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Novel biotransformation of betulin to produce betulone by *Rhodotorula mucilaginosa*

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ABSTRACT

In this study, betulin was biotransformed to betulone by a strain of yeast. A primary screening was carried out using 47 microbial strains isolated from soil, that was able to grow in the presence of betulin as sole carbon source. The strain of yeast was identified as *Rhodotorula mucilaginosa* by Bruker MALDI Biotyper and 26S rDNA analysis. Under the optimum conditions, this yeast converted 52.65% of the added 4 mg/ml betulin to two products. One was betulone detected by high performance liquid chromatography (HPLC), isolated by preparative HPLC and characterized by high resolution mass spectrum (HRMS), ^{13}C NMR and ^{1}H NMR spectra. The other was a semi-volatile aromatic compound identified as 11,14-Octadecadienoic acid methyl ester by GC–MS. In the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging system, the activity of betulone was about 2 times higher than that of betulin.

1. Introduction

Betulin is a pentacyclic triterpene alcohol with a lupane skeleton. It can be isolated from various plants in small amounts, but up to 30% dry weight from the birch bark by extraction with high boiling hydrocarbon solvents or with water azeotropes of alcohols [1]. Betulin has attracted more attention because it has some pharmacological activities, such as antiviral, anti-inflammatory, antitumor activities [2], and protective effects against cadmium (Cd)-induced cytotoxicity [3]. The latest research by Tang et al. showed that betulin can inhibit sterol regulatory element-binding proteins (SREBPs) pathway, decreases the biosynthesis of cholesterol and fatty acid, improves insulin sensitivity and reduces atherosclerotic plaques [4].

In recent years, derivatization of betulin has attracted much attention, because its derivatives had more potential activities, such as betulinic acid and its derivatives [5,6]. Betulin has three positions in its structure, namely secondary hydroxy group at position C-3, primary hydroxy group at position C-28 and alkene moiety at position C-20, where chemical modifications can be easily performed to yield derivatives. Studies at the positions C-3 and C-28 demonstrate that chemical modifications of the parent structure of betulin can produce potentially important derivatives, which can act as antitumor and antiviral agents [5–7].

Biotransformation is defined as an enzymatic reaction catalyzed by the microorganisms. Some advantages over chemical synthesis are observed such as higher stereo- and regio-selectivity, as well as milder reaction conditions, less cost and lower pollution. Furthermore, some reactions that cannot be fulfilled in chemical approaches are facile process by microbial transformation [8,9]. In the earlier research, the brown-rot fungus *Gloeophyllum odoratum* shows the ability to transform betulin [10]. Chen *et al.* found *Armillaria luteovirens Sacc* QH could transform betulin to betulinic acid [11], which has been reported to have anti-cancer, anti-HIV, anti-melanoma, anti-leukemia, anti-viral, anti-inflammatory, antiseptic, antimicrobial, antimalarial, antileishmanial, antihelmintic and antifeedent activities [12–14].

Betulone, lup-20(29)-ene-28-ol-3-one, is a known triterpenoid and can be isolated from *Excoecaria agallocha* L. [15]. Betulone showed positive result in Liebermann–Burchard test for sterols [16] and was also reported to possess the antifouling activity against cyprid larvae, antimycobacterial and antiparasitic activity [17–19]. Furthermore, betulone has recently been utilized as a key agent for the synthesis of novel anticancer triterpenoid derivatives [20–25]. In this study, we reported that a *Rhodotorula mucilaginosa* from local soil could transform betulin to betulone, which exhibits about two-fold increase in antixodant activity. The biotransformation was optimized and 52.65% of betulin in the transformation broth was successfully converted to the product by *R. mucilaginosa.*

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2. Materials and methods

2.1. Chemicals and reagents

Standard betulin (99%) was purchased from Nanjing Spring & Autumn Biological Engineering Co. Ltd (China), and dissovled in dimethylsulphoxide (DMSO) (4 mg/ml) as a stock solution for experiments. Methanol (HPLC grade) was obtained from Siyou (Tianjin, China). Other chemicals used in the study were of analytical grade.

2.2. Screening and isolation of microorganisms

Each soil sample (0.5 g) in our campus (Zhengzhou, China) were dissolved in 100 ml sterile water, 1 ml of this suspension was added to 50 ml screening media containing betulin 0.5%, $(NH_4)_2SO_4$ 0.3%, KCl 0.1%, NaCl 0.2%, $MgSO_4$ 0.02%, KH_2PO_4 0,05%, pH 7.0 for 3 days. 0.5 ml culture broth was inoculated to solid medium (screening culture with agar) and cultivated for 3 days. The strains were isolated and maintained on potato dextrose agar (PDA) plates for further test.

The seed culture of each single colony was grown in a 250 ml flask containing 50 ml fermentation medium (3.0 g/L beef extract, 10 g/L soy peptone, 5.0 g/L NaCl) and 30 °C on a rotary shaker incubator at 150 rev/min for 24 h. The flask culture experiments were performed in a 250 ml flask containing 50 ml the media after inoculating with 4% (v/v) of the seed culture. The pH and agitation rates were controlled at 7.0 and 150 rpm, respectively. After the 24 h culture, 1 ml of the prepared substrate solution betulin (4 mg/ml) was added to each flask and these flasks were maintained under the same cultivation conditions for additional 24 h. Culture control was run with the inoculation of microorganism, while with the addition of the same amount of DMSO instead of substrate. So, there was microorganism growing while no substrate biotransformation in culture control. Substrate control was run without the inoculation of microorganism, and the other culture conditions were the same with the biotransformation experiment.

2.3. Identification of the strain

The morphology of strain was observed through a microscope (Olympus ZX31, Olympus Corporation). The strains was identified by matrix-assisted laser desorption ionization time-of-flight–time-of-flight tandem mass spectrometry (MALDI-TOF MS). The extraction of genomic DNA, PCR amplification of 26S rDNA gene, and sequencing of the purified PCR products were carried out by Sangon Biotech (Shanghai) Co. Ltd.

2.4. Optimization of the condition (initial pH, temperature, time) of biotransformation

To investigate the effect of initial pH on biotransformation products in shake flask cultures, the different initial pH was adjusted (4.0–8.0) by addition of 1.0 M NaOH or HCl. The temperature change (16–37 °C) was conducted in the temperature controlled shaking incubator. The biotransformation time (1–3 d) after the injection of the substance was examined.

2.5. HPLC analysis method

After fermentation, the culture broth was extracted three times by equivalent volume of ethyl acetate, and all the organic layers were combined. After that, the extracted solutions were concentrated under vacuum condition at rotavapor at 40 °C. Finally, the collected residues were dissolved in methanol and filtered through 0.22 μm millipore filter, then analyzed by RP-HPLC (Waters, USA). The HPLC system used throughout current study consists of Waters 1525 pump, 2998 photodiode array detector, Symmetry C18 (4.6 mm × 75 mm i.d., Waters) column. Samples were analyzed under following condition: the flow rate was set at 1.0 ml/min under room temperature, mobile phase was composed of methanol–water (contains 0.15% formic acid) in the ratio 85:15 (v:v). The wavelength was set at 206 nm. To generate calibration curve, betulin was dissolved in methanol (2 mg/ml), serially diluted to various concentrations (0.125, 0.25, 0.5 and 1 mg/ml) and kept at 4 °C in darkness. Before analysis by HPLC, the solutions were filtered with 0.22 μm Millipore filter and analyzed by HPLC (Waters, USA). The calibration curve was constructed by plotting the peak area versus the ratio of their corresponding concentrations.

2.6. Biotransformation in a bioreactor

The fermentation medium was inoculated with 4% (v/v) of the seed culture and then cultivated in a 5-L stirred-bank (Infors, Switzerland). Unless otherwise specified, fermentations were performed under the following conditions: temperature, 30 °C; agitation speed, 150 rpm; initial pH, 6.0; working volume, 3.5 L. After 24 h, substrate (200 mg) was added, fermented for another 24 h. The culture broth was centrifuged and the supernatants were analyzed by HPLC assay.

2.7. Purification and identification of biotransformation products

Biotransformation products were isolated by preparative HPLC (Waters, USA). The HPLC system used throughout current study consisted of Waters 600 pump, 2498 photodiode array detector, Sunfire C18 (10 mm × 250 mm i.d., Waters) column. Samples were analyzed under following condition: the flow rate was set at 4.0 ml/min at room temperature, mobile phase was composed of methanol–water in the ratio 88:12 (v:v). The wavelength was set at 206 nm.

The structures of biotransformation products were elucidated by means of spectral measurements. ^1H and ^{13}C NMR spectra were recorded at 400 MHz on a DPX 400 Bruker instrument in deuteriochloroform ($CDCl_3$). High resolution mass spectrum was performed on an Agilent 1290 LC/6540 Q-TOF MS system.

GC–MS analysis of the semi-volatile compound was carried out on an Agilent 5975C mass select detector (Agilent Corporation of America) directly coupled to a HP 7890 gas chromatograph with a 30 m hp-5 column. All injections were performed in splitless mode with 1.0 μL volume; the oven was held at an initial temperature of 50 °C for 2 min before increasing to 300 °C at 10 °C/min; the final temperature was held for 5 min. The temperature of the GC–MS transfer line was 250 °C in the electron impact (EI) mode (70 eV), scanning from m/z 30–550 in one scan. The mass spectral identification of the semi-volatile aromatic compounds was carried out by comparing to the NIST 02 (USA Agilent Corporation). Qualitative analysis (mass spectral data) was verified by comparing the retention indices and mass spectra of compounds.

2.8. DPPH radical scavenging assay

The antioxidant activity, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Shimada *et al.* [20]. The different amount of the compound sample was dissolved in ethanol/water to make different concentration solution. Two milliliters of 0.1 mmol/L DPPH dissolved in ethanol were added to 2 ml of the compound sample solution. The absorbance was measured at 517 nm after 30 min of incubation at room temperature. Ethanol instead of DPPH was used for the control while ethanol instead of sample was used for the blank. The scavenging

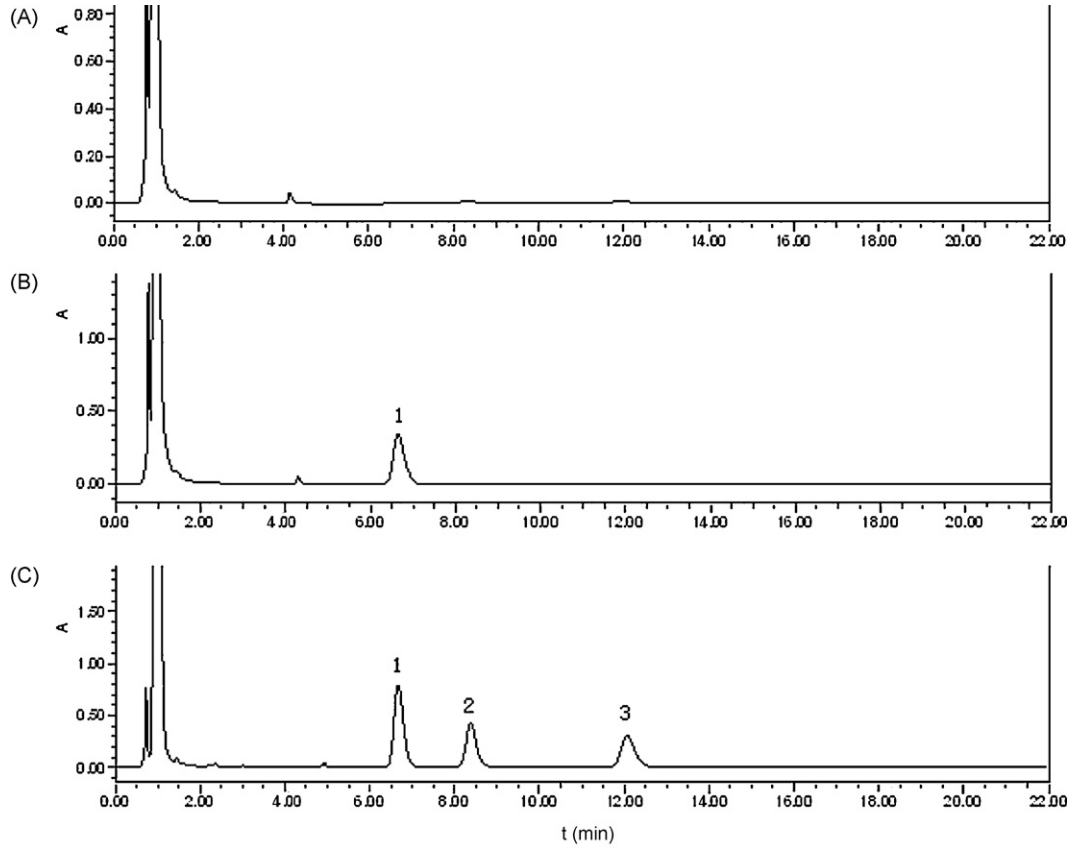

Fig. 1. HPLC-UV chromatograms of betulin and its produce product biotransformed by *Rhodotorula mucilaginosa*. (A) The chromatogram of culture control; (B) the chromatogram of substrate control and (C) the chromatogram of biotransformation sample. In B and C, peak 1 was betulin, peak 2 (product 1) and 3 (product 2) were two biotransformation products.

activity of DPPH radicals by the sample was calculated according to the following equation: DPPH radical scavenging activity (%) = $[1 - (A_{sample} - A_{blank})/A_{control}] \times 100\%$. Where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, blank (without compound) and control (without compound).

3. Results and discussion

3.1. Screening strains for the transformation of betulin

Approximately 47 strains could grow on the medium, among which only strain F10 could covert betulin into two new products detected by HPLC (Fig. 1). By comparing Fig. 1(C) with Fig. 1(A) and (B), it was concluded that the peaks at the retention time of 0–2 min were medium composition and metabolites of strain F10. The peak 1 at the retention time of 6.9 min in Fig. 1(B) and (C) was identified to be the substrate of betulin. Except for the medium composition and the substrate of betulin, there was two new peaks (2 and 3) at the retention time of 8.5 and 12.2 min occurred in the substrate control in Fig. 1(C), which should be the transformed products of betulin.

3.2. Identification of strain F10

Strain F10 was assigned to the *Rhodotorula* genus on the basis of its typical colony appearance and microscopical morphology. Strain identification was carried out with MALDI Bruker Daltonik Biotyper software (Bruker Daltonik GmbH, Bremen, Germany) by comparing the mass spectrum of each strain with the mass spectra

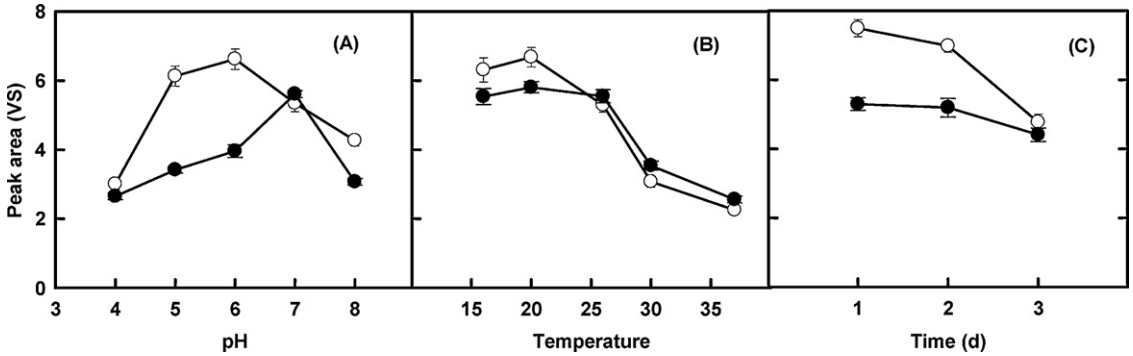

Fig. 2. Effect of initial pH, temperature, and time on the biotransformation process. The production yield was represented by the peak area on the chromatogram of HPLC. Symbols: (○) product 1; (●) product 2.

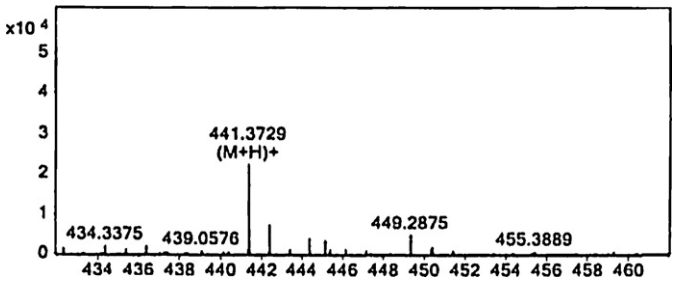

Fig. 3. The HRMS spectra of the biotransformation product 1.

of the reference strains available. The 26S rDNA sequence analysis of F10 showed 100% similarities with that of *R. mucilaginosa* SN27 (Gen-Bank Accession no. FJ515247.1). Therefore, strain F10 was identified as a member of *R. mucilaginosa* species.

3.3. Optimal conditions of the transformation of betulin

In order to investigate the effect of initial culture pH on transformation production, *R. mucilaginosa* was cultivated in the medium with different initial pH (4.0–8.0) in shake flask cultures. Maximum transformation product was obtained at an initial pH 6.0 though the byproduct was investigated at an initial pH of 7.0 (Fig. 2(A)). The optimal transformation temperature was investigated and found to be 20 °C (Fig. 2(B)). The optimal transformation time was determined at the end of exponential phase (24 h), where after, the transformation capacity decreased (Fig. 2(C)).

3.4. Isolation and purification of biotransformation products

In a 5-L mini-jar fermentor system, 52.65% of betulin in the transformation broth was converted to the product by *R. mucilaginosa.* By extraction, concentration and HPLC preparative

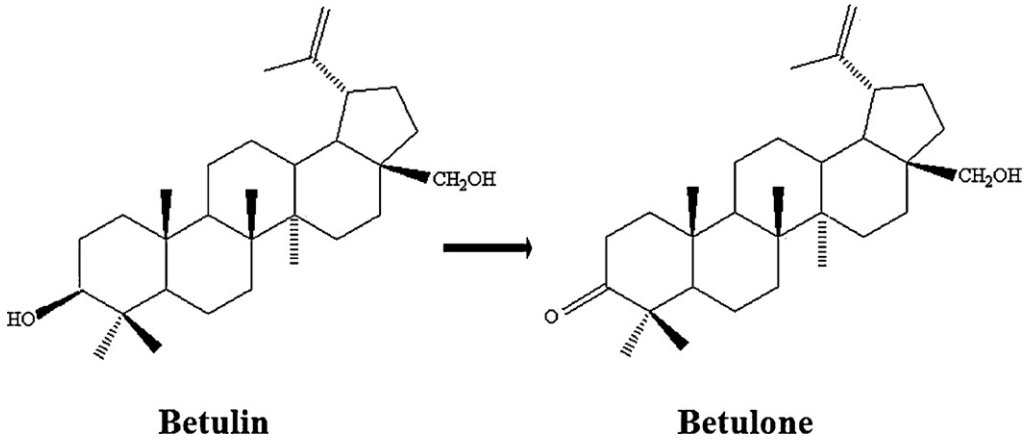

Fig. 4. Betulin was biotransformed to betulone.

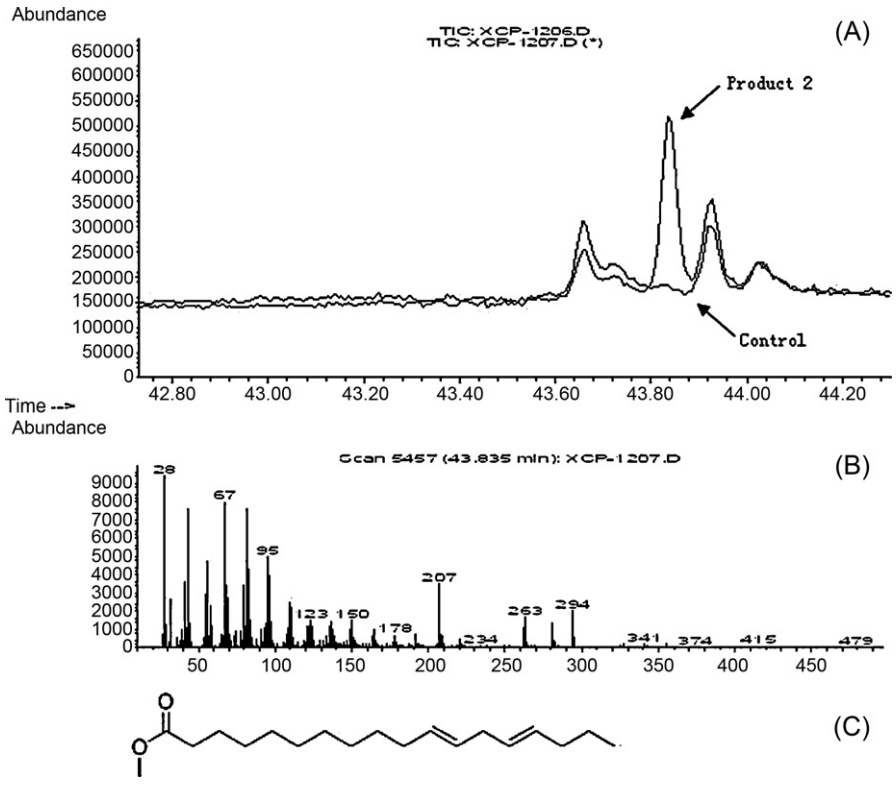

Fig. 5. GC–MS chromatogram (A), MS spectra (B) and the structure (C) of transformed product 2 by *Rhodotorula mucilaginosa.*

Table 1
^{13}C and ^{1}H NMR spectroscopic data for product 1, betulone.

^{13}C NMR (400 MHz, $CDCl_3$)	^{1}H NMR (400 MHz, $CDCl_3$)
δ: 218.382, 150.420, 109.798, 60.542, 54.902, 49.741, 48.679, 47.775, 47.775, 47.378, 42.778, 40.860, 39.609, 37.412, 36.877, 34.167, 33.968, 33.504, 29.715, 29.106, 27.019, 26.641, 25.197, 21.362, 21.058, 19.662, 19.094, 15.987, 15.796, 14.696	δ: 4.6859 (H-29), 4.5872 (H-29), 3.7927 (H-28), 3.3345 (H-28), 2.4710 (H-2), 2.4156 (H-2), 2.3883 (H-19), 1.6848 (H-30), 1.0740 (H-23), 1.0623 (H-26), 1.0262 (H-24), 0.9921 (H-27), 0.9285 (H-25)

Table 2
Antioxidative activity of betulone and betulin in the radical-scavenging system using DPPH.

Sample concentration (mg/ml)	DPPH scavenging rate (%)	
	Betulin	Betulone
0.5	0.87 ± 0.02	1.51 ± 0.05
1.0	2.95 ± 0.16	4.62 ± 0.12
2.0	8.35 ± 0.21	18.32 ± 0.25

purification, 35 mg product 1 (purity 99.0%) and 24 mg product 2 (purity 99.5%) were obtained.

LC/ESI-MS analysis of the transformed product 1 exhibited the $[M+H]^+$ peaks at m/z 441.3729 (relative abundance: 99.5%) (Fig. 3), the molecular formula of transformed product was determined to be $C_{30}H_{48}O_2$. The ^{13}C NMR and ^{1}H NMR data (Table 1) indicated that the keto (=O) group locates at the 3′-position. Therefore, the product was identified as betulone (Fig. 4) with the spectroscopic data identical to those reported in the literature [15]. The most reasonable explanation is that the transformation of betulin by *R. mucilaginosa* removed two hydrogen atoms from the parent betulin. The GC–MS (Fig. 5(A) and (B)) was used to identify the semi-volatile compound (product 2) as 11,14-Octadecadienoic acid methyl ester (Fig. 5(C)). The metabolic process is still unknown and the metabolic flux is under further investigation.

3.5. Antioxidative activity of betulone

In the radical-scavenging system of DPPH, the activity of betulone is more than about 2 times higher than that of betulin (Table 2). The effect was concentration-dependent, with scavenging potencies increasing with concentration. This result showed that the present of keto group seemed essential for higher DPPH radical scavenging ability [26].

4. Conclusion

In this study, a novel biotransformation process of betulin to betulone was developed. A yeast strain was screened from soil that could catalyze betulin and identified to be *R. mucilaginosa*. An initial pH 6.0, 20 °C for 1 d was favorable for the accumulation of betulone. Because the yield of betulone is still low, the scale-up of fermentation processes for betulone in a 5-L bioreactor is in progress. According to chemical structure, the 3-OH group in betulin is relatively less active due to more steric hindrance compared to the 28-OH. Chemical synthesis of betulone normally takes 3 steps: selective protection of 28-OH by pyranyl (THP) group and oxidation of the free 3-OH to ketone followed by the removal of THP. The reported biotransformation selectively oxidizes 3-OH without protection of the 28-OH, resulting in betulone in one step. Furthermore, betulone was investigated to show antioxidative activity. Investigation of other biological activities of betulone, such as anticancer activity, anti-HIV, anti-hyperlipidemic are also undergoing in our laboratory.

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