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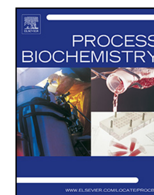

Short communication

# Biotransformation of betulin to betulone by growing and resting cells of the actinobacterium *Rhodococcus rhodochrous* IEGM 66

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ABSTRACT

The ability of *Rhodococcus* actinobacteria to transform betulin to betulone was proved and reported for the first time. Betulone, the product of regioselective oxidation of a 3β-hydroxyl group of betulin, is a useful intermediate in the synthesis of novel biologically active compounds. Of 56 strains of *Rhodococcus* tested, *Rhodococcus rhodochrous* IEGM 66 was selected because it had the highest betulin-transforming ability. It was shown that *R. rhodochrous* IEGM 66 growing cells transformed 0.5 g/L betulin to betulone with 45% conversion rate within 240 h. A substantial reduction in the time of betulin (0.5 g/L) biotransformation was achieved by using resting cells, which catalyzed the production of 75% betulone after 96 h. At higher initial betulin concentrations (1.0–3.0 g/L), resting cells catalyzed 40–60% betulone production within 24 h.

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## 1. Introduction

Betulin (**1**), a pentacyclic lupane triterpenoid, is one of the main compounds of birch bark extract (*Betula* spp.). Betulin is used to synthesize biologically active (anti-inflammatory, hepatoprotective, antitumor, antiviral, antimalarial, antimicrobial, etc.) derivatives [1–3]. Biocatalysis allows for the production of pharmacologically important products with high stereo- and regioselectivity in a one-stage process under mild and eco-friendly reaction conditions. Attempts have been made to use microorganisms in the biological transformation of betulin. A few papers have reported the biotransformation of betulin using eukaryotic microorganisms [4,5], in particular, the mycelial fungi *Armillaria*, *Aspergillus*, *Chaetomium*, *Dothideomycetes*, and the unicellular fungus (yeast) *Rhodotorula*. The processes of oxidative betulin cleavage to 4,28-dihydroxy-3,4-secolup-20(29)-en-3-oic acid by *Chaetomium longirostre* IFO 9873 [6] and betulin oxidation to betulinic acid by *Armillaria luteo-virens* Sacc QH, *Aspergillus foetidus* Zu-G1, and *A. oryzae* Sacc QH were described [7,8]. Recently, the first papers on regioselective oxidation of betulin to betulone (**2**) by *Rhodotorula mucilaginosa* F10 [9] and *Dothideomycete* sp. HQ 316564 [10] have been published.

Betulone, a 3-oxo derivative of betulin, is gaining interest as one of the useful intermediates in the synthesis of biologically active compounds [11–14]. The process of the chemical synthesis of cytotoxic derivatives using betulone as a key building block has been described [11,12]. Using microorganisms enables the one-step oxidation of a secondary hydroxyl group of betulin to an oxo group without affecting the native hydroxyl group at C-28. It avoids complicated protection and deprotection steps, which are essential in the three-step chemical synthesis of betulone.

The reported biotransformation of betulin to betulone by growing yeast and fungal cells is characterized by low (0.057 and 0.1 g/L, respectively) initial betulin concentrations. The biotransformation process with *Dothideomycete* sp. HQ 316564 proceeded for 144 h, while an opportunistic pathogen *R. mucilaginosa* F10 produced betulone along with a secondary metabolite 11,14-octadecadienoic acid methyl ester. Previously reported biotransformation processes were not effective therefore it is essential to search for novel microbial cultures that efficiently convert betulin to betulone.

*Rhodococcus* actinobacteria are one of the most extensively developed groups of prokaryotic microorganisms used in industrial biotechnology. Typical bacterial growth, lability of their metabolic system, biosurfactant production, the ability to grow on basal media, and high transforming activities [15–17] all make

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rhodococci a promising biocatalyst for oxidative betulin biotransformation. In the present work, data on betulin biotransformation by growing and resting cells are presented for the first time.

## 2. Materials and methods

### 2.1. Microorganisms and the substrate

In this work, 56 strains used were from the Regional Specialized Collection of Alkanotrophic Microorganisms (acronym IEGM; member of the World Federation for Culture Collection, WFCC #768; www.iegm.ru/iegmcol) representing the species *R. erythropolis* (21 strains), *R.* '*longus*' (5 strains), *R. opacus* (5 strains), *R. rhodochrous* (5 strains), and *R. ruber* (20 strains).

Betulin was produced by extraction from birch bark followed by recrystallization using a mixture of isopropanol:water (9:1, v/v) for both procedures. The purity was above 95% as shown by GC–MS analysis. All other chemicals used in this study were of the highest commercially available grade.

### 2.2. Preliminary screening

All experiments were performed in 250 ml Erlenmeyer flasks containing 100 ml of medium with shaking (160 rpm) at 28 °C. The mineral medium K was used containing (per L) 1 g $KNO_3$; 1 g $K_2HPO_4$; 1 g $KH_2PO_4$; 1 g NaCl; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.02 g $CaCl_2 \cdot 2H_2O$; and 0.001 g $FeCl_3$. The initial pH of the medium was adjusted to 6.8 with 0.1 N HCl. The yeast extract (0.1%) was added to the mineral medium. Betulin (0.5 g/L) dissolved in 5 ml of dimethyl sulfoxide (DMSO) was used as a carbon source. Optical densities (OD) of cell suspensions were measured using a Lambda EZ201 UV spectrophotometer (Perkin-Elmer, USA) at 600 nm. Suspensions of rhodococcal cells ($OD_{600}$ 1.0) pre-grown on agar (Oxoid, UK) for 2 days were used to inoculate media. The process of betulin biotransformation by growing cells was studied in LB broth (Sigma, USA) or in the mineral medium K enriched with glucose (1.0%), glycerol (1.0%), or *n*-hexadecane (1.0 vol.%). Betulin (0.5 g/L) was added after 48 h of cell cultivation. The betulin biotransformation by growing cells proceeded for 240 h. Abiotic controls contained the sterile culture medium plus the same amount of the substrate used. Culture controls consisted of fermentation blanks in which bacteria were grown under identical conditions without substrate.

### 2.3. Biotransformation by resting cells

*R. rhodochrous* IEGM 66 was grown either in LB broth or the mineral medium K supplemented with glucose (1.0%), glycerol (1.0%), or *n*-hexadecane (1.0 vol.%) for 48 h. Cells were harvested by centrifugation at 3000 rpm for 10 min and washed three times with Clark-Labs phosphate-alkaline buffer (pH 7) [18]. The washed cells were resuspended in 100 ml of the same buffer (pH 6, 7, 8, or 9) to achieve final $OD_{600}$ values of 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, or 2.8. The cell mass was determined by a calibration curve relating the optical density ($OD_{600}$) and dry cell weight (DCW, g/L). Betulin was dissolved in DMSO and added to cell suspensions at a concentration of 0.5, 1.0, 2.0, or 3.0 g/L. Preliminary biotransformation experiments with resting cells were performed for 48 h. The dynamics of betulone production by resting cells were monitored by sampling at 24 h intervals over the course of 96 h. Abiotic controls contained sterile buffer solutions supplemented with similar amounts of the initial substrate.

### 2.4. Respirometry analysis

The oxygen uptake rates with resting cells were measured using a six-channel MicroOxymax® respirometer (Columbus Instruments, USA) connected to a PC. The experiments were performed in 300 ml bottles containing 100 ml of cell suspension in the buffer ($OD_{600}$ 2.6) and supplemented with betulin at a concentration of 0.5, 1.0, 2.0, or 3.0 g/L. The experiments were carried out with constant stirring (300 rpm) using an RT 10 magnetic stirrer (Power IKAMAG, Germany) at 25 ± 2 °C. Respiration rates (μl/min) were measured at 42-min intervals over the course of 96 h. Cell suspension without betulin was used as a control.

### 2.5. Isolation and identification of biotransformation metabolites

The culture media were acidified with an aqueous solution of 10% HCl and extracted three times with an equal volume of ethyl acetate. The combined organic layers were washed with an aqueous solution of 1% $Na_2CO_3$ and then with distilled water to a final pH of 7.0. The ethyl acetate extract was dried over anhydrous $Na_2SO_4$ and concentrated under a vacuum. The biotransformation products were monitored by thin layer chromatography (TLC) using silica gel 60 plates (Merk, Germany). The sample spots were visualized by treating plates with ethyl acetate:*n*-hexane (1:4, v/v) and spraying with an aqueous solution of 5% $H_2SO_4$ followed by heating at 95–100 °C for 2–3 min. The chromatography–mass-spectrometry analyses of the biotransformation products were performed using an Agilent 6890N/5975B chromatograph (Agilent Technologies, USA) equipped with a HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm; 70 eV electron impact). Helium was used as a carrier gas. The ethyl acetate extracts (0.2 μl) were injected and analyzed by separation of the carrier gas flow (from 1:1 to 11:1). The column temperature was adjusted to 150 °C and programmed at 50 °C/min to 300 °C. The mass spectra (MS) were recorded in the range of 40–460 *m/z* and compared with those from the NIST08 Library.

### 2.6. Preparative-scale betulin biotransformation

Preparative-scale biotransformation of betulin by *R. rhodochrous* IEGM 66 resting cells was carried out in 250 ml Erlenmeyer flasks containing 100 ml of cell suspension in the phosphate-alkaline buffer ($OD_{600}$ 2.6) with shaking (160 rpm at 28 °C). Betulin (0.3 g) dissolved in 3 ml of DMSO was added. The biotransformation products were extracted three times with equivalent volumes of ethyl acetate after 24 h, and a crude extract was produced (0.28 g).

### 2.7. Purification and identification of betulone

The resulting crude extract (0.28 g) was separated by column chromatography using silica gel (60–200 μm) (Merck, Germany) with the compound:sorbent ratio of 1:50 and with *n*-hexane:ethyl acetate (9:1, v/v) as the eluent. The $^1$H NMR spectrum of the solution in $CDCl_3$ was recorded on a Varian Mercury Plus 300 MHz NMR spectrometer (Varian Inc., USA) (the internal standard was hexamethyldisiloxane). The melting point was measured using an OptiMelt MPA100 instrument (Stanford Research Systems Inc., USA).

Betulone (lup-20(29)-en-28-ol-3-one) (**2**) (0.11 g, 36.8% yield). White powder; mp 108.8 °C (*n*-hexane–ethyl acetate, 5:1) (lit.: mp 94–96 °C (*n*-hexane) [19], 175–176 °C [20]). $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 0.92 (3H, s), 0.98 (3H, s), 1.01 (3H, s), 1.05 (3H, s), 1.06 (3H, s), 1.67 (3H, s), 2.37–2.48 (3H, m, H-19 + 2H-2), 3.33 and 3.79 (2H, 2d, $J$ = 10.8 Hz, $H_2$-28), 4.57 and 4.68 (2H, 2 broad s, $H_2$-29). MS, *m/z* (relative intensity): 440.4 (9.51, M+) [21].

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