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Antioxidant activity of liquid cultured *Inonotus obliquus* polyphenols using tween-20 as a stimulatory agent: Correlation of the activity and the phenolic profiles

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ABSTRACT

Polyphenols are major functional components of the medicinal mushroom *Inonotus obliquus*. Compared with the wide application of surfactants in medicinal mushroom polysaccharide production, using surfactants to enhance mushroom polyphenol production and antioxidant activity is a relatively new approach. For the first time, in the present study, we evaluated the effect of Tween-20 on the growth-associated generation of different compositions and antioxidant activity expression of exo-polyphenol product (EPC) and endo-polyphenol product (IPC) of liquid cultured *I. obliquus*. The IC_{50} values against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals of EA-EPC, NB-EPC, and IPC from the Tween-20 (5.0 g/l on Day 1) adding medium were between 40 and 47 mg/l, significantly ($p < 0.05$) lower than the control group (96–112 mg/l). Phenolic acids, *i.e.,* ferulic acid and gallic acid, and flavonoids, *i.e.,* epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), and naringin, were the main components in EPC and IPC analyzed by HPLC. The production of ferulic acid, ECG, EGCG, and naringin was significantly increased by 106.0%, 157.7%, 37.3%, 75.2% in EA-EPC, 372.9%, 133.7%, 117.3%, 19.4% in NB-EPC, and 10.4%, 102.7%, 57.7%, 322.2% in IPC. The antioxidant activity expression of EA-EPC, NB-EPC, and IPC from both the control and Tween-20 media was highly correlated with the growth-associated generation of these flavonoids during fermentation. The significantly higher antioxidant activity of EPC and IPC from the Tween-20 medium compared with the control was also attributed to the higher content of the flavonoids. The strategy of supplementation of surfactants has the potential for other medicinal mushroom fermentation processes to enhance the production of highly bioactive flavonoids.

1. Introduction

Reactive radicals and oxygen species can induce oxidative stress and break down the balance in human body, and oxidized products may increase the chance of having some cancers, degenerative disorders or other diseases [1]. Phenolic compound-rich foods such as mushrooms and plants have antioxidant activity, that is, inhibitory effect of oxidation [2], thus reduce damage of human body [3]. *Inonotus obliquus* (*I. obliquus*), belonging to the family Hymenochaetaceae in the Basidiomycota, is such a well-known medicinal mushroom with hypoglycaemic, immunomodulating, anti-cancer, anti-viral, and antioxidant activities [4,5]. The phenolic compounds of *I. obliquus* from both wild sclerotia and liquid cultures have attracted much attention due to their superb antioxidant, anti-inflammatory and antitumor agents [6–9].

Many studies have been conducted on the optimization of submerged culture conditions [10–16], as the production of mushroom mycelial and phenolic compounds under submerged fermentation is subjective to the mushroom's growth environment. Recently, there is an increasing interest in the search for chemical agents to stimulate mushroom mycelial polysaccharide production under submerged fermentation. These stimulatory agents include vegetable oils, fatty acids, and surfactants (originally used as antifoam agents), most of which researchers reported effective in promoting the exopolysaccharide (EPS) production of fungi [17–22]. Our previous studies investigated the feasibility of using fatty acids (oleic acid, linoleic acid, plamitic acid, and stearic acid), surfactants (Tween-20, Tween-80, CHAPS, TritonX-100, PEG 4000), and organic solvents (chloroform, toluene, acetone, methanol, ethanol) to enhance the production of exo-/endo-polysaccharides (EPS/IPS) and exo-/endo-polyphenols (EPC/IPC) of *I. obliquus* under submerged fermentation [23,24]. The effect of agents from different categories on the cellular growth and production of EPS, IPS, EPC, and IPC varied. Tween-80 and oleic acid were proved to be the most effective

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in enhancing the EPS and IPS production, respectively [23]. All the fatty acids and surfactants were favorable for the production of both the EPC and IPC. As known, phenolic compounds include a large number of subclasses, such as flavonoids, phenolic acids, stilbenes, lignans, tannins, and oxidized polyphenols, displaying a great diversity of structures. Individual phenolic compound could show markedly different antioxidant effects as a result of synergism, antagonism, co-antioxidation and the presence of oxidation retarders [25]. Although linoleic acid was the most effective agent among the 14 tested chemicals including Tween-20 in the screening experiment, linoleic acid significantly inhibited the mushroom growth as a disadvantage factor [24]. More importantly, considering the fact that the antioxidant activity is known to be more dependent on the phenolic compositions of polyphenol products than on the total phenolic content [26], Furthermore, the phenolic compositions of polyphenol products are highly likely to be subjective to the addition of stimulatory agents. Therefore, in the present study, we further investigated the effect of the two groups of fatty acids and surfactants on the antioxidant activity based on their better effects in enhancing the production of polyphenol products. What we found was that Tween-20, rather than linoleic acid [24], was the most effective in enhancing the antioxidant activity of polyphenol product. It was then desirable to carry out a thorough study to optimize Tween-20 addition concentration and addition time. Under the optimal condition, for the first time, we found that both the generation of different compositions of polyphenol products and activity expression were growth-associated, and that the higher antioxidant activity was related to the more active compositions of polyphenol products from the Tween-20-containing medium than the control.

2. Materials and methods

2.1. Screening of chemical agents

Although 4 fatty acids, 5 surfactants, and 5 solvents were used before for screening of best chemicals in increasing the production of EPC and IPC [24], as far as the antioxidant activity and active compositions of EPC and IPC was concerned, the DPPH radical-scavenging activity of EPC and IPC from the 4 fatty acids- and 5 surfactants-containing media was simply compared rather than a system optimization for quick selection of a potential chemical to enhance the antioxidant activity of EPC and IPC at the same time, to increase the production. The solvents were excluded because they were less effective in enhancing the production and possible toxic property for the mushroom growth. The chemical agents include (1) fatty acids: oleic acid, stearic acid, linoleic, acid and palmitic acid; (2) surfactants: Tween-20, polyoxyethylene sorbitan monooleate (Tween-80), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), polyoxyethylene octyl phenyl ether (TritonX-100), and polyethylene glycol-4000 (PEG-4000).

2.2. Inoculum preparation and liquid fermentation

I. obliquus (CBS314.39) was maintained on malt extract agar slants and cultivated for about 2 weeks at 25 °C, then stored at 4 °C, and subcultured every 3 months. The mycelia from a malt extract agar slant were inoculated in liquid medium (g/100 ml): glucose 2, peptone 0.3, yeast extract 0.2, KH_2PO_4, 0.1; $MgSO_4$, 0.15; and $CaCl_2$ 0.01, and then culture at 28 °C on a rotary shaker with a speed of 150 rpm for 4–5 days.

The harvested seed culture was added into 250 ml Erlenmeyer flasks containing 100 ml of control medium or stimulatory agent-containing medium, incubated at a ratio of 9% (v/v), then cultivated in a rotary shaker at 150 rpm at 28 °C for 9 days. Triplicate cultures in each group were performed. The media used in this work in the following list.

Control medium (g/100 ml) contained: corn flour, 5.3; peptone, 0.3; $CoCl_2$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.005; $CuSO_4 \cdot 5H_2O$, 0.002; $CaCl_2$ 0.05, KH_2PO_4, 0.1; $ZnSO_4 \cdot 2H_2O$, 0.001; K_2HPO_4, 0.05; $ZnSO_4 \cdot 7H_2O$, 0.001; and $MnCl_2 \cdot 4H_2O$, 0.009, pH=6.0. Corn flour and peptone, as the best carbon source and nitrogen source, were demonstrated by response surface methodology (RSM) in our previous work [27]. For the screening experiments, one of the 10 chemical agents at 1 g/l was added into the control medium on Day 0.

2.3. Extraction and determination of EPC and IPC

The fermentation broth and mycelia were isolated by suction filtration using a buchner funnel. Mycelia were washed three times with distilled water, dried in oven and then ground to a powder. The powder was disrupted by sonication at 20-s intervals for 10 min in 7 volume of 70% aqueous acetone (v/v) with ice bath and then extracted three times with 20 ml of 70% aqueous acetone (v/v) for 24 h. The aqueous acetone solutions were combined, concentrated, and freeze-dried to give IPC extracts.

The culture broth was concentrated under vacuum and then stored at 4 °C with addition of 95% ethanol to remove the polysaccharides. The aqueous solution was extracted with 1 volume ×3 of chloroform to remove impurities firstly, and the remaining aqueous solution was extracted with 1 volume × 3 of ethyl acetate (EA) and then with n-butanol (NB). The EA and NB extracts were concentrated under vacuum and lyophilized to remove organic solvents, and then to give EA-soluble polyphenol product (EA-EPC) and NB-soluble polyphenol product (NB-EPC).

Folin–Ciocalteu reagent method was used to measured total phenolic content. Gallic acid equivalents (GAE) was used as a standard curve generated with 0–60 mg GAE/l [28].

2.4. Assay for DPPH radical-scavenging capacity

The extracted EPC and IPC samples were dissolved in water in a concentration gradient (20–150 mg(GAE)/l), and DPPH-methanol (0.4 mM) was prepared. Then each 2.4 ml of the extract solution was mixed with 0.8 ml of DPPH-methanol solution separately, followed by mixing each adequately, standing in the dark for 30 min and measuring the absorbance at 517 nm as A_x. A_0 was measured in a mixture of the same amount of methanol and DPPH. The absorbance of samples containing the same amount of methanol and the extract solution was recorded as A_{x0} [29]. This activity was given as DPPH scavenging rate and was calculated according to the following equation:

$$Scavenging\,rate(\%) = \frac{[A_0 - (A_X - A_{X0})]}{A_0} \times 100 \quad (1)$$

The concentration of phenolic compound extracts inhibiting 50% DPPH radicals was defined as the IC_{50}.

2.5. Identification of individual phenolic compounds

The compositions of EA-EPC, NB-EPC and IPC extracts were identified by HPLC–DAD–ESI–MS/MS (Agilent, Santa Clara, CA, USA) at 280, 320 nm and 360 nm. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 100% acetonitrile (solvent B). The non-linear gradient elution used was as follows: 95% A at 0 min, 90% A at 20 min, and 5% A at 40 min. Quantification was achieved corresponding external standards such as gallic acid, ferulic acid, naringin, EGCG, ECG [26].