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Biotransformation of ginsenoside Rb1 to ginsenoside Rd by highly substrate-tolerant *Paecilomyces bainier* 229-7

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ABSTRACT

Paecilomyces bainier 229-7 was obtained after UV irradiation for 8 min in the presence of 0.4% LiCl and selection on potato dextrose media containing 30 mg/mL saponin from Panax notoginseng leaves (SPNL). The mutant produces ginsenoside Rd from ginsenoside Rb1 with a bioconversion rate as high as 94.9% under optimized culture conditions in shake flasks when supplied with 20 mg/mL of SPNL. Scale-up in 10-L fermenter resulted in an 89% bioconversion rate. Ginsenoside Rd was purified from the culture medium by a macroporous resin with a chromatographic purity of 92.6%. These results suggest that *P. bainier* 229-7 could be useful for the preparation of ginsenoside Rd in the pharmaceutical industry.

1. Introduction

Ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), is a traditional herbal medicine that has been used for thousands of years in Asian countries for its various medicinal functions, including tonic, adaptogenic, immunomodulatory and anti-aging effects (Borchers et al., 1998; Kiefer and Pantuso, 2003; Lee et al., 2005; Wu et al., 1992; Zhang et al., 1994). These beneficial effects are attributed mainly to triterpene glycosides, known as ginsenosides, among which protopanaxadiol has recently attracted great interest (Chen et al., 2002; Yokozawa et al., 2004; Yokozawa and Liu, 2000). Protopanaxadiol is composed of ginsenoside Rb1, Rb2, Rb3, Rd and CK (Attele et al., 1999; Yuan et al., 2002). Of those, ginsenoside Rd has been reported to enhance the differentiation of neural stem cells (Shi et al., 2005), markedly inhibit Ca^{2+} entry in vascular smooth muscle cells (Guan et al., 2006), induce COX-2 and prostaglandin E2 production (Jeong et al., 2007), and protect and rescue rat intestinal epithelial cells from irradiation-induced apoptosis (Tamura et al., 2008). Therefore, ginsenoside Rd might be a new promising drug. Unfortunately, the content of ginsenoside Rd in wild ginseng is so low (less than 0.4%) that isolation of Rd from natural products is extremely difficult and costly.

Rb1, the major component of ginsenosides, is structurally similar to Rd, but has two glucose residues at position C-20, rather than one. Therefore it can be transformed to Rd by removing the glucose residue from position C-20 by mild acid hydrolysis or alkaline cleavage. Since these methods are not efficient and environmentally friendly (Han et al., 1982; Wang et al., 2007), efforts have been focused on biotransformation of Rb1 to pharmacologically active Rd using crude or purified enzymes extracted from plants or microorganisms (Son et al., 2008; Yu et al., 2004; Zhang et al., 2002; Zhao et al., 2009a). However, the current enzymes are not stable enough to meet industrial requirements. Some microbes can transform Rb1 to Rd (Chen et al., 2008; Cheng et al., 2006; Chi and Ji, 2005; Kim et al., 2005; Zhao et al., 2009b), but yields were low due to inhibition of Rd production by other ginsenosides, low substrate tolerance, requirement for pure substrates, and further transformation of Rd to other compounds.

We have previously reported on a filamentous fungus *Paecilomyces bainier* 229 that possesses a series of ginsenoside-hydrolyzing β-glucosidases (Yan et al., 2008a,b) and effectively transforms ginsenoside Rb1 to ginsenoside compound K (CK) (Zhou et al., 2008b). In the course of research aiming to further improve substrate concentration and increase substrate tolerance of the strain, we obtained a mutant that transforms ginsenoside Rb1 to Rd with high selectivity and substrate tolerance. Culture conditions required for biotransformation of ginsenoside Rb1 to Rd under high

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substrate concentration were optimized and applied in a 10-L fermenter. Ginsenoside Rd was purified from the culture medium by a novel purification method.

2. Methods

2.1. Materials

The saponin from *Panax notoginseng* leaves (SPNL) containing 62.08% Rb1 and 0.12% Rd ginsenosides from Kunming Shanghima Biotech Co. Ltd., Yunnan, China was used as substrate in the current investigation. Standard ginsenosides Rb1, F2 and Rd were from the National Institute for the Control of Pharmaceutical and Biological Products of China. Ginsenoside compound K (CK) was prepared in our laboratory and its structure was confirmed by X-ray crystallography (Zhou et al., 2006). Methanol and acetonitrile of HPLC grade were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Microorganism

P. bainier 229 was stored at 4 °C on potato dextrose agar (PDA) containing 2% (w/v) glucose, 2% (w/v) agar and 20% (v/v) potato extract prepared by adding 200 g freshly sliced potatoes to 1000 mL of tap water, boiling for about 30 min, filtering through cottoncloth and adjusting to a final volume of 1000 mL.

Fungal spore suspensions were prepared by washing 5-d-old culture slants with sterile 0.9% NaCl and shaking vigorously for 1 min. Spores were counted with a haemocytometer and adjusted approximately to 10^7 spores per mL.

2.3. Mutagenesis

Spore suspensions in 0%, 0.3%, 0.4%, or 0.5% (w/v) LiCl were transferred to 9-cm plates with a iron clip as stir bar of magnetic stirrer and irradiated with a 30 W UV lamp for 2, 4, 6 and 8 min. Aliquots of 0.1 mL spore suspension from each plate were transferred into a 10 × 150 mm tube containing 4 mL sterile potato glucose broth supplemented with 30 mg/mL SPNL, and cultured at 28 °C. After 7–12 days, 0.1 mL from each tube with visible mycelia was spread on a series of PDA plates containing 30 mg/mL SPNL and cultured at 28 °C for 4 days. A total of 600 colonies were selected and purified by culturing on new PDA plates at 28 °C for 4 days. These 600 purified colonies were cultured in 250-mL shake flasks with 30 mL of P-medium to biotransform SPNL to screen mutants that produce high ginsenoside Rd. The P-medium for mutant screening contained 30 g sucrose, 30 g soybean powder, 1 g wheat bran powder, 2 g $MgSO_4{\cdot}7H_2O$, 1 g $CaCl_2$, 1 g $(NH_4)_2SO_4$ in 1 L of distilled water (Zhou et al., 2008b). Based on TLC and HPLC analysis results, strain 229-7 was selected for further studies.

2.4. Biotransformation in flasks

Two-hundred-fifty mL flasks containing 30 mL of sterilized (20 min, at 121 °C) medium (20 g glucose, 20 g peanut powder, 0.5 g $NaNO_3$, 1 g KH_2PO_4, 2 g Na_2HPO_4, 0.2 g $FeSO_4$, 0.1 g $ZnSO_4$, 0.2 g $CuSO_4$, 1 g $CaCl_2$, 0.5 g $MgSO_4{\cdot}7H_2O$ in 1 L of distilled water, initial pH of 5.0) were inoculated with 3% (v/v) of fungal spore suspensions from mutant 229-7 and incubated 28 °C on a rotary shaker at 220 rpm. After 36 h, SPNL was added to a final concentration of 20 mg/mL. After an additional 72 h, the culture was extracted with ethanol and concentrated to dryness as previously described (Zhou et al., 2008b). The solid residue was dissolved in methanol and applied to thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

The stability of *P. bainier* 229-7 to produce ginsenoside Rd was investigated in shake flasks simultaneously inoculating five different flasks, respectively with five different inocula of spores that had been sub-cultured consecutively on PDA plates for one to five times, respectively.

2.5. Process optimization in flasks

Single-factor experiments were performed to optimize the medium for Rd production by the mutant 229-7 on the basis of P-medium, the optimal medium for the parent strain 229. Carbon sources (glucose, sucrose, starch, maltose and lactose, at concentrations of 0.5%, 1%, 2%, 3% and 4%, w/v), organic nitrogen sources (peanut powder, soybean powder, peptone, corn steep liquor and yeast powder, at concentrations of 0.5%, 1%, 2%, 3% and 4%, w/v) and inorganic nitrogen sources ($NaNO_3$, urea and $(NH_4)_2SO_4$, at concentrations of 0%, 0.05%, 0.1% and 0.2%, w/v) were tested one by one. Subsequently, KH_2PO_4, Na_2HPO_4, $CaCl_2$ and $MgSO_4{\cdot}7H_2O$ at concentrations of 0%, 0.05%, 0.1% and 0.2%, w/v and trace elements $FeSO_4$, $ZnSO_4$ and $CuSO_4$ instead of 0.1% wheat bran powder, at concentrations of 0%, 0.01%, 0.02% and 0.03%, w/v were investigated. Substrate concentrations between 10 mg/mL and 60 mg/mL and the effect of surfactants Tween 20, Tween 80, SDS, Triton X-100 and Span 60 at concentrations of 0.05%, 0.1%, 0.15%, 0.2%, 0.25% and 0.3%, w/v were also investigated.

2.6. Scale-up fermentation

Seed cultures were prepared by inoculating 2 mL of spore suspension into a 500-mL flask with 150 mL of P-medium and cultivation at 220 rpm for 24 h at 28 °C. A total 600 mL of seed culture was required as inocula for a 10-L fermenter (BioFlo 110, New Brunswick Scientific Inc. Co., Edison, NJ, USA) with a 6-L working volume of optimized medium (20 g glucose, 20 g peanut powder, 0.5 g $NaNO_3$, 1 g KH_2PO_4, 2 g Na_2HPO_4, 0.2 g $FeSO_4$, 0.1 g $ZnSO_4$, 0.2 g $CuSO_4$, 1 g $CaCl_2$, 0.5 g $MgSO_4{\cdot}7H_2O$ in 1 L of distilled water, initial pH of 5.0), and cultured at an agitation rate of 200–300 rpm at 28 °C. After 47 h, 20 mg/mL SPNL was added to initiate biotransformation. Cultivation was continued for an additional 72 h. The pH was controlled by addition of 1 N HCl.

2.7. Isolation of ginsenoside Rd

Mycelia were collected by vacuum filtration and extracted with 6 L of 60% ethanol for 24 h. The extract was collected by filtration, and the pellet was washed with 1 L of 60% ethanol, then extracted and washed again with 6 L and 1 L 50% ethanol, respectively. The ethanol extracts were pooled together and purified as described in Table 1.

2.8. Analytic methods

2.8.1. TLC

TLC was carried out using a Silica Gel G 60 plate (Huiyou Silica Gel Development Co. Ltd. in Yantai, China) and a solvent system of chloroform:methanol:H_2O (65:35:10, v/v/v) as lower phase. After TLC, the samples were visualized by spraying with 10% H_2SO_4 solution followed by heating at 110 °C for 10 min.

2.8.2. HPLC

Samples after biotransformation were quantitatively analyzed by HPLC (Waters 2487 detector, 1525 binary pump; Waters, Milford, MA, USA) using a C18 column (Waters Symmetry, 150 mm × 4.6 mm, 5 μm) with column temperature of 35 °C, and mobile phase of acetonitrile–water (48:52, v/v) at flow rate of 1.0 mL/min. Metabolites were detected by absorption at 203 nm.

Table 1
The purification procedures of ginsenoside Rd.[a]

Steps	Operating processes	Sample collected
Crude separation	Resin: HP20 (2 L) Equilibrate: 25% ethanol–water, flow rate: 0.5 BV/h Loading sample: the combined extracts diluted by 13.5 L water, flow rate: 0.5 BV/h Re-quilibrate: 2 BV of 40% ethanol–water, flow rate: 2 BV/h Elution: 6 BV of 60% ethanol–water, flow rate: 6 BV/h	About 11 L elute
Dehydration	Resin: HP20 (2 L) Equilibrate: 25% ethanol–water, flow rate: 0.5 BV/h Loading sample: 11 L eluate diluted by water to 25% ethanol, flow rate: 0.5 BV/h Elution: ethanol, flow rate: 6 BV/h, and then evaporated under reduced pressure	Crude Rd sample
Refine	Resin: H41 (2 L) Equilibrate: 45% acetone–water, flow rate: 0.5 BV/h Loading sample: 49.8 g crude Rd sample dissolved in 300 mL ethanol, diluted by 900 mL water, flow rate: 0.5 BV/h Re-quilibrate: 3 BV of 45% acetone–water, flow rate: 0.5 BV/h Elution: 1.5 BV of 50% acetone–water, flow rate: 0.5 BV/h	About 2.5 L elute

[a] All samples were monitored by TLC. BV: Bed volume.

Each sample was analyzed in triplicate. Bioconversion rate of ginsenoside Rd was calculated as follows:

$$\text{Bioconversion rate } (\%) = \frac{\text{Weight of Rd/MW of Rd}}{\text{Weight of Rb1/MW of Rb1}}$$

where MW is the molecular weight, i.e., MW of Rb1 is 1108 and of Rd is 929.All the experiments were conducted in triplicate and the results were expressed as mean values ± SD.

3. Results and discussion

3.1. Breeding of ginsenoside Rd biotransformation strain

The inhibitory effect of the ginsenoside substrates on biotransformation strains is a ubiquitous problem in ginsenoside biotransformation, and increased substrate tolerance would be expected to increase biotransformation efficiency. The mutagenesis experiments produced about 600 mutant strains that were able to grow well in the presence of 30 mg/mL of SPNL. Among these mutants, 11 were able to transform ginsenoside Rb1 in SPNL to ginsenoside Rd (Fig. 1). Mutant 229-7 had the highest biotransformation efficiency according to TLC and HPLC analysis results. The HPLC analysis confirmed the biotransformation of ginsenoside Rb1 to Rd by this mutant (data not shown). Although some microbes, such as *Acremonium strictum* (Chen et al., 2008), *Caulobacter leidyia* (Cheng et al., 2006) and some food microorganisms (Chi and Ji, 2005), have been reported to be able to transform gensenoside Rb1 to Rd, all of them had low specificity and further biotransformed Rd to F2, CK or other ginsenosides. Mutant 229-7 is highly substrate-tolerant since it was able to grow well at SPNL concentration of 60 mg/mL (data not shown). It is known that the biotransformation pathway of ginsenoside Rb1 by parent strain *P. bainier* 229 is Rb1 → Rd → F2 → CK, where ginsenoside Rd is just an intermediate of this pathway (Yan et al., 2008a; Zhou et al., 2008a). Since *P. bainier* 229-7 was only able to transform ginsenoside Rb1 to Rd, it appears that the mutation affected production of downstream products. Studies are underway to determine the nature of the mutation(s).

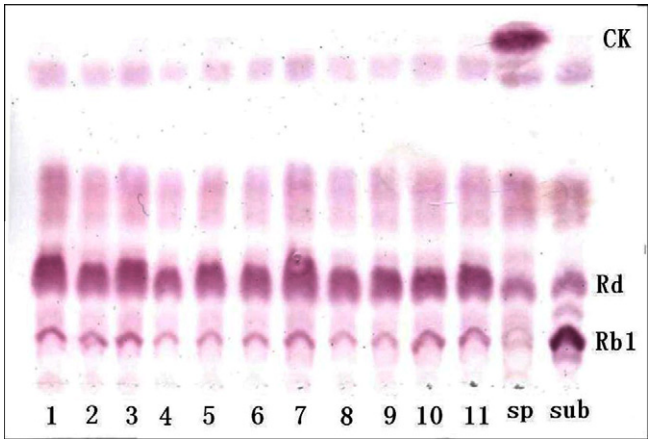

Fig. 1. TLC analyses of biotransformation products by parent and mutant strains. 1–11: mutant strains; sp: parent strain *Paecilomyces bainier* 229; sub: substrate SPNL. Mutant strains 1–11 mainly transform ginsenoside Rb1 in SPNL to ginsenoside Rd instead of CK, which mainly produced by parent strain *Paecilomyces bainier* 229.

The five consecutive spore subcultures produced similar amounts of ginsenoside Rd in shake flasks and their bioconversion rates ranged from 74.2% to 81.2%, indicating that the genotype of strain 229-7 was stable.

3.2. Optimization of fermentation medium

The bioconversion rate of mutant 229-7 was increased from 25.1% to 39.5% when 2% glucose as carbon sources instead of 3% sucrose was used. Of all organic nitrogen sources examined, 2% peanut powder was the best for biotransformation and increased the bioconversion rate by about 18% as compared with that of 2% glucose-containing medium with 3% soybean powder as nitrogen sources. The bioconversion rate changed little among different inorganic nitrogen sources, compared with the change caused by different organic nitrogen sources. Addition of 0.05% $NaNO_3$, instead of 0.1% $(NH_4)_2SO_4$, only increased the bioconversion rate by about 4%. Altogether, under optimized carbon and nitrogen sources, the bioconversion rate was increased from 25.1% to 61.2%.

Addition of 0.1% KH_2PO_4, 0.2% Na_2HPO_4, 0.02% $FeSO_4$, 0.01% $ZnSO_4$, 0.02% $CuSO_4$, 0.1% $CaCl_2$ and 0.05% $MgSO_4{\cdot}7H_2O$ instead of 0.1% wheat bran powder, 0.2% $MgSO_4{\cdot}7H_2O$ and 0.1% $CaCl_2$, increased the bioconversion rate from 61.2% to about 85%. Thus, the obtained optimal medium contained 20 g glucose, 20 g peanut powder, 0.5 g $NaNO_3$, 1 g KH_2PO_4, 2 g Na_2HPO_4, 0.2 g $FeSO_4$, 0.1 g $ZnSO_4$, 0.2 g $CuSO_4$, 1 g $CaCl_2$, 0.5 g $MgSO_4{\cdot}7H_2O$ in 1 L of distilled water.

3.3. Effect of substrate concentration

The maximum bioconversion rate of about 85% was obtained at an SPNL concentration of 20 mg/mL (Fig. 2). At 60 mg/mL, the maximum bioconversion rate was still as high as 71.32%. The relative stability of the maximum bioconversion rate further confirmed the high-substrate tolerance of mutant 229-7; however, the biotransformation period increased with increasing SPNL concentration (Fig. 2). For concentrations of 10 and 20 mg/mL, only 72 h were needed to reach the maximum bioconversion rate. At concentrations of 30–60 mg/mL, the bioconversion rates during the first 72 h were far below those at lower concentrations and optimal biotransformation time was delayed to 5–7 d. Therefore 20 mg/mL SPNL was selected, which is significantly higher than the ginseno-

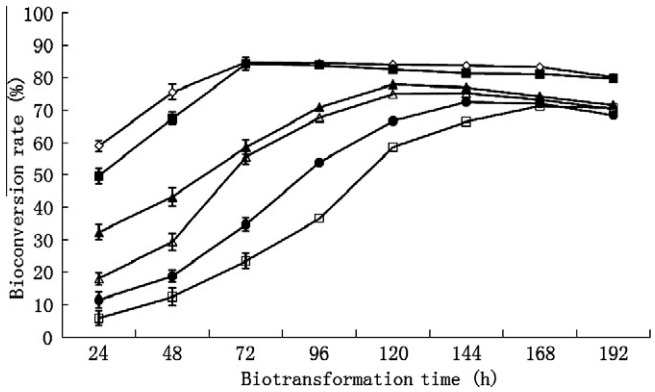

Fig. 2. Effect of substrate concentration on biotransformation for ginsenoside Rd production by *Paecilomyces bainier* 229-7. SPNL was added to the flasks containing mycelia of *Paecilomyces bainier* 229-7 preincubated for 36 h at a final concentration of 10 (◇), 20 (■), 30 (▲), 40 (△), 50 (●) and 60 (□) mg/mL, respectively. The fermentation conditions were the same as those described in Section 2.4. Samples were collected at different biotransformation time and analyzed. Data were expressed as mean ± SD from three independent experiments.

side Rb1 concentrations of 0.25 and 0.02 mg/mL used in biotransformation of ginsenoside Rb1 to Rd by the phytopathogenic fungus *Cladosporium fulvum* (Zhao et al., 2009b) and *A. strictum* (Chen et al., 2008), respectively. The biotransformation time of these two strains was 7–8 d, longer than that in our experiment. Furthermore, the substrate used in these two reports was pure ginsenoside Rb1. SPNL which contained 62.08% Rb1 (determined by HPLC) and was extracted from stems and leaves of *P. notoginseng* (Burk.) F.H. Chen (Araliaceae), a Chinese herb often known by its common name, Tienchi ginseng, is much cheaper.

3.4. Effect of surfactant

Surfactant can improve the permeability of cell membrane (King et al., 1991) and consequently promote the transportation of SPNL into the cells and enhance the biotransformation. Therefore, different surfactants were added following SPNL addition under optimized fermentation conditions. Triton X-100 and SDS showed a negative effect because of its toxicity on mycelium growth. Tween 80 and Span 60 were less toxic but barely increased the bioconversion rate. Tween 20 at the concentration of 0.25% (w/v) increased gensenoside Rd yield by 10% and the bioconversion rate reached 94.9%.

3.5. Scale-up fermentation

The changes of fermentation parameters, agitation rate, dO_2, pH and temperature are shown in Fig. 3. At 16 h, the concentration of dissolved O_2 (dO_2) was 78%, pH rose to 6.5 and maintained stable. Since dO_2 declined continuously during fast mycelia growth, the agitation rate had to be increased to 300 rpm to maintain dO_2 at above 30% at 36 h. At 47 h, pH was adjusted to 5.0 (the optimal pH for biotransformation) and then SPNL solution (120 g in 850 mL sterile water) along with 18 g of Tween 20 was added to the fermentor at flow rate of 200 mL/h.

The pH was maintained at 5.0 during biotransformation. The dO_2 declined to below 30% because of substrate addition even at an agitation rate of 300 rpm. Later, the agitation rate could be reduced to 180 rpm to maintain the dO_2 between 30% and 50%. At 119 h, biotransformation was terminated and a total volume of 7.5 L culture was obtained. The final concentration of ginsenoside Rd was 10.2 mg/mL and the bioconversion rate reached 89%, slightly lower than that obtained in the shake flask culture. This

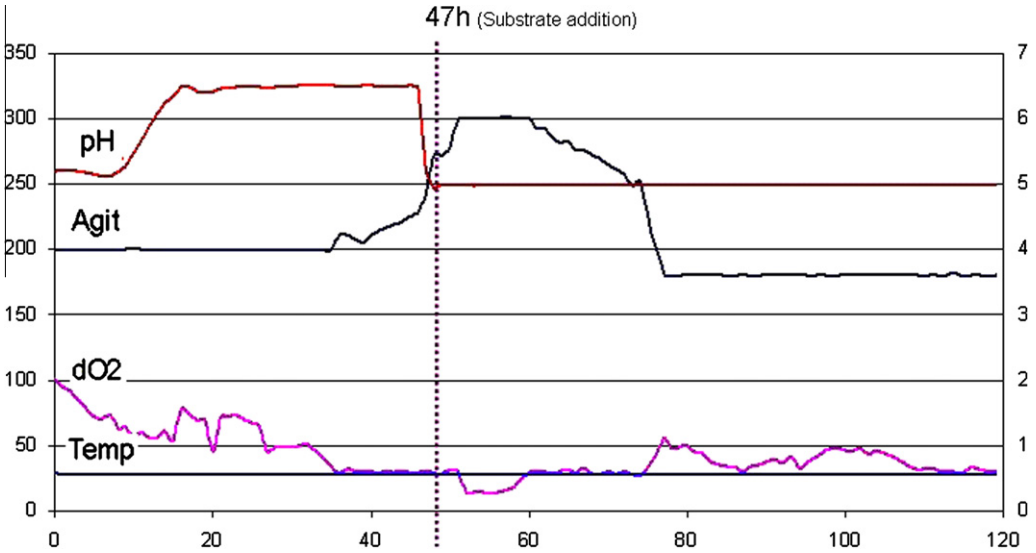

Fig. 3. The dynamic changes of fermentation parameters in scale-up fermentation. The *X*-axis shows the fermentation time (h), the right *Y*-axis shows pH scale, and the left *Y*-axis indicates agitation rate (rpm), dO_2 (%) and temperature (°C). SPNL containing 63.8% Rb1 and 16.3% Rd was added at 47 h in scale-up fermentation.

Table 2
Purification profile of ginsenoside Rd.

Step	Volume (L)	Concentration (mg/mL)	Yield (g)	Recovery (%)	Chromatographic purity (%)
Ethanol extracts	14	5.7	79.8	100	40.9
HP20 crude separation	11	5.3	58.3	73.1	82.5
HP20 dehydration	–	–	49.8	62.4	–
H41 refine	2.5	13.5	33.8	42.4	92.6

outcome was likely due to a lack of control over dO_2. Further studies on the control and optimization of scale-up fermentation process are therefore necessary.

3.6. Purification of ginsenoside Rd

The 14 L of ethanol extract from the culture contained 5.7 mg/mL ginsenoside Rd with a chromatographic purity of 40.9% as determined by HPLC (Table 2). The ethanol extracts were purified with HP20 resin to remove most of the pigments, and 11 L of Rd solution (5.3 mg/mL) with chromatographic purity of 82.5% was obtained. After HP20 dehydration, crude Rd was refined with H41 resin, and 33.8 g ginsenoside Rd with chromatographic purity of 92.6% was obtained. To our knowledge, this is the first report on isolation and purification of ginsenoside Rd from fermentation medium. Further studies are undergoing in our laboratory to improve the product purity and recovery by optimizing such as concentration and volume of feeding sample and eluent.

4. Conclusions

The highly substrate-tolerant mutant *Paecilomyces bainier* 229-7 selectively transformed ginsenoside Rb1 to Rd with a high bioconversion rate. Purification of Rd with macroporous resin was easy and cost-effective. These desirable properties make the mutant *Paecilomyces bainier* 229-7 a promising strain for ginsenoside Rd preparation in the pharmaceutical industry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.04.102.

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