Journal of Bioscience and Bioengineering
VOL. 118 No. 6, 728–731, 2014

www.elsevier.com/locate/jbiosc

Stimulated production of steroids in *Inonotus obliquus* by host factors from birch

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Received 18 March 2014; accepted 29 May 2014
Available online 12 July 2014

Steroids was considered as one of the bioactive components in *Inonotus obliquus*, while this kind of secondary metabolites are less accumulated in cultured mycelia. In this study, effect of extracts from bark and core of host-related species, birch (*Betula platyphylla* Suk.), on steroid production of *I. obliquus* in submerged culture were evaluated. The results showed that all dosages (0.01 and 0.1 g/L) of aqueous extracts and methanol extracts from birch bark and birch core possessed significantly stimulatory effect on steroid production of *I. obliquus* ($P < 0.05$). Among the eight extracts, the aqueous extract (0.01 g/L) from birch bark gave the highest steroid production (225.5 ± 8.7 mg/L), which is 97.3% higher than that of the control group. The aqueous extract (0.01 and 0.1 g/L) from birch bark could simultaneously stimulated mycelial growth and steroid content, while the methanol extract from birch bark only elevated the steroid content. High performance liquid chromatography analysis showed that productions of betulin, ergosterol, cholesterol, lanosterol, stigmasterol, and sitosterol in *I. obliquus* simultaneously increased in the presence of aqueous extract and methanol extract from birch bark. The results presented herein indicate that extracts from birch bark could act as an inducer for steroid biosynthesis of *I. obliquus*.

[**Key words:** *Betula platyphylla*; Birch; *Inonotus obliquus*; Steroids; Submerged fermentation]

The medicinal mushroom *Inonotus obliquus* (Pers.:Fr.) Pilát (Hymenochaetaceae) grows on silver birch trees (*Betula platyphylla*) forming a hard dark-brown sclerotia. *I. obliquus* has been used for cancer treatment in Russia since the 17th century (1). In recent years, extracts from this fungus have exhibited various biological activities, including hypoglycaemic, anti-viral, anti-fungal, and anti-tumor activities (2,3). Thus, a great demand for the sclerotia of *I. obliquus* is observed due to its potent bioactivities. However, sclerotia of *I. obliquus* grows and accumulates bioactive metabolites very slowly in nature since it is restricted to cold conditions. In addition, artificial cultivation of this fungus usually takes several months and it is difficult to control the quality of product. As an alternative method, growing it by submerged culture can provide fungal biomass of consistent quality (4).

Recent studies have shown that both fruiting bodies and mycelia of *I. obliquus* contain many bioactive metabolites, such as steroids, triterpenoids, polysaccharides, and phenolic compounds (5–7). Hereinto, steroids in *I. obliquus* were considered as one of the main active compounds for their effective therapy of many diseases (2,8,9). Ethanol extract of *I. obliquus* containing triterpenoids and steroids also presented a strong antioxidant effect (8). Several methods including high performance liquid chromatography (HPLC), and gas chromatography combined with mass spectrometry (GC–MS) have been used to analyze different steroids in *I. obliquus* (10,11). On the other hand, steroids were less accumulated in cultured mycelia while accumulated in a large amount in the wild (12). Therefore, it is of interest to investigate the effect of host factor from birch on the steroid synthesis in mycelia of *I. obliquus* in submerged culture.

In this study, extracts of birch core and birch bark were prepared with solvents with different polarity. Then effects of various extracts on the mycelial growth and steroid production of *I. obliquus* were evaluated. Furthermore, synthesis of six steroids (betulin, ergosterol, cholesterol, lanosterol, stigmasterol, sitosterol) in the mycelia of *I. obliquus* were studied in the culture media contained extracts with potent stimulatory activity on the steroid production of *I. obliquus*.

MATERIALS AND METHODS

Submerged fermentation of *I. obliquus* *I. obliquus* JNPF-IO01 strain was obtained from the Laboratory of Pharmaceutical Engineering, Jiangnan University (Jiangsu, China). Mycelia of *I. obliquus* were inoculated into basal culture medium composed of 2.5% glucose, 0.3% tryptone, 0.15% $MgSO_4$ and 0.3% KH_2PO_4 at pH 4.0. The shaking flask culture was carried out in a 250 mL Erlenmeyer flask containing 50 mL of medium, at 25°C for 120 h by shaking at 150 rpm/min. *I. obliquus* cultured in the basal medium served as control. Dimethyl sulfoxide (DMSO, 300 μL/L) or different extracts (0.01 and 0.1 g/L) from birch core and birch bark were added to the basal medium at corresponding dosages and incubated for 12 days.

Preparation of extracts from birch core and birch bark Birch core and birch bark as finely ground pieces were obtained the Liaoning province in the northeast of China. The birch core and birch bark were finely ground to powder form, and dried at 60°C. Fifty grams each of the birch core and birch bark powders were extracted sequentially with 500 mL petroleum ether (PE), ethyl acetate (EAC) and methanol (MT) three times for 12 h at room temperature. Then, the residues were extracted with water three times for 30 min at 95°C. All the extracts were

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.05.022

filtered through a Whatman no. 2 filter (Maidstone, UK), concentrated to paste by vacuum distillation using the rotary evaporator and vacuum controller to maintain the desired pressure and temperature. The dried extracts were dissolved in DMSO to form 500 mg/mL stock solution and stored at −20°C before being used. The extracts were diluted in culture media to the final concentrations (0.01 and 0.1 g/L). Final concentrations of DMSO in all groups were less than 300 μL/L.

Determination of polysaccharides, polyphenols and triterpenoids in birch extracts Content of crude polysaccharides in various birch extracts was determined using the phenol-sulfuric acid method (13). Contents of total polyphenols and triterpenoids in various birch extracts were determined using the Folin–Ciocalteau method (14) and the method of Song and Yen (15), respectively.

Determination of dry cell weight Mycelia from the culture broth of *I. obliquus* were obtained by filtration through a mesh (pore size, 30 μm), washed with distilled water, and collected by centrifugation at 5000 rpm (twice). The mycelia were freeze dried, weighed to calculate the dry cell weight (DCW), and maintained at 4°C until further analysis.

Analysis of intracellular steroids Contents of betulin, ergosterol, cholesterol, lanosterol, stigmasterol, and sitosterol in the mycelia of *I. obliquus* were determined using the method described by Gao (11). Mycelia (0.1 g) of *I. obliquus* was accurately weighted and extracted with 1 mL methanol by ultrasonic for 2 h. The methanol solutions were filtered through a 0.45 μm Millipore filter unit. Then 20 μL of each sample solution was analyzed by HPLC. HPLC was performed on an UltiMate 3000 system (Dionex, USA). The detecting wavelength was set at 202 nm. Separations were obtained with a column (Waters XBridge C18, 5 μm, 4.6 × 250 mm) eluted at a flow rate of 1.4 mL/min with stepwise gradient elution using eluents A and B (A: CH_3OH; B: H_2O) according to the following A–B profile: 0–10 min, 90% A and 10% B; 10–40 min, 97% A and 3% B. The column temperature was maintained at 30°C. Steroid production (mg/L) was calculated by multiplying the steroid content (mg/g) by dry cell weight (g/L) of *I. obliquus*.

Statistical analysis Data are expressed as means ± standard deviation (SD). Differences in measured variables between experimental and control group were assessed by using one-way analysis of variance (ANOVA). Results were considered statistically significant at $P < 0.05$.

RESULTS

Component analysis of birch extracts Birch core and birch bark were extracted with PE, EAC, MT, and water, successively. The extraction yields are presented in Table 1. The yields of MT extract from birch core and birch bark were 2.58 ± 0.08% and 4.07 ± 0.33%, respectively, which are highest in all the extracts. Contents of crude polysaccharides, polyphenols and triterpenoids in the extracts from birch bore and birch bark were assayed. Contents of crude polysaccharides in aqueous extract of birch bark was 2.1-fold than that of aqueous extract of birch core. In this study, no triterpenoid was detected in aqueous extract, and PE, EAC and MT extracts had no detectable trace of polysaccharides.

Effect of birch extracts on steroid production of *I. obliquus* *I. obliquus* was cultured in the presence and absence of the extracts from birch core and birch. As shown in Fig. 1, DCWs, steroid contents, and steroid productions in the control group and DMSO group showed no significant difference ($P > 0.05$), which indicates DMSO at a dosage of 300 μL/L showed no effect on the mycelial growth and steroid production of *I. obliquus*. The aqueous extract (0.01 g/L) of birch bark significantly promoted biomass growth of *I. obliquus* ($P < 0.05$), while the PE extract (0.01 and 0.1 g/L) from birch bark significantly inhibited mycelial growth of *I. obliquus* ($P < 0.05$) (Fig. 1A). All dosages of the extracts from birch core showed no significant effect on mycelial growth of *I. obliquus* ($P > 0.05$) (Fig. 1A).

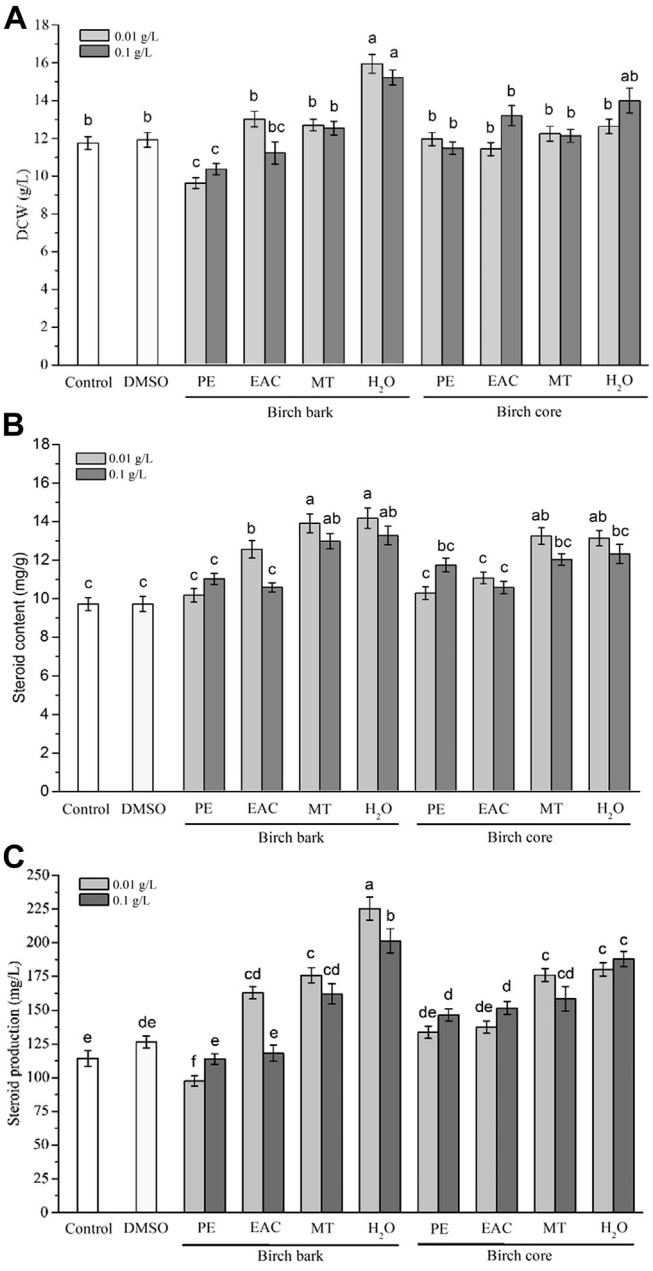

FIG. 1. Effects of extracts from birch core and birch bark on DCW (A), steroid content (B) and steroid production (C) of *I. obliquus*. *I. obliquus* was cultured in basal medium contained extracts from birch core and birch bark with high (0.1 g/L) and low (0.01 g/L) concentration. Values were expressed as mean ± SD ($n = 3$). Same letters in the columns indicate non-significant differences ($P < 0.05$).

All dosages of MT extract and aqueous extract from birch bark, and lower dosage of MT extract and aqueous extract from birch core showed significantly stimulatory effect on steroid synthesis of *I. obliquus* ($P < 0.05$) (Fig. 1B). The maximum of steroid content

TABLE 1. Polyphenol, triterpenoid and polysaccharide contents of extracts from birch core and birch bark.

	Birch core				Birch bark			
	PE	EAC	MT	H_2O	PE	EAC	MT	H_2O
Extraction yields (%)	1.22 ± 0.06	0.72 ± 0.03	2.58 ± 0.08	1.53 ± 0.06	1.93 ± 0.08	0.74 ± 0.09	4.07 ± 0.33	3.22 ± 0.08
Polyphenol content (mg/g extraction)	27.37 ± 0.15	19.30 ± 0.08	74.96 ± 0.17	89.38 ± 0.19	75.19 ± 0.16	21.99 ± 0.14	121.54 ± 0.18	133.43 ± 0.20
Triterpenoid content (mg/g extraction)	212.56 ± 0.29	322.41 ± 0.30	91.55 ± 0.13	nd	379.50 ± 0.32	116.78 ± 0.14	59.61 ± 0.12	nd
Polysaccharide content (mg/g extraction)	nd	nd	nd	124.27 ± 0.03	nd	nd	nd	264.98 ± 0.26

nd, not detectable.