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Optimization for betulin production from mycelial culture of *Inonotus obliquus* by orthogonal design and evaluation of its antioxidant activity

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ABSTRACT

This paper is concerned with optimization of submerged culture conditions for the mycelial growth and betulin production by *Inonotus obliquus* by one-factor-at-a-time and orthogonal experiment design. Among the variables of medium components, glucose, yeast extract, and $MgSO_4$ were identified to be the most suitable carbon, nitrogen, and mineral sources, respectively. The optimal temperature and initial pH for mycelial growth and betulin production were found to be 25 °C and 6.0, respectively. Subsequently, the concentration of glucose, yeast extract, and $MgSO_4$ were optimized using the orthogonal experiment design. The optimal concentration for the enhanced production are determined as 30 g/L glucose, 3 g/L yeast extract, 5 mmol/L $MgSO_4{\cdot}7H_2O$ for mycelial yield, and 30 g/L glucose, 3.5 g/L yeast extract, 5 mmol/L $MgSO_4{\cdot}7H_2O$ for betulin production, respectively. The subsequent verification experiments confirmed the validity of the models. Under optimal culture conditions, the maximum betulin concentration in a 5-L stirred-tank bioreactor reached to 69.37 mg/L. Furthermore, the morphological parameters of the pellets were characterized by their mean diameter, circularity, roughness and compactness. It was proved that mycelial growth and pellet morphology (*i.e.* compactness, mean diameter and roughness) may be the critical parameters affecting betulin production. In addition, betulin showed the potential antioxidant capacities on scavenging DPPH radical and hydroxyl radical.

1. Introduction

Betulin is one of pentacyclic triterpenoids, which possesses many pharmacological effects, such as antiviral, anti-inflammatory, antitumor activities [1], and protective effects against Cadmium (Cd)-induced cytotoxicity [2]. In particular, betulin can be used as such or after chemical modification as a starting compound for other useful biologically active derivatives, such as betulinic acid, which possess various interesting pharmacological properties.

I. obliquus otherwise known as chaga (Family, Hymenochaetaceae), is a black parasitic fungus that grows on living trunks of the mature birch in northern climates such as Russia. Recent research also proved that *I. obliquus* has comprehensive pharmacological effects [3]. In previous investigations on the chemical constituents of this mushroom, lanostane-type triterpenoids, such as trametenolicacid, inotodiol and inonotsuoxides were reported to have antitumor and antifungal activities [4]. Some phenolic compounds, such as betulin, were also found in this mushroom and shown the most positive therapeutic effects [5,6]. Gao et al. extracted betulin by ultrasonic method from fruiting bodies and mycelial of *I. obliquus* and found betulin extraction production in mycelial was higher than that in fruiting bodies [7]. Yin et al. investigated the extraction of betulin from fruiting body of *I. obliquus* by applying high intensity pulsed electric fields [8]. However, available literature reviewed no information on the nutritional requirements and environmental conditions for submerged culture of *I. obliquus* for betulin production.

In the present study, the one-factor-at-a time method and orthogonal matrix design was used to optimize the submerged culture conditions to simultaneously produce the mycelial biomass and betulin by *I. obliquus*. Meanwhile, the morphology of *I. obliquus* was characterized and the favorable mycelial form for betulin production was determined during the fermentation period. Then, *in vitro* antioxidant capacity of betulin was also investigated.

2. Materials and methods

2.1. Chemicals and reagents

Standard betulin (99%) was purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd. Methanol (HPLC grade) was obtained from Siyou (Tianjin, China). Other chemicals used in the study were of analytical grade.

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2.2. Preparation of standard solution

Stock solution of betulin in 0.125 mg/ml was dissolved in methanol and kept at 4 °C in darkness. Before analysis by HPLC, the solution was filtered with 0.22 μm Millipore filter.

2.3. Microorganisms and culture conditions

I. obliquus was originally isolated form a mountainous district in Jilin Province of PR China, then phylogenetically identified by ITS-5.8S rDNA sequencing analysis and preserved at the Henan Province Microbiological Culture Collection Center (HPMCC no. 1945363). The fungus was maintained on potato dextrose agar (PDA) slants at 4 °C. *I. obliquus* was initially grown for 6 days on PDA medium in Petri dish. A disc (5 mm) was cut from the margins of 6 days old culture of the agar plate culture and then transferred into the seed medium. The seed culture was grown in a 250 ml flask containing 50 ml medium (30.0 g/L glucose, 3.0 g/L bran extract) and 28 °C on a rotary shaker incubator at 150 rev/min for 4 d. Then, the mycelium pellets of seed culture were grinded with the sterile glass beads. The flask culture experiments were performed in a 250 ml flask containing 50 ml medium after inoculating with 4% (v/v) of the seed culture (about 0.3 g/L dry mycelium). The pH and agitation rates were controlled at 6.0 and 150 rpm, respectively. To investigate the effect of initial pH on mycelial growth and the yield of betulin in shake flask cultures, the different initial pH was adjusted by addition of 1.0 M NaOH or HCl, and the temperature change were conducted in the temperature controlled shaking incubator.

2.4. Bioreactor fermentation

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, 25 °C; agitation speed, 150 rpm; initial pH, 6.0; working volume, 3.5 L. All experiments were performed in triplicate to ensure the trends observed were reproducible.

2.5. Characterization of the morphology

The morphological properties of the samples collected were evaluated using an image analyzer (DT2000 System, China) with software linked to a light microscope (Nikon, Japan) through a CCD camera. Samples were fixed with an equal volume of fixative (13 ml of 40% formaldehyde, 5 ml glacial acetic acid, 200 ml of 50% ethanol). An aliquot (0.1 ml) of each fixed sample was transferred to a slide, air dried, and then stained with methylene blue (0.3 g of methylene blue, 30 ml of 95% ethanol in 100 ml water). For each sample, the morphology of pellet was characterized by measuring the area and perimeter of the pellet core and the maximum diameter of the pellet. Normally, a magnification of 40 was used. The morphology of the pellets was characterized by their mean diameter, circularity, roughness and compactness. The circularity was estimated as the ratio of the Fieret's minimum diameter to the Fieret's maximum diameter of the pellets or aggregates. The compactness was estimated as the ratio of the projected area of the hyphae in a clump to the projected convex area of that clump, the latter being the area after filling internal voids and concavities in the clump's external perimeter. In addition, the roughness (R) was measured using the following equation: $R = (\text{pellet/aggregate perimeter})^2/(4\pi \times \text{pellet area})$.

2.6. Analytical methods

After cultivation for several days, mycelium was harvested from the cultivation broth by centrifuging at 10,000 r/min for 10 min at 4 °C and washed twice with distilled water. Mycelium was dried through vacuum freeze drying. Then mycelium (0.1 g) was accurately weighed and extracted with 2 ml isopropanol by ultrasonic for 2 h. The isopropanol solution was dried under vacuum condition at rotavapor at 40 °C. Finally, the collected residues were dissolved in 1 ml methanol, filtered with a 0.22-μm millipore filter and analyzed by 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

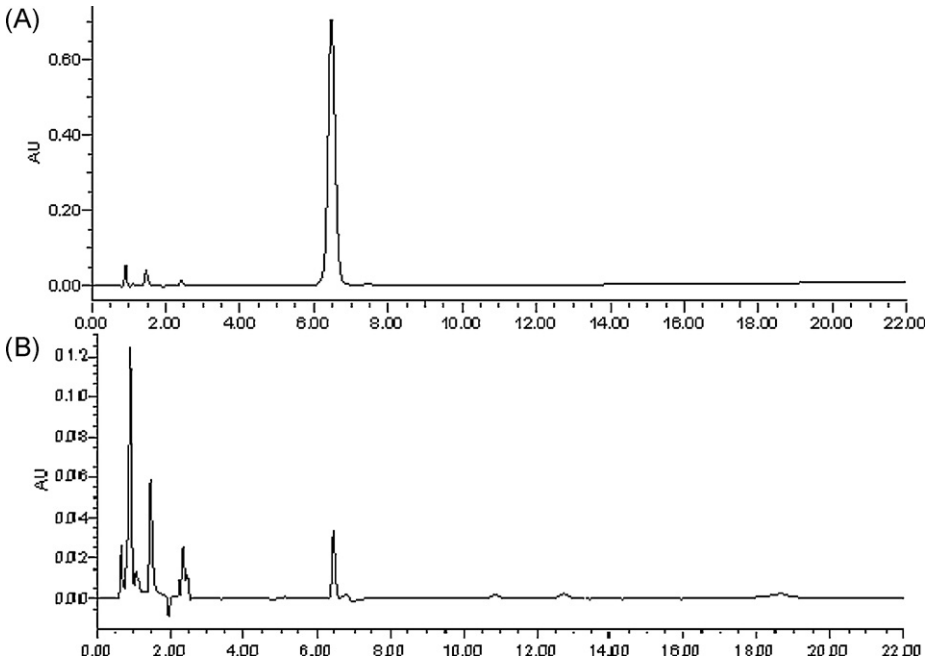

Fig. 1. Chromatogram of standard betulin (A) and chromatogram of extracted betulin from *I. obliquus* (B).