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ORIGINAL PAPER

Highly selective biotransformation of ginsenoside Rb_1 to Rd by the phytopathogenic fungus *Cladosporium fulvum* (*syn. Fulvia fulva*)

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Abstract Fourteen phytopathogenic fungi were tested for their ability to transform the major ginsenosides to the active minor ginsenoside Rd. The transformation products were identified by TLC and HPLC, and their structures were assigned by NMR analysis. *Cladosporium fulvum*, a tomato pathogen, was found to transform major ginsenoside Rb_1 to Rd as the sole product. The following optimum conditions for transforming Rd by *C. fulvum* were determined: the time of substrate addition, 24 h; substrate concentration, 0.25 mg ml^{-1}; temperature, 37°C; pH 5.0; and biotransformation period, 8 days. At these optimum conditions, the maximum yield was 86% (molar ratio). Further, a preparative scale transformation with *C. fulvum* was performed at a dose of 100 mg of Rb_1 by a yield of 80%. This fungus has potential to be applied on the preparation for Rd in pharmaceutical industry.

Keywords Biotransformation · *Cladosporium fulvum* (*syn. Fulvia fulva*) · Ginsenoside Rb_1 · Ginsenoside Rd

Introduction

Ginsenosides are the major active components of ginseng [8]. It has been reported that the protopanaxadiol-type ginsenoside Rd has the ability to protect neurons from neurotoxic chemicals [6], enhance the differentiation of neural stem cells [9] and prevent the contraction of blood vessels [12]. Rd is a potential drug candidate. However, it is difficult to separate Rd from ginseng because of its low concentration and to prepare it by chemical synthesis because of its complex structure. A possible pathway for preparation of Rd is through transforming structurally related compounds to it.

The amount of the major ginsenoside Rb_1 is high in ginseng, and it has the same aglycone (protopanaxadiol) as Rd (Fig. 1). Rb_1 has one more sugar residue at the C-20 position than Rd. Theoretically, Rd can be obtained by hydrolysis of Rb_1 by removing a glucose residue at position C-20. Chemical transformation usually has poor selectivity and generates more environmental pollution. Biotransformation has more potential for conversion because of its high specificity and environmental compatibility. Some studies have looked for suitable microbes or enzymes that can transform Rb_1 into Rd [1–3, 5, 7, 10]. However, most of them have lack of specificity and can further transform Rd into F_2 and compound K (C–K) or Rg_3 and Rh_2 (Fig. 1), which resulted in a low yield of Rd. We now report a high specificity transformation of Rb_1 to Rd by a phytopathogenic fungus *C. fulvum*. This biotransformation has great potential to be applied in the preparation of Rd in the pharmaceutical industry.

Materials and methods

Materials and microorganisms

Standard ginsenosides were purchased from Chengdu Mansite Biotechnology Co. Ltd, China. A mixture of ginsenosides Rb_1, Rb_2 and Rc, and their pure single compounds, were prepared from Chinese white ginseng roots (5 years

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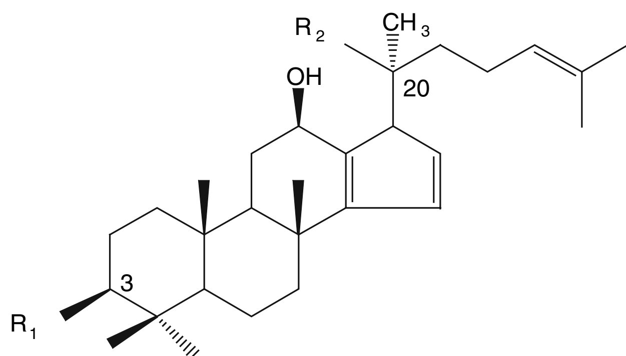

Ginsenoside	R_1	R_2
Rb_1	O-glc(2→1)glc	O-glc(6→1)glc
Rb_2	O-glc(2→1)glc	O-glc(6→1)arap
Rc	O-glc(2→1)glc	O-glc(6→1)araf
Rd	O-glc(2→1)glc	O-glc
GXVII	O-glc	O-glc(6→1)glc
CO	O-glc	O-glc(6→1)arap
Mb	O-glc	O-glc(6→1)araf
20(S)-Rg_3	O-glc(2→1)glc	OH
F_2	O-glc	O-glc
Rh_2	O-glc	OH
Compound K	OH	O-glc

glc: β-D-glucopyranosyl arap: α-L-arabinopyranosyl

araf: α-L-arabinofuranosyl

Fig. 1 Chemical structures of protopanaxadiol ginsenosides

old) cultivated in Fusong, Jilin Province of China, according to Chen et al. [1] and identified by ^{13}C-NMR.

Phytopathogenic fungi *Cladosporium fulvum* (*syn. Fulvia fulva*), *Cylindrocarpon destructans*, *Botrytis cinerea*, *Gloeocercospora sorghi*, *Alternaria porri*, *Fusarium oxysporum* f. sp. *lycoporsici, Fusarinm solani, Colletotrichum coccodes, Alternaria alternata, Alternaria solani, Fusarium oxysporum, Cladosporium cucumerinum, Plasmopara viticola,* and *Fusarium graminearum* were isolated from plants cultivated locally and gifted by the Jilin Academy of Agricultural Sciences.

Cultivation and biotransformation

The testing spores were incubated in V8 juice liquid medium (per litre: 200 ml of V8 juice, 2 g of $CaCO_3$) with shaking (130 rpm) at 28°C. After 8 days, the spores were harvested from the cultures by filtration through absorbent gauze and then centrifugation at 8,000*g* at 15°C for 20 min. The spores were washed once with 20 mM acetate buffer (pH 5.0) to remove the residual media and resuspended in 20 mM acetate buffer (pH 5.0).

For biotransformation, the spores were incubated in 20 mM acetate buffer (pH 5.0) with shaking (130 rpm) at 28°C. After 24 h, the ginsenoside (in 20 mM acetate buffer and filtered through a 0.2 μm filter) was added to the flasks containing spores (final spore concentration: 5×10^6 spores ml^{-1}). The culture was incubated for 10 days and then filtered and centrifuged. The supernatant was extracted with *n*-butanol. The *n*-butanol extract was concentrated and dried under vacuum. The residue was resuspended in 37% acetonitrile in water (v/v) and subjected to HPLC analysis. The conversion yield of the product was determined based on the peak area ratio in the HPLC analysis.

Preparative scale biotransformation

A solution of ginsenoside Rb_1 (100 mg) in 20 mM acetate buffer (10 ml, pH 5.0) was added to the same buffer containing the spores of *C. fulvum* preincubated for 24 h to give a final substrate concentration of 0.25 mg ml^{-1} and a final spore concentration of 5×10^6 spores ml^{-1}. After 8 days of incubation on a rotary shaker (130 rpm) at 37°C, the mixture was filtered and the filtrate was extracted with *n*-butanol. The *n*-butanol extract was evaporated under vacuum and the residue was further purified by preparative HPLC. The pure metabolite was identified by HPLC and ^{13}C-NMR.

Analysis

Thin layer chromatography (TLC) was carried out using a silica gel G 60 plate and a developing solvent consisting of chloroform, methanol and water (65:35:10, v/v/v, lower phase). The ginsenosides developed on the plate were stained by spraying with 5% (v/v) sulphuric acid in ethanol, and then heating at 110°C for 5 min. High performance liquid chromatography (HPLC) analysis was performed using Shim-pack PREP-ODS (H) columns (4.6 mm × 250 mm, 5 μm) connected to an HPLC system (Shimadzu, Japan), eluted at 1.0 ml min^{-1} with acetonitrile/water, and monitored via absorbance at 203 nm. Preparative HPLC was performed with a preparative Shim-pack PREP-ODS (H) column (20 mm × 250 mm, 5 μm), and eluted with acetonitrile/water (37.5:62.5, v/v) at 5 ml min^{-1}. ^{13}C-NMR spectra were carried out on a Bruker Av 600 NMR spectrometer at 150 MHz with CD_3OD as the solvent and TMS as the internal standard.

Results and discussion

Screening of ginsenoside Rd-producing fungi

Fourteen phytopathogenic fungi were initially tested for their ability to convert protopanaxadiol-type ginsenosides into Rd. For efficiency, a mixture of ginsenosides

Rb_1, Rb_2 and Rc was used as the substrate. HPLC and TLC were used to monitor the biotransformation process. The results indicated that all of the tested fungi can transform one or more protopanaxadiol-type ginsenosides. The possible metabolites of each biotransformation are listed in Table 1. Among the fourteen fungi, the tomato pathogen *C. fulvum* showed the most selective activity. This fungus transformed ginsenoside Rb_1 to the minor ginsenoside Rd as a final product. *C. fulvum* has potential to be used in industry to prepare ginsenoside Rd with high yield from major ginsenoside Rb_1 and was chosen for further study.

Biotransformation selectivity of *C. fulvum*

To verify the selectivity of *C. fulvum*, single compounds Rb_1, Rb_2 and Rc were used in the biotransformation of *C. fulvum*. As shown in Fig. 2, 86% of ginsenoside Rb_1 was transformed to Rd as the sole metabolite after 10 days of incubation. The metabolites of Rd, including ginsenoside Rg_3, F_2, Rh_2 and C–K, were not detected. *C. fulvum* did not transform Rb_2 and Rc under the same conditions. These results confirmed that *C. fulvum* had high selectivity to cleave the β-(1→6)-glucosidic linkage at position C-20 of Rb_1, without attack on other glycosidic linkages in protopanaxadiol-type ginsenosides. Therefore, Rb_1 can be selectively converted into Rd with the conversion terminating at Rd. The high selectivity is eminently suitable for industrial production.

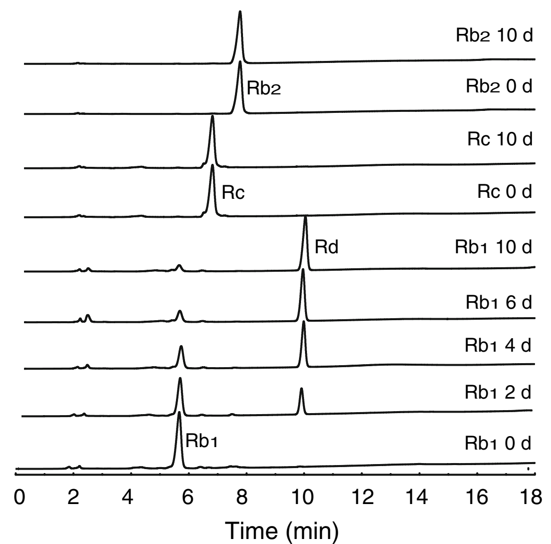

Fig. 2 HPLC analysis of biotransformation products of *C. fulvum*. A solution of ginsenosides in 20 mM acetate buffer (pH 5.0) was added to the flasks containing spores of *C. fulvum* preincubated for 24 h. The mixtures were incubated with shaking (130 rpm) at 37°C for 1–10 days. HPLC analysis was performed with a linear gradient from 35:65 to 50:40 (acetonitrile/water, v/v) for 15 min

Table 1 Biotransformation products of ginsenoside Rb_1, Rc and Rb_2 by 14 phytopathogenic fungi

Microbes	Transformed ginsenoside											
	Metabolites of Rb_1				Metabolites of Rc				Metabolites of Rb_2			
	Rd	GXVII	F_2	C–K	Rd	Mb	F_2	C–K	Rd	CO	F_2	C–K
C. fulvum	+	−	−	−	−	−	−	−	−	−	−	−
B. cinerea	+	−	+	−	−	−	−	−	−	−	−	−
G. sorghi	+	−	+	−	−	−	−	−	−	−	−	−
A. porri	+	−	+	−	−	−	−	−	−	−	−	−
C. coccodes	+	−	+	+	−	−	−	−	−	−	−	−
F. oxysporum	+	−	+	+	+	−	+	−	+	−	+	−
C. cucumerinum	+	−	+	+	+	−	+	+	+	−	+	+
F. graminearum	+	−	+	+	+	−	+	+	+	−	+	+
C. destructans	−	+	+	+	−	+	+	−	−	+	+	−
P. viticola	−	+	+	−	−	+	+	−	−	+	+	−
A. solani	−	+	+	+	−	+	+	−	−	+	+	−
A. alternata	−	+	+	−	−	+	+	−	−	+	+	−
F. oxysporum f. sp. *Lycoporsici*	−	+	+	+	−	+	+	−	−	+	+	−
F. solani	−	+	+	+	−	+	+	−	−	+	+	−

A solution of ginsenosides in 20 mM acetate buffer (pH 5.0) was added to the cultures preincubated for 24 h. The mixtures were incubated with shaking (130 rpm) at 28°C for 10 days. HPLC analysis was performed with a linear gradient elution of acetonitrile/water from 35:65 to 50:40 (v/v) for 15 min

Rd ginsenoside Rd, *GXVII* gypenoside XVII, *F_2* ginsenoside F_2, *C–K* compound K, *Mb* ginsenoside Mb, *CO* compound O, + products detected, − no product detected

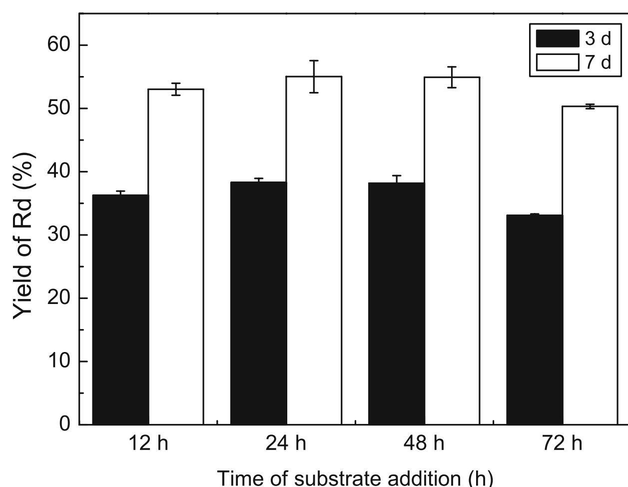

Fig. 3 Effects of the time of substrate addition on biotransformation of *C. fulvum*. A solution of Rb_1 in 20 mM acetate buffer (pH 5.0) was added to the flasks containing spores of *C. fulvum* preincubated for 12, 24, 48 and 72 h. The mixtures were incubated with shaking (130 rpm) at 28°C for 3 or 7 days. HPLC analysis was performed using isocratic elution with acetonitrile/water (37.5:62.5, v/v) for 20 min. Data were expressed as mean ± SD from three independent experiments

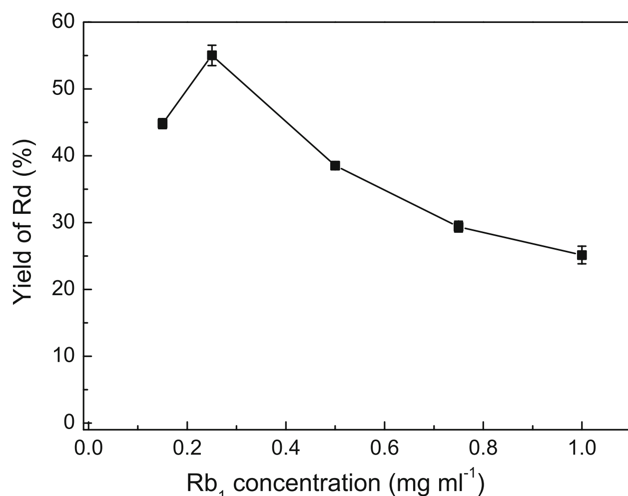

Fig. 4 Effects of substrate concentration on biotransformation of *C. fulvum*. A solution of Rb_1 in 20 mM acetate buffer (pH 5.0) was added to the flasks containing spores of *C. fulvum* preincubated for 24 h at a final concentration of 0.0, 0.15, 0.25, 0.5, 0.75 and 1.0 mg/ml. The mixtures were incubated with shaking (130 rpm) at 28°C for 7 days. HPLC analysis was performed as described in Fig. 3. Data were expressed as mean ± SD from three independent experiments

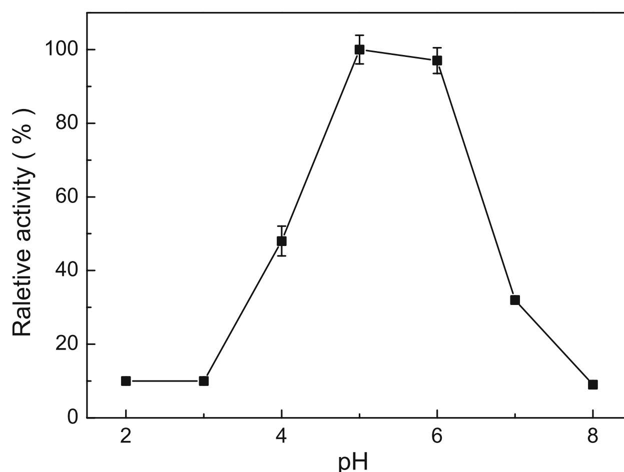

Fig. 5 Effects of pH on biotransformation of *C. fulvum*. A solution of Rb_1 in 20 mM acetate buffer was added to the flasks containing spores of *C. fulvum* preincubated for 24 h. The mixtures were incubated at different pH with shaking (130 rpm) at 28°C for 7 days. HPLC analysis was performed as described in Fig. 3. The maximum yield obtained (56%) was defined as 100%. Data were expressed as mean ± SD from three independent experiments

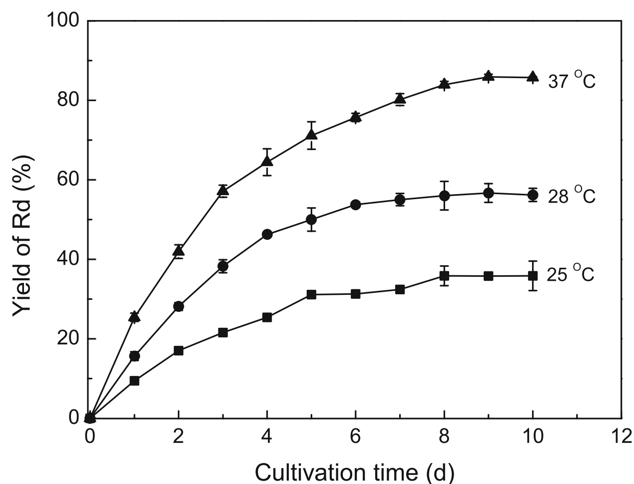

Fig. 6 Effects of cultivation time and temperature on biotransformation of *C. fulvum*. A solution of Rb_1 in 20 mM acetate buffer (pH 5.0) was added to the flasks containing spores of *C. fulvum* preincubated for 24 h. The mixtures were incubated with shaking (130 rpm) at 25, 28 and 37°C for 1–10 days, respectively. HPLC analysis was performed as described in Fig. 3. Data were expressed as mean ± SD from three independent experiments

Optimisation of conditions for preparative scale biotransformation

To better apply this biotransformation to the preparation of ginsenoside Rd, we evaluated the effects of time of substrate addition, substrate concentration, cultivation time, temperature and pH on the converting yield. Ginsenoside Rb_1 was added to a flask containing spores from *C. fulvum* preincubated for different times (12, 24, 48 and 72 h). As shown in Fig. 3, the conversion rates arrived at their highest level when the time of substrate addition was between 24 and 48 h. Thus, 24 h preincubation was used in the following optimisations. The effect of substrate concentration, pH and temperature on the biotransformation of Rb_1 was significant. The optimum substrate concentration was 0.25 mg ml^{-1} (Fig. 4) and the optimum pH for biotransformation was 5.0–6.0 (Fig. 5). As shown in Fig. 6, the

Table 2 ^{13}C-NMR data for ginsenoside Rb_1, Rb_2, Rc and Rd (150 MHz, solvent: CD_3OD)

Carbon site	Rc	Rb_2	Rb_1	Rd	Carbon site	Rc	Rb_2	Rb_1	Rd
Aglycone moiety					3-O-inner-Glc				
C-1	40.54	40.54	40.54	40.53	C-2	81.35	81.35	81.36	81.36
C-2	28.70	28.70	28.70	28.67	C-3	77.85	77.98	77.97	77.96
C-3	91.69	91.69	91.68	91.63	C-4	71.85	71.84	71.85	71.85
C-4	40.88	40.89	40.88	40.88	C-5	78.18	78.18	78.22	78.61
C-5	57.84	57.85	57.85	57.82	C-6	63.10	63.10	63.11	62.80
C-6	19.54	19.54	19.54	19.53	3-O-outer-Glc				
C-7	36.15	36.15	36.15	36.14	C-1	105.68	105.68	105.68	105.67
C-8	41.28	41.28	41.28	41.29	C-2	76.69	76.82	77.06	76.60
C-9	51.38	51.39	51.39	51.34	C-3	78.62	78.80	78.81	78.81
C-10	38.22	38.22	38.22	38.20	C-4	71.85	71.84	71.96	71.85
C-11	31.13	31.13	31.13	31.31	C-5	78.18	77.98	77.06	77.96
C-12	68.56	69.53	70.55		C-6	63.26	63.41	63.41	63.10
C-13	50.00	50.02	50.00	50.07	20-O-inner-Glc				
C-14	52.72	52.72	52.71	52.79	C-1	98.34	98.42	98.40	98.57
C-15	31.01	31.01	31.01	31.00	C-2	75.62	75.56	75.43	75.62
C-16	26.21	26.21	26.25	26.15	C-3	78.62	77.98	77.97	78.52
C-17	53.21	53.19	53.23	53.45	C-4	71.85	72.23	72.03	72.24
C-18	16.62	16.62	16.62	16.55	C-5	76.60	76.60	76.60	78.19
C-19	16.99	16.99	16.99	17.00	C-6	68.56	69.53	71.79	63.40
C-20	85.26	85.35	85.31	85.27	20-O-outer-Glc				
C-21	27.55	27.58	27.55	27.53	C-1			105.27	
C-22	37.13	37.15	37.09	36.96	C-2			75.59	
C-23	22.75	22.71	22.82	23.17	C-3			78.22	
C-24	126.32	126.24	126.31	126.31	C-4			72.03	
C-25	132.58	132.67	132.59	132.63	C-5			78.22	
C-26	24.15	24.19	24.21	24.54	C-6			61.89	
C-27	18.28	18.28	18.33	18.24	20-O-outer-Ara				
C-28	31.80	31.79	31.81	31.93	C-1	110.19	104.85		
C-29	17.04	17.04	17.04	17.03	C-2	83.47	72.00		
C-30	17.44	17.57	17.57	17.56	C-3	78.81	74.27		
3-O-inner-Glc					C-4	86.06	69.26		
C-1	104.76	104.76	104.77	104.77	C-5	61.89	66.17		

biotransformation yield increased more slowly at 25 and 28 than at 37°C. The biotransformation yield of Rb_1 to Rd reached 86% after 8 days of incubation, which is the maximum yield for the conditions tested in this work.

Preparative scale biotransformation and identification of ginsenosides

The preparative scale biotransformation of ginsenoside Rb_1 by *C. fulvum* was performed at a dose of 100 mg under the optimum conditions, yielding 68 mg of pure product. The product was confirmed by ^{13}C-NMR to be Rd. The yield of Rd by preparative biotransformation was still 80%. This indicated that the biotransformation has potential for industry application.

The ^{13}C-NMR data of Rb_1, Rb_2, Rc and Rd listed in Table 2 were identified using those reported in the literatures [1, 3, 4, 11]. The analysis of NMR data showed that the number of sugar residues decreased from four sugar residues in Rb_1 to three sugar residues in the metabolite. The signal for position C-20 of the metabolite shifted upfield from δ85.31 to δ85.27. This finding further confirmed that the metabolite of biotransformation of Rb_1 by *C. fulvum* was ginsenoside Rd.

In summary, a β-glucosidase-producing phytopathogenic fungus, *C. fulvum*, was found to specifically cleave the β-(1→6)-glucosidic linkage at position C-20 of ginsenoside Rb_1. Therefore, it can convert Rb_1 to Rd with a high yield. This biotransformation has the potential for application in industry. Further work has been undertaken to

enhance the enzyme activities so that the production cycle can be shortened and the yield can be increased.

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References

1. Chen LQ, Kim MK, Lee JW, Lee YJ, Yang DC (2006) Conversion of major ginsenoside Rb_1 to ginsenoside F_2 by Caulobacter leidyia. Biotechnol Lett 28:1121–1127. doi:10.1007/s10529-006-9059-x
2. Chi H, Ji GE (2005) Transformation of ginsenosides Rb_1 and Re from Panax ginseng by food microorganisms. Biotechnol Lett 27:765–771. doi:10.1007/s10529-005-5632-y
3. Dong A, Ye M, Guo H, Zheng J, Guo D (2003) Microbial transformation of ginsenoside Rb_1 by *Rhizopus stolonifer* and *Curvularia lunata*. Biotechnol Lett 25:339–344. doi:10.1023/A:1022320824000
4. Dou DQ, Chen YJ, Liang LH, Pang FG, Shimizu N, Takeda T (2001) Six new dammarane-type triterpene saponins from leaves of *Panax ginseng*. Chem Pharm Bull (Tokyo) 49:442–446. doi:10.1248/cpb.49.442
5. Kim MK, Lee JW, Lee KY, Yang DC (2005) Microbial conversion of major ginsenoside Rb_1 to pharmaceutically active minor ginsenoside Rd. J Microbiol 43:456–462
6. Lee JK, Choi SS, Lee HK, Han KJ, Han EJ, Suh HW (2003) Effects of ginsenoside Rd and decursinol on the neurotoxic responses induced by kainic acid in mice. Planta Med 69:230–234. doi:10.1055/s-2003-38475
7. Luan H, Liu X, Qi X, Hu Y, Hao D, Cui Y, Yang L (2006) Purification and characterization of a novel stable ginsenoside Rb_1-hydrolyzing β-D-glucosidase from China white jade snail. Process Biochem 41:1974–1980. doi:10.1016/j.procbio.2006.04.011
8. Park JD, Rhee DK, Lee YH (2005) Biological activities and chemistry of saponins from *Panax ginseng* C. A Meyer Phytochem Rev 4:159–175. doi:10.1007/s11101-005-2835-8
9. Shi Q, Hao Q, Bouissac J, Lu Y, Tian S, Luu B (2005) Ginsenoside-Rd from Panax notoginseng enhances astrocyte differentiation from neural stem cells. Life Sci 76:983–995. doi:10.1016/j.lfs.2004.07.026
10. Son JW, Kim HJ, Oh DK (2008) Ginsenoside Rd production from the major ginsenoside Rb_1 by β-glucosidase from Thermus caldophilus. Biotechnol Lett 30:713–716. doi:10.1007/s10529-007-9590-4
11. Teng RW, Li HZ, Chen JT, Wang DZ, He YN, Yang CR (2002) Complete assignment of H-1 and C-13 NMR data for nine protopanaxatriol glycosides. Magn Reson Chem 40:483–488. doi:10.1002/mrc.1033
12. Zeng S, Guan YY, Liu DY, He H, Wang W, Qiu QY, Wang XR, Wang YD (2003) Synthesis of 12-epi-ginsenoside Rd and its effects on contractions of rat aortic rings. Chin Pharmacol Bull 19:282–286