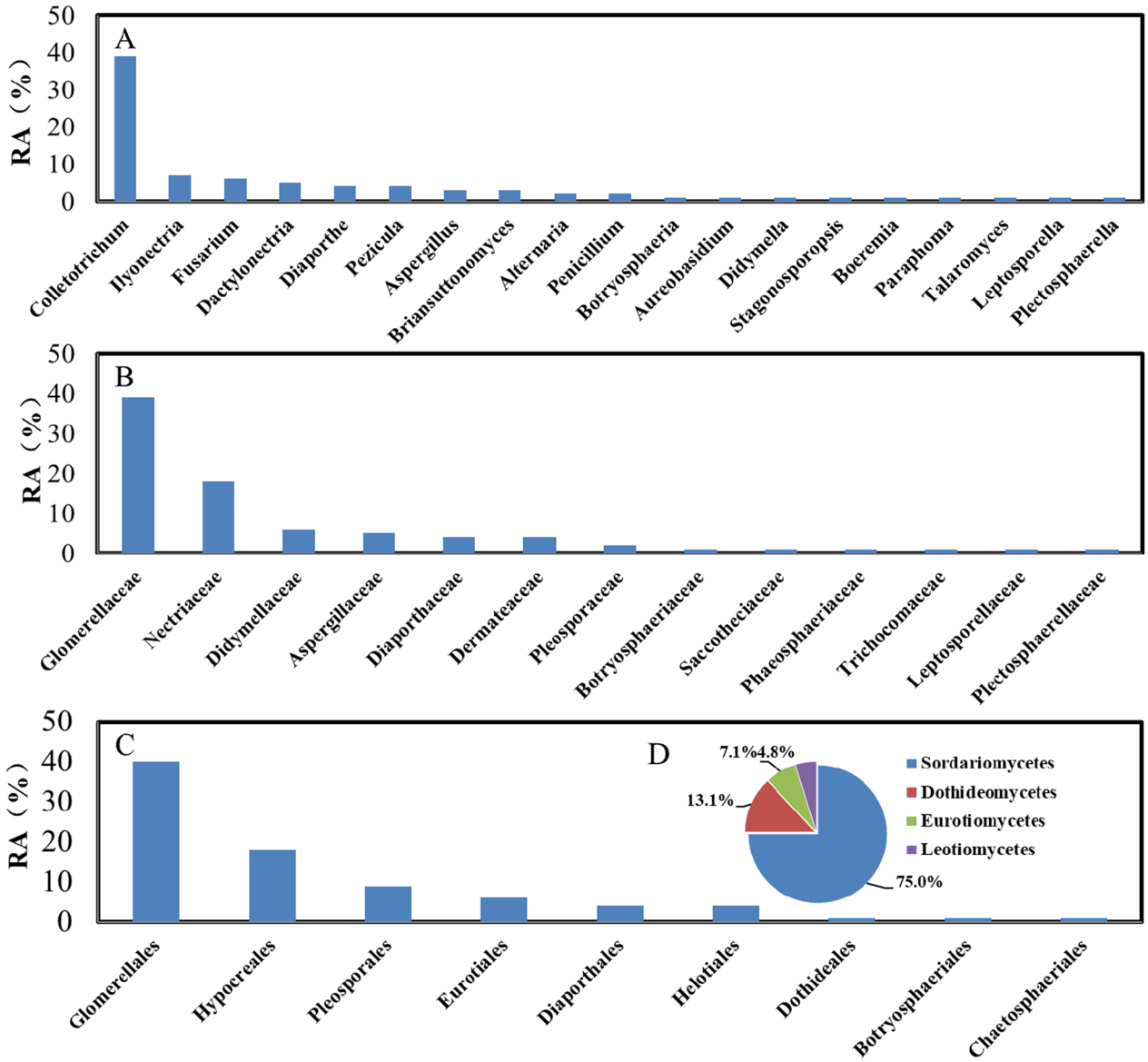


Fig. 2 The relative abundance (RA, %) of endophytic fungi at the genus (**A**), family (**B**), order (**C**), and class (**D**) levels

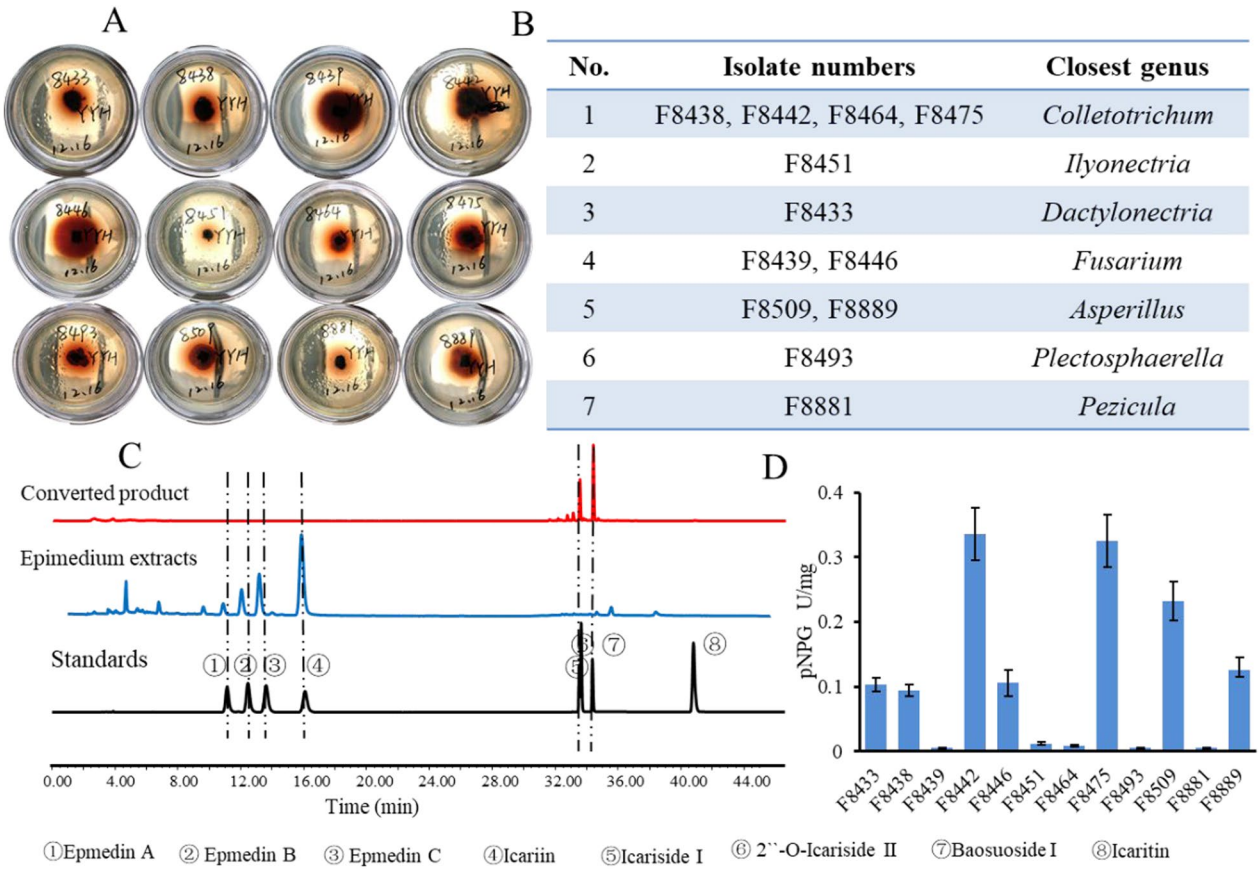
the BLAST in NCBI GenBank (Supplementary information files (1)), and phylogenetic trees were constructed using the maximum likelihood method (Fig. 1C).

Relative abundance (RA) analyses of endophytic fungi from *E. brevicornum Maxim*

The relative abundances (RAs) of these isolates at the genus, family, order, class, and phylum levels are shown in Fig. 2A–D, respectively. At the genus level, *Colletotrichum* (RA, 46.43%) was most abundant, followed by *Ilyonectria* (RA, 8.33%) and *Fusarium* (RA, 7.14%). At the family level, Glomerellaceae (RA, 46.43%), Nectriaceae (RA, 21.43%), and Didymellaceae (RA, 7.14%) were the three most abundant groups in this study. At the phylum level, all fungi isolated from *E. brevicornum Maxim.* were identified as Ascomycota.

Analysis of the biotransformation characteristics of endophytic fungi exhibiting β-glucosidase activity and *Epimedium*

As shown in Fig. 3A, 12 out of the 84 endophytic fungi presented the greatest capacity for β-glucosidase production. Among these endophytes, strains F8433, F8438, F8439, and F8442 belong to the genus *Colletotrichum*; strains F8439 and F8446 belong to genera of *Fusarium*; strains F8509 and F8889 belong to the genus *Aspergillus*;



No.	Isolate numbers	Closest genus
1	F8438, F8442, F8464, F8475	*Colletotrichum*
2	F8451	*Ilyonectria*
3	F8433	*Dactylonectria*
4	F8439, F8446	*Fusarium*
5	F8509, F8889	*Asperillus*
6	F8493	*Plectosphaerella*
7	F8881	*Pezicula*

Fig. 3 Screening of β-glucosidase-producing endophytic fungi and analysis of their properties for the bioconversion of the major flavonol glycosides from the extracts of epimedium. **A** Screening of β-glucosidase produced on esculin-R2A agar. **B** The characteristics of the β-glycosidase-producing isolates and their taxonomic status. **C** HPLC spectra of the flavonol glycoside standards and bioconversion products from 7 strains with β-glucosidase activity. **D** Comparison of the activity of the *p*NPG enzyme in different strains

and strains F8451, F8433, F8493, and F8881 belong to the genera *Ilyonectria*, *Dactylonectria*, *Plectosphaerella*, and *Pezicula*, respectively (Fig. 3B). To evaluate the ability of these endophytic fungi with β-glucosidase activity to biotransform epimedium flavonoids, the composition of epimedium flavonoids in the initial and post fermentation broths on a shaker was determined via HPLC (Fig. 3C). The results revealed that (1) the total flavonoids from the epimedium extracts included mainly major glycosylated epimedium flavonoids, such as epimedin A, epimedin B, epimedin C, and icariin; (2) ten endophytic fungi with β-glucosidase activity (excluding F8881 and F8439) had the capacity to biotransform major epimedium flavonoids into 2``-O-icariside II and baohuoside I; and (3) the activity of β-glucosidase was significantly different among the different strains (Fig. 3D). Although strains F8475 and F8442 presented relatively high β-glycosidase activity, they belong to the genus *Colletotrichum*, which is an opportunistic pathogen [29]. Considering its β-glycosidase activity and safety characteristics, the strains F8509 and F8889, which were initially characterized as *A. ochraceus* and *A. protuberus*, respectively, showed greater potential for use in functional enzyme preparation.

The enzymatic properties of the F8509 and F8889 fermentation broths were comprehensively studied to evaluate their application potential. As shown in Fig. 4A and Fig. 4B, the optimal temperature and pH were determined to be 60 °C and pH 5.0, respectively. The effects of organic solvents on the fermentation broth were evaluated, as shown in Fig. 4C–E. The results revealed that the glycosidases derived from the two strains tolerated certain organic solvents, and the glycosidase activity reached 70% of the normal enzyme activity in 10% methanol, ethanol and DMSO. In general, natural products are insoluble in water, and methanol, ethanol, and DMSO are commonly used as cosolvents. Therefore, the organic solvent tolerance of enzymes is an important characteristic index of enzymes, which determines the application

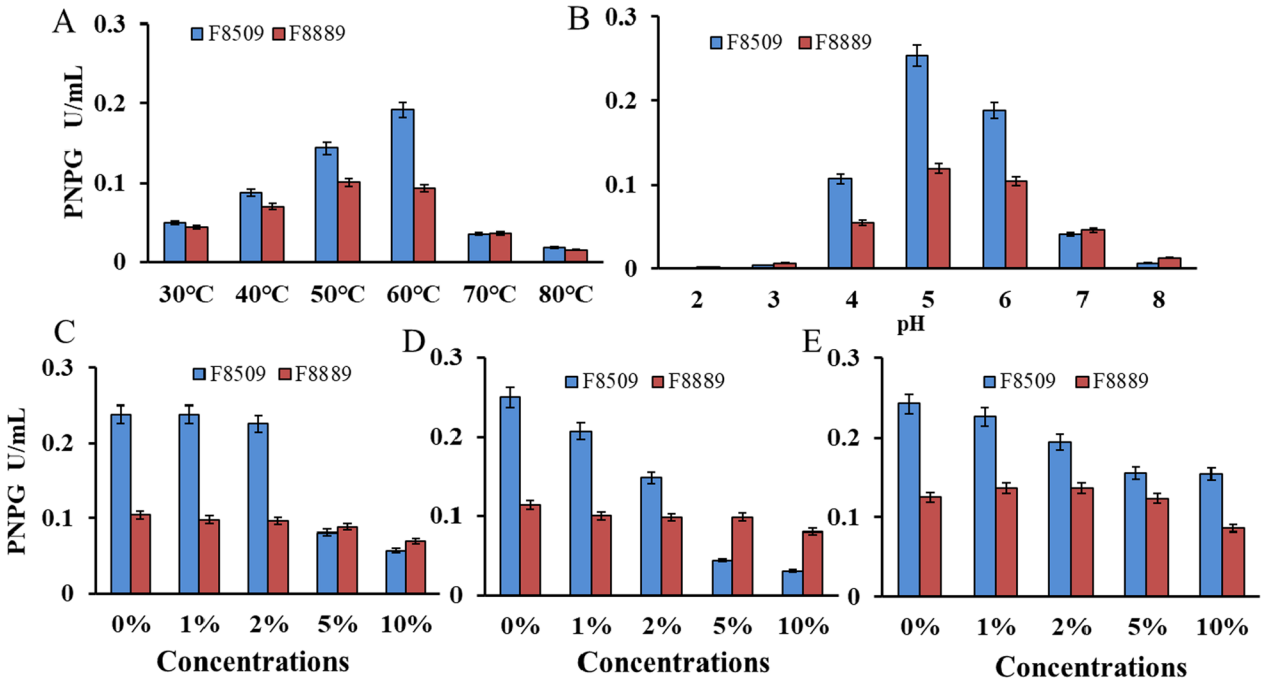


Fig. 4 Effects of reaction temperature (**A**), pH (**B**) and organic solvents (**C** methanol, **D** ethanol, **E** DMSO) on *p*NPG enzyme activity

potential of enzymes in natural biotransformation where they are insoluble in water [30].

Strain F8509 was subsequently grown in a 10 L fermenter. After five days of cultivation, the β-glucosidase activities of the supernatant and freeze-dried fermentation broth were determined, and the activities of the β-glycosidases were 0.23 ± 0.02 U/mL and 78.24 ± 2.48 U/g, as shown in Fig. 5A. Further research revealed that the glycosylated flavonoids from 100 g/L epimedium extracts were biotransformed completely into minor deglycosylated flavonoids within 90 min by supplementation with 1 g/L crude enzyme (Fig. 5B). Among the initial epimedium extracts, the flavones of the epimedium extracts were epimedin A (7%), epimedin B (16%), epimedin C (25%) and icariin (52%) (Fig. 5C). After biotransformation, only the high-value components 2``-O-icariside II (55%) and baohuoside I (45%) were retained in the extracts (Fig. 5D).

HPLC analysis of flavonoid compounds

As shown in Fig. 6A, a series of epimedium flavonoid glycoside standards with similar structures were effectively separated via a gradient of acetonitrile and water in an HPLC system (Waters, Alliance Separations module 2695, 2998 detector; Waters, Milford, MA, USA) with a C18 column (YMC-Pack ODS-AQ, 250 mm × 4.6 mm, 5 μm; YMC, Japan). However, acetonitrile is somewhat toxic, and the HPLC separation method of the gradient mobile phase is also not conducive to separation and purification in preparation because of process instability and high equipment requirements. Therefore, to achieve high-efficiency separation of the main target component and impurities of the biotransformation products (Fig. 6B), HPLC separation methods were optimized by using ethyl alcohol and water as the mobile phase and changing the composition of the mobile phase under isocratic conditions using a C18 column (YMC-Pack ODS-AQ, 250 mm × 2.6 mm, 5 μm, YMC, Japan). Ultimately, the flow rate was set to 1 mL/min, and the ratio of ethyl alcohol to water used as the mobile phase was 70:30. The target components and impurities were also efficiently isolated via isocratic HPLC (Fig. 6C). Furthermore, the analytical method was optimized in a Waters 2695 system via semipreparative reversed-phase high-performance liquid chromatography (Waters, Alliance Separations module 1525, 2998 detector; Waters, Milford, MA, USA) using a C18 column (YMC-Pack ODS-AQ, 250 mm × 10 mm, 20 μm, YMC, Japan). The separation and purification results were consistent with the semipreparative reversed-phase high-performance liquid chromatography results obtained from the Waters 2695 (Fig. 6D). In addition, the method was stable for multiple separations and purifications (Fig. 6E). The solutions containing the components of peaks 3 and 4 were collected in the semipreparative liquid phase preparation process, and the molecular weights were analysed via mass

A

No.	Sample	β-glucosidase activity (U/mL or U/g)	Volume/Weight (mL or g)	Total enzyme activity (U)
1	Fermentation broth	0.23 ±0.02	5000	1150±100
2	Freeze-dried crude enzymes	78.24±2.48	10	782.4±24.8

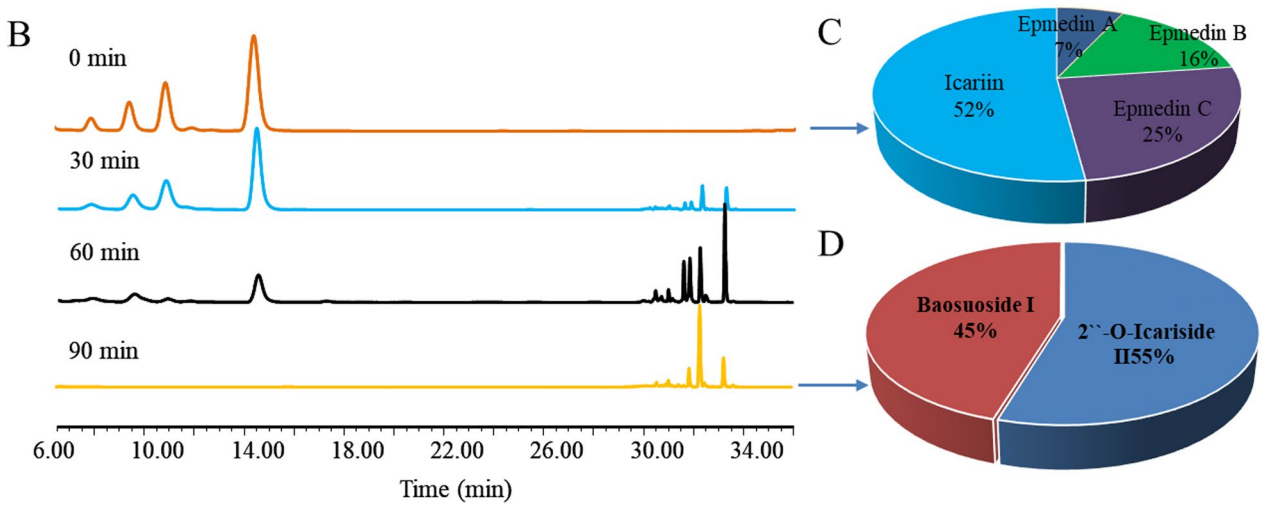


Fig. 5 Enzyme preparation and transformation process. **A** Enzyme activity of fermentation broth and freeze-dried crude enzyme. **B** HPLC analysis of the bioconversion products at different biotransformation times. The composition of flavonoids in the initial epimedium extracts (**C**) and the converted product (**D**)

spectrometry (Fig. 6F). The results revealed that one of the compounds obtained from peaks 3 and 4 had a peak of (M+H)+at m/z 659.2297 and 513.1734, respectively, which was consistent with the molecular weights of the 2``-O-icariside II and baohuoside I standard substances. Sugar groups on the highly glycosylated flavonoids were gradually hydrolysed to yield low-glycosylation flavonoids.

Discussion

Because β-glucosidase is deeply involved in the biosynthesis and transformation of natural plant products, endophytes with glycosidase activity have great potential for promoting the accumulation of natural plant products and improving plant quality. Although the endophytic fungi of epimedium have been studied previously [31], the screening of endophytic fungi with glycosidase activity in epimedium has not been reported. In addition, the endophytic fungal community composition is significantly influenced by the host geographic area, habitat environment and growth cycle [32]. Therefore, it is still highly important to investigate resources and screen the functional strains of endophytes from different regions and different plant species.

Epimedium brevicornum Maxim. is widely distributed in northwestern China, including Gansu, Shaanxi, Ningxia and He'nan Provinces [33]. Many active compounds, including epimedin A, epimedin B, epimedin C, icariin and baohuoside I, have been identified from epimedium, among which the prenylflavonoid flavonoids icariin, epimedin C and baohuoside I are considered the major bioactive components and are used as marker compounds for quality control [34]. In this study, the main epimedium flavonoid glycosides identified were epimedin A, epimedin B, epimedin C, and icariinin epimedium extracts (Fig. 3C), which was in accordance with previous studies [33]. Compared with other large flavonoid glycosides, deglycosylated baohuoside I has greater pharmacological activity and bioavailability.

Compared with icariin, baohuoside I, although present in low amounts (<0.15%) in the raw material of the epimedium extracts, exhibited a wider range of pharmacological activities [4]. Baohuoside I has been demonstrated to have a significant therapeutic effect on various diseases, such as sexual dysfunction, osteoporosis, and cancer, because it has better bioavailability in vivo and is more easily absorbed by the capillaries of intestinal epithelial cells because of its lower polarity. Baohuoside I restored erectile function caused by bilateral cavernous

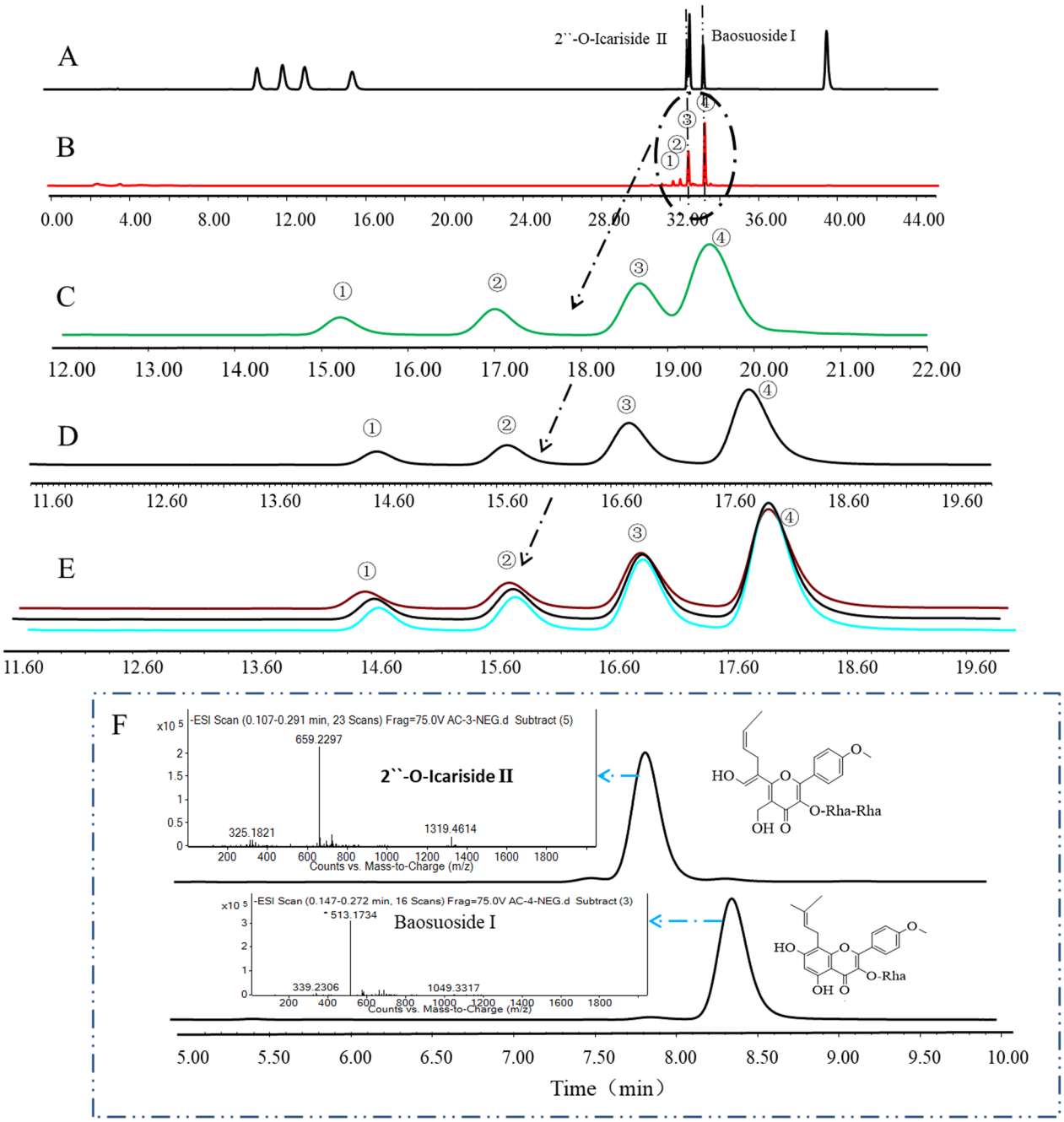


Fig. 6 Optimization of the bioconversion product separation and purification process and identification of the conversion product structure. Gradient HPLC was used for the detection of flavonol glycoside standards (**A**) and bioconversion products (**B**). Separation of the conversion products (**C**) and separation process optimization (**D**) by equi-HPLC. Stability verification of the purification process (**E**) and mass spectrum analysis of the purification products (**F**)

nerve injury (BCNI) in rats by promoting the differentiation of adipose-derived stem cells into Schwann cells and improving erectile dysfunction (BCNI) [35–37]. It has been suggested that baohuoside I has antiosteoporotic activity, which is associated with the induction of bone marrow stromal cell differentiation into osteoblasts while inhibiting adipocyte formation, regulating immune functions, and providing antioxidant activity [38].

Most epimedium flavonoids are extracted from epimedium plants, but this method fails to meet the increasing market demand for baohuoside I. The preparation of baohuoside I by acid hydrolysis has been gradually eliminated

Table 1 Summary of the transformation pathways of baohuoside I from different glycosylated flavonoids

No	Enzyme Name	Taxon (Accession number)	Transformation Pathway	References
1	β-glucosidase (Tpebgl3)	*Thermomotoga petrophila* DSM 13995	Icariin → baohuoside I	[30]
2	β-glucosidase (IagBgl1)	*Ignisphaera aggregans*	Icariin → baohuoside I	[4]
3	β-1,3-glucanase (CtLam55)	*Chaetomium thermophilum*	Icariin → baohuoside I	[10]
4	β-glucosidase (Dth3)	*Dictyoglomus thermophilum* DSM3960	Epimedin A → baohuoside I; Icariin → baohuoside I	[39]
5	Dextranase	–	Icariin → baohuoside I	[40]
6	Cellulase	–	Icariin → baohuoside I	[41]
7	β-xylosidase	*Dictyoglomusthermophilum DSM3960*	Epimedin B → baohuoside I	[42]
8	–	*Aspergillus ochraceus* F8509	Epimedin A → icariin → baohuoside I Epimedin B → icariin → baohuoside I Epimedin C → 2``-O-icariside II	In this study
9	–	*Aspergillus protuberus* F8889	Epimedin A → icariin → baohuoside I Epimedin B → icariin → baohuoside I Epimedin C → 2``-O-icariside II	In this study

–: Not determined

because of its low efficiency and amount of environmental pollution, and baohuoside I has been replaced by bioenzymes for green biomanufacturing [12]. Table 1 summarizes recent research on the bioconversion of baohuoside I, mainly using biological enzymes. According to the previous studies, a single enzyme can only complete the biotransformation of baohuoside I from a single substrate, and the simultaneous transformation of multiple substrates is difficult [4, 10, 30, 39–42].

Endophytes are the main biological sources of whole-cell catalysis and form mixed enzyme systems for plant natural product biotransformation during long-term interactions [43]. In previous studies, only a few strains were shown to be able to directly convert the main components of epimedium extract into rare and highly active baohuoside I. In our study, we isolated endophytic fungi from *E. brevicornum Maxim.* and screened their β-glucosidase activities. This study provides a target strain for the efficient whole-cell catalysis of rare and highly active baohuoside I and icaritin.

At present, whole-cell catalysis can be used to inexpensively and quickly transform low-value biomass into high-value products, but some technical obstacles remain, including the screening of core enzymes, the construction of multienzyme expression systems, and the transport of substances across cell membranes [44]. Therefore, the recovery of key enzymes and the construction of the required engineered microorganisms remain challenging tasks. Omics techniques have been widely used to identify novel functional genes, whereas metabolic engineering and metabolic flux analysis help establish complete and efficient natural product synthesis pathways in microbial cell factories. These advanced technologies will greatly promote the efficient production of Chinese herbal active ingredients such as epimedium flavones in the near future [12].

The separation and purification of similar natural products is difficult, and high-performance liquid chromatography is an important method for solving the problem of natural products with similar structures. In previous studies, acetonitrile and water were used for the liquid phase separation of icariin via gradient separation methods [30], which caused certain difficulties in the preparation of large quantities of the liquid phase. First, acetonitrile is more expensive, and the use of solvents for the preparation of large amounts and the separation costs are high. Second, acetonitrile has a certain degree of toxicity, and residue is retained during the product preparation process, resulting in operator toxicity. Third, the use of gradient separation, which places greater requirements on the preparation equipment, results in considerable separation difficulties. Therefore, the use of low-toxicity and low-cost organic solvents and equal gradient separation methods are urgently needed for the manufacturing of a large number of natural products. In addition, the liquid phase separation conditions were optimized for the conversion products. This provides a reliable technical method for the preparation of high-purity baohuoside I. The possible biotransformation pathway is shown in Fig. 7.

Conclusion

In this study, the diversity and epimedium biotransformation potential of the endophytic fungus *E. brevicornum Maxim.* were investigated for the first time. Our results revealed that *E. brevicornum Maxim.* harboured abundant fungal endophyte resources, and a total of 84 representative morphotype strains were isolated and

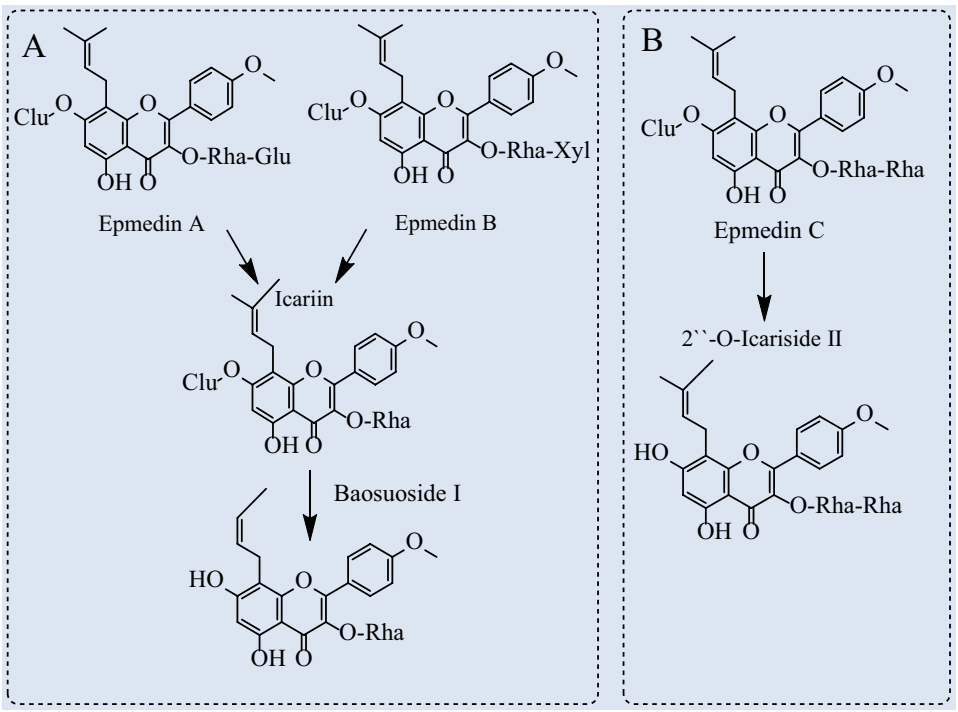


Fig. 7 Schematic diagram of the direct biotransformation of high-glycosylation flavonoids (epimedin A, epimedin B, and epimedin C) into low-glycosylation flavonoids (icariin, baohuoside I, 2``-O-icariside II) by glucosidase

identified. Among the 84 isolates, 10 strains exhibited strong β-glucosidase activity and exhibited the ability to biotransform epimedium via various glycoside-hydrolysing pathways. In addition, the liquid phase separation conditions were optimized to efficiently obtain the conversion products, and methanol and water were finally used for separation under equal flow. In this study, endophytes with biocatalytic activity for rare baohuoside I bio fabrication were identified, and an efficient isolation method was created. More importantly, this study also demonstrated the important value of endophytes in the biotransformation of rare natural products.

Materials and methods

Materials

The standard epimedin A, epimedin B, epimedin C, icariin, icariside I, 2``-O-icariside II, baohuoside I, icaritin and epimedium extracts were purchased from Chengdu DeSiTe Biological Technology Co., Ltd. Artificial substrates, including *p*NP-β-D-galactopyranoside (pNPG) and esculin, were purchased from Aladdin Chemical Co. Ltd. Methanol and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). All other reagents and chemicals were at least of analytical grade.

Plant collection and isolation of fungal endophytes

In August 2022, wild and healthy *E. brevicornum Maxim.* plants were randomly collected from Yingpan town, Shaanxi Province, China (33°48′25′′ N, 108°56′19′′ E, elevation, 1057 m), and identified by the associate researcher XinWei Shi at Xi'an Botanical Garden of Shaanxi Province. Plant sample collection was performed according to a previously described method [45]. The endophytic fungal isolation procedure was carried out within 24 h of sample collection according to a previously described protocol [45]. Finally, the pure isolates were stored at − 80 °C in a 20% glycerol solution at the Engineering Center of QinLing Mountains Natural Products, Shaanxi Provincial Institute of Microbiology.

Molecular identification and phylogenetic analyses

The mycelia of the endophytic fungi, which were purified and cultured on PDA media plates at 28 °C for 7 days, were pulverized in liquid nitrogen with a mortar and pestle. The genomic DNA was extracted using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction

Kit (Dalian, China). Genomic DNA was then used as the template for PCR amplification of the nuclear ribosomal DNA internal transcribed spacer (ITS) using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCG G-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) according to a previously described method [25]. All the sequences were aligned by MEGA 5.05 using an alignment prepared with Clustal W (Arizona State University, Tempe, USA), and positions containing gaps were deleted to avoid potential biases in the phylogenetic analysis. Phylogenetic trees were constructed using the maximum likelihood method on the basis of the Tamura–Nei model using MEGA software 5.05.

Screening of β-glucosidase-producing endophytic fungi

The β-glucosidase activities were screened via the methods described in a previous study [46]. The isolates, which were subsequently grown on a PDA plate at 28 °C for 5 days, were inoculated on esculin-R2A agar. Endophytic fungi with β-glucosidase activities can hydrolyse esculin and appear as colonies surrounded by a reddish-brown to dark brown zone. Esculin-R2A plates without inoculated fungi were used as controls. The esculin-R2A agar contained 1 g/L esculin, 0.5 g/L ferric citrate and 15.2 g/L R2A agar and was autoclaved at 121 °C for 20 min. All the assays were performed in triplicate.

Transformation of total epimedium extracts

The endophytic fungi with β-glucosidase activity were precultured on PDA media for 7 d at 28 °C and then inoculated into 250 mL flasks containing 30 mL of fermentation culture mixture (20 g of glucose, 10 g of yeast powder, 0.5 g of $NaNO_3$, 1 g of KH_2PO_4, 2 g of Na_2HPO_4, 0.2 g of $FeSO_4$, 0.1 g of $ZnSO_4$, 0.2 g of $CuSO_4$, 1 g of $CaCl_2$, 0.5 g of $MgSO_4{\cdot}7H_2O$ in 1 L of distilled water, initial pH of 6.5) and incubated at 28 °C on a rotary shaker at 180 rpm. The total epimedium extracts were first dissolved in methanol at a final concentration of 2 g/L and then added to an equal volume of culture broth. The extracts were incubated with the endophytic fungi and cultivated on a rotary shaker at 180 rpm for 72 h. The fungi were continually cultured for 48 h under these conditions, and 2 mL of fungal suspension was aseptically removed from a shake flask culture and centrifuged to remove the precipitate. The residue was dissolved in methanol, and analysis of the biotransformation ability of the total epimedium extracts was carried out via high-performance liquid chromatography.

Enzyme activity assay

An activity assay was performed in a mixed solution containing 5 mM *p*NPG at pH 5.0 (50 mM citric acid–potassium phosphate buffer) and an equal volume of fermentation broth at 50 °C for 30 min, and the reactions were terminated by adding double the volume of 2 M Na_2CO_3 solution. The activity of the enzyme was measured spectrophotometric ally at 405 nm, and a calibration curve was designed using *p*NP as a standard [47, 48]. One unit (U) was defined as the amount of fermentation broth required to release 1 μmol *p*NP per minute. Enzyme activity was calculated as follows:

$$U = \frac{C \times V}{t \times v} \times N$$

Note: *C*, concentration of *P*-nitrophenol, mmol; V, volume of the reaction system, mL; *N*, dilution ratio; *t*, reaction time, min; *v*, supernatant liquid volume, mL.

Enzyme characterization assay

The subsequent detection method was the same as that described above. The biological characteristics of the enzyme were evaluated by changing the pH and reaction temperature of the reaction system and adding organic solvents to the reaction system. Specifically, enzyme activity was measured at pH values ranging from 2.0 to 8.0 to evaluate the effect of the optimal pH of fermentation broth on β-glucosidase activity. The effect of temperature on fermentation broth activity was investigated by measuring the activity at temperatures ranging from 30 to 80 °C (10 °C interval). The effects of organic solvents on β-glucosidase activity in the fermentation broth were determined by adding 0%, 1%, 2%, 5% and 10% DMSO, alcohol or methanol. All the assays were performed in triplicate.

Enzyme preparation and transformation process

The endophytic fungi with β-glucosidase activity were precultured on PDA media for 7 days at 28 °C, inoculated into 250 mL flasks containing 50 mL of potato dextrose broth and incubated at 28 °C on a rotary shaker for 3 days at 180 rpm. The obtained culture was inoculated into a 10 L fermentation bioreactor (Yangge Bioengineering Equipment Co., Ltd, Shanghai, China) containing 5 L of fermentation culture mixture (20 g of glucose, 10 g of yeast powder, 0.5 g of $NaNO_3$, 1 g of KH_2PO_4, 2 g of Na_2HPO_4, 0.2 g of $FeSO_4$, 0.1 g of $ZnSO_4$, 0.2 g of $CuSO_4$, 1 g of $CaCl_2$, and 0.5 g of $MgSO_4.7H_2O$ in 1 L of distilled water, initial pH of 6.5) and incubated at 28 °C at 400 rpm. After five days of cultivation, the fermentation mixture was centrifuged to remove the precipitate, and the supernatant was freeze-dried using a Heto PowerDry LL3000 Freeze Dryer (Thermo, USA). The obtained enzyme and epimedium extracts were dissolved in 50 mM citric acid–potassium phosphate buffer and subjected to catalysis in a water bath at 50 °C. Samples were collected and freeze-dried every 30 min, after which the conversion product was determined by HPLC.

Analytical methods

Eight standard epimedium flavonoids were first prepared from a 1 g/L methanol solution, and the mixed standard samples containing eight standard epimedium flavonoids were diluted to 100 mg/L with a methanol solution. Using the HPLC method described by An et al. (2022) with minor modifications [47], all samples were quantitatively analysed by HPLC (Waters, Alliance Separations module 2695, 2998 detector; Waters, Milford, MA, USA) using a C18 column (YMC-Pack ODS-AQ, 250 mm × 4.6 mm, 5 μm, YMC, Japan) with a column temperature of 30 °C. The mobile phases were A (water) and B (acetonitrile). Gradient elution started with 72% solvent A (water) and 28% solvent B (acetonitrile) in the first 25 min, followed by solvent A changing from 72 to 30% and solvent B changing from 28 to 70% from 26–30 min, solvent A remaining at 30% and solvent B remaining at 70% from 31–42 min, and finally solvent A changing from 30 to 72% and solvent B changing from 70 to 28% from 43–45 min. All samples were detected by absorption at 270 nm, with an injection volume of 10 μL.

1. Optimization of the HPLC separation and purification process

Using a C18 column (YMC-Pack ODS-AQ, 250 mm × 2.6 mm, 5 μm, YMC, Japan), the HPLC separation methods were optimized for high-efficiency separation of the transformation products using ethyl alcohol and water as the mobile phase by changing the composition of the mobile phase under isocratic conditions (Waters, Alliance Separations module 2695, 2998 detector; Waters, Milford, MA, USA).

Purification was performed via semipreparative reversed-phase high-performance liquid chromatography (Waters, Alliance Separations module 1525, 2998 detector; Waters, Milford, MA, USA) using a C18 column (YMC-Pack ODS-AQ, 250 mm × 10 mm, 20 μm, YMC, Japan) with a column temperature of 30 °C. All samples were detected by absorption at 270 nm, with an injection volume of 100 μL.

2. Mass spectrometry (MS)

The MS instrument was operated in negative ion mode and was set to total ion chromatogram mode with the following mass conditions: capillary voltage = 1.0 kV, low collision energy = 6 V, source temperature = 100 °C, desolvation temperature = 500 °C, and desolvation gas flow = 800 L/h (Waters I-Class Vion IMS Qtof). Data acquisition and processing were conducted using Masslynx version 4.2 (Waters, Manchester, UK).

Statistical analyses

All the results are expressed as the means ± SEMs. Graphs were prepared using Excel 2010 (Microsoft, USA).

Supplementary Information

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Supplementary material 1.

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Author contributions

S-JM conceived and performed the experiments and drafted the manuscript with input from W-JX. CA optimized the HPLC separation and purification conditions. HD and Q-WZ isolated the endophytic fungi. CL and YL extracted the genome and amplified the genes. X-WS collected the plant samples. J-JZ and J-JS revised the manuscript. All the authors have read and agreed to the published version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Ma HP, He XR, Yang Y, Li MX, Hao DJ, Jia ZP. The genus *Epimedium*: An ethnopharmacological and phytochemical review. J Ethnopharmacol. 2011;34(3):519–41.
2. Qian HQ, Wu DC, Li CY, Liu XR, Han XK, Peng Y, Zhang H, Zhao BY, Zhao Y: A systematic review of traditional uses, phytochemistry, pharmacology and toxicity of *Epimedium koreanum* Nakai. J Ethnopharmacol. 2024; 318.

3. Gao Y, Shi W, Tu C, Li P, Zhao GY, Xiao XH, Wang JB, Bai ZF: Immunostimulatory activity and structure-activity relationship of epimedin B from *Epimedium brevicornmu* Maxim. Fronti Pharmacol. 2022; 13.
4. Xie JC, Xu H, Jiang JC, Zhang N, Yang J, Zhao J, Wei M. Characterization of a novel thermostable glucosetolerant GH1 beta-glucosidase from the hyperthermophile Ignisphaera aggregans and its application in the efficient production of baohuoside I from icariin and total epimedium flavonoids. Bioorg Chem. 2020;104: 104296.
5. Yan HM, Song J, Jia XB, Zhang ZH. Hyaluronic acid-modified didecyldimethylammonium bromide/d-a-tocopheryl polyethylene glycol succinate mixed micelles for delivery of baohuoside I against non-small cell lung cancer: in vitro and in vivo evaluation. Drug Delivery. 2017;24(1):30–9.
6. Cheng LY, Zhang H, Cui HY, Cheng JM, Wang WY, Wei B, Liu F, Liang H, Shen XL, Yuan QP: A novel α-L-Rhamnosidase renders efficient and clean production of icaritin. J Clean Prod. 2022; 341.
7. Li Q, Ge L, Zheng DY, Zhang XM, Zhao LG: Screening and characterization of a GH78 α-L-rhamnosidase from *Aspergillus terreus* and its application in the bioconversion of icariin to icaritin with recombinant β-glucosidase. Enzyme Microbial Tech. 2022; 153.
8. Chen W, Liu GG, Zhang Y: Production of Rhamnosyl Icariside II by snailase hydrolysis of Epimedium wushanense extracts. Heliyon. 2024; 10(1).
9. Wang L, Xu DZ, Dai QX, Chu CC, Li D, Liu G. Research progress in the preparation of pharmaceutical formulations based on supercritical fluid technology. Chinese Sci Bull. 2021;66(10):1187–94.
10. Shen YP, Wang M, Zhou JW, Chen YF, Xu LL, Wu MR, Xia GH, Tam JP, Yu JN, Teng XY *et al*: Eco-efficient biphasic enzymatic hydrolysis for the green production of rare baohuoside I. Enzyme Microbial Tech. 2019; 131.
11. Xie JC, Zhao J, Zhang N, Xu H, Yang J, Ye J, Jiang JC: Efficient production of isoquercitin, icariin and icariside II by a novel thermostable a-l-rhamnosidase PodoRha from Paenibacillus odorifer with high a-1, 6-/a-1, 2-glycoside specificity. Enzyme Microbial Tech. 2022; 158.
12. Zhang XL, Tang BL, Wen SJ, Wang YT, Pan CX, Qu LB, Yin YL, Wei YJ: Advancements in the Biotransformation and Biosynthesis of the Primary Active Flavonoids Derived from Epimedium. Molecules; 2023, 28(20).
13. Mohamad NR, Marzuki NHC, Buang NA, Huyop F, Wahab RA. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnol Biotec Eq. 2015;29(2):205–20.
14. Li ZY, Liu XC, Wu ZS, Huang XJ, Long HY, Yue JS, Cao SS, Fan DD. Study on one-step purification and immobilization of glycosyltransferase by modified Fe_3O_4 for the synthesis of rare ginsenoside Rh2. ACS Appl Mater Interfaces. 2024;16(12):14561–72.
15. Wachtmeister J, Rother D. Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale. Curr Opin Biotechnol. 2016;42:169–77.
16. Lyu Y, Zeng WZ, Du GC, Chen J, Zhou JW. Efficient bioconversion of epimedin C to icariin by a glycosidase from *Aspergillus nidulans*. Bioresour Technol. 2019;289:121612.
17. Lin Y, Chen WW, Ding B, Guo M, Liang M, Pang H, Wei YT, Huang RB, Du LQ. Highly efficient bioconversion of icariin to icaritin by whole-cell catalysis. Microb Cell Fact. 2023;22:64.
18. Jia M, Chen L, Xin HL, Zheng CJ, Rahman K, Han T, Qin LP: A Friendly Relationship between Endophytic Fungi and Medicinal Plants: A Systematic Review. Front Microbiol. 2016, 7.
19. Vasundhara M, Kumar A, Reddy MS: Molecular Approaches to Screen Bioactive Compounds from Endophytic Fungi. *Front Microbiol*. 2016, 7.
20. Xu Z, Tian J, L Gan, Y Tian. Discovery of the Endophytic Fungi from *Polygonum cuspidatum* and Biotransformation of Resveratrol to Pterostillbene by the Endophyte *Penicillium* sp. F5. *Appl Biochem Micro*, 2020, 56, pages 313–320.
21. Lin WX, Jiang Q, Dong YM, Xiao YW, Wang Y, Gao BL, Zhu D. Plant endophytic fungi exhibit diverse biotransformation pathways of mogrosides and show great potential application in siamenoside I production. Bioresour Bioprocess. 2024;11:42.
22. Cui L, Wu SQ, Zhao CA, Yin CR. Microbial conversion of major ginsenosides in ginseng total saponins by *Platycodon grandiflorum* endophytes. J Ginseng Res. 2016;40(4):366–74.
23. Cheng LY, Zhang H, Cui HY, Davari MD, Wei B, Wang WY, Yuan QP. Efficient enzyme-catalyzed production of diosgenin: inspired by the biotransformation mechanisms of steroid saponins in *Talaromyces stollii* CLY-6. Green Chem. 2021;23(16):5896–910.
24. Park B, Hwang H, Lee J, Sohn SO, Lee SH, Jung MY, Lim HI, Park HW, Lee JH. Evaluation of ginsenoside bioconversion of lactic acid bacteria isolated from kimchi. J ginseng res. 2017;41(4):524–30.
25. An C, Ma SJ, Shi XW, Xue WJ, Liu C, Ding H: Diversity and Antimicrobial Activity of Endophytic Fungi Isolated from *Chloranthus japonicus* Sieb in Qinling Mountains, China. *Int J Mol Sci.* 2020, 21(17).
26. Ma T, Hu Y, Russo IM, Nie Y, Yang T, Xiong L, Ma S, Meng T, Han H, Zhang X, Bruford MW, Wei F. Walking in a heterogeneous landscape: dispersal, gene flow and conservation implications for the giant panda in the Qinling Mountains. Evol Appl. 2018;11(10):12686.
27. Zhang Y, Li J, Wang Y, Liang Q. Taxonomy of *Epimedium* (Berberidaceae) with special reference to Chinese species. Chin Herb Med. 2021;14(1):20–35.
28. Yao L, Wang J, He J, Huang L, Gao W. Endophytes, biotransforming microorganisms, and engineering microbial factories for triterpenoid saponins production. Crit Rev Biotechnol. 2021;41(2):249–72.
29. Liu F, Ma ZY, Hou LW, Diao YZ, Wu WP, Damm U, Song S, Cai L. Updating species diversity of *Colletotrichum*, with a phylogenomic overview. Stud Mycol. 2022;101:1–56.
30. Lu S, Zou K, Guo B, Pei J, Wang Z, Xiao W, Zhao L. One-step purification and immobilization of thermostable β-glucosidase on Na-Y zeolite based on the linker and its application in the efficient production of baohuoside I from icariin. Bioorg Chem. 2022;121:105690.
31. Zhu ZB, Fan JY, Guo QS, Liu ZL, Zhu GS: The growth and medicinal quality of Epimedium wushanense are improved by an isolate of dark septate fungus. *Pharm biol.* 2015, 53(9).
32. Fan LL, Li YL, Wang XL, Leng FF, Li SW, Zhu N, Chen K, Wang YG. Culturable endophytic fungi community structure isolated from *Codonopsis pilosula* roots and effect of season and geographic location on their structures. *Bmc Microbiol.* 2023, 23(1).
33. Li B, Lima MRM, Nie YH, Xu L, Liu X, Yuan HC, Chen C, Dias AC, Zhang XY: HPLC-DAD Fingerprints Combined With Multivariate Analysis of Epimedii Folium From Major Producing Areas in Eastern Asia: Effect of Geographical Origin and Species. *Front Pharmacol.* 2021, 12.
34. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China; China Medical Science Press: Beijing, China, 2020; Volume 1.
35. Ge PY, Guo YX, Shen J. Icariside II facilitates the differentiation of ADSCs to SCs via let-7i/STAT3 axis to preserve erectile function. Biol Res. 2019;52:54.
36. Khan M, Maryam A, Qazi JI, Ma TH. Targeting Apoptosis and Multiple Signaling Pathways with Icariside II in Cancer Cells. Int J Biol Sci. 2015;11(9):1100–12.
37. Zheng T, Zhang TB, Zhang WX, Lv KL, Jia DH, Yang F, Sun YY, Lian J, Wang R. Icariside II facilitates the differentiation of ADSCs to schwann cells and restores erectile dysfunction through regulation of miR-33/GDNF axis. Biomed Pharmacother. 2020;125:109888.
38. Xi YH, Jiang TW, Yu JM. Xue MT, Xu N, Wen JK, Wang WH, He HL, Ye XJ. Preliminary studies on the anti-osteoporosis activity of Baohuoside I. *Biomed. Pharmacother*. 2019, 115, 108850.
39. Zhang SS, Lu CN, Cao SP, Li Q, Wu GW, Zhao LG: Efficient production of icariin and baohuoside I from *Epimedium Folium* flavonoids by fungal α-L-rhamnosidase hydrolysing regioselectively the terminal rhamnose of epimedin C. *Biotechnol Biof Biop.* 2023, 16(1).
40. Yang QX, Wang L, Zhang LX, Xiao HB. Baohuoside I production through enzyme hydrolysis and parameter optimization by using response surface and subset selection. J Mol Catal B-Enzym. 2013;90:132–8.
41. Cui L, Zhang ZH, Sun E, Jia XB, Qian Q: Effect of β-cyclodextrin complexation on solubility and enzymatic hydrolysis rate of icariin. *J Nat Sci, Biol Med.* 2013, 4(1).
42. Li Q, Wu T, Zhao LG, Pei JJ, Wang ZZ, Xiao W. Highly Efficient Biotransformation of Astragaloside IV to Cycloastragenol by Sugar-Stimulated β-Glucosidase and β-Xylosidase from *Dictyoglomus thermophilum*. J Microbiol Biotechnol. 2019;29(12):1882–93.
43. Choudhary M, Gupta S, Dhar MK, Kaul S. Endophytic Fungi-Mediated Biocatalysis and Biotransformations Paving the Way Toward Green Chemistry. Front Bioeng Biotech. 2021;6:664705.

44. Lin B, Tao Y. Whole-cell biocatalysts by design. Microb Cell Fact. 2017;16:106.
45. An C, Ma SJ, Shi XW, Xue WJ, Liu C, Ding H: Isolation, diversity, and antimicrobial activity of fungal endophytes from *Rohdea chinensis* (Baker) N.*Tanaka* (synonym *Tupistra chinensis* Baker) of Qinling Mountains, China. *Peerj.* 2020, 8.
46. An C, Ma SJ, Shi XW, Liu C, Ding H, Xue WJ: Diversity and Ginsenoside Biotransformation Potential of Cultivable Endophytic Fungi Associated With *Panax bipinnatifidus* var. *bipinnatifidus* in Qinling Mountains, China. *Front Pharmacol.* 2022, 13.
47. Saroj P, Manasa P, Narasimhulu K. Biochemical characterization of thermostable carboxymethyl cellulase and β-Glucosidase from *Aspergillus fumigatus* JCM 10253. Appl Biochem Tech. 2022;194:2503–27.
48. Kim SA, Jeong EB, Oh DK. Complete bioconversion of protopanaxadiol-type ginsenosides to compound K by extracellular enzymes from the isolated strain *Aspergillus tubingensis*. J Agric Food Chem. 2021;69(1):315–24.

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