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Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*

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Abstract

This research deals with the production of water-soluble polysaccharides from *Ganoderma applanatum* by comparing the productivity of endo- and exopolysaccharides under various cultural conditions. Both maltose and glucose proved to be the effective carbon sources for polysaccharide production. High yield of exopolysaccharide (EPS) required moderate temperature (25 °C), high carbon concentrations (60 g/l), and short culture period (8–12 days). In contrast, endopolysaccharide (PPS) production required lower culture temperature (10–15 °C), low level of C/N ratio and minimal 10 day culture period. *G. applanatum* accumulated more PPS in their bodies at lower temperature in death phase. Meanwhile more EPS were produced at moderate temperature and in the stationary growth phase. The molecular weight of EPS from *G. applanatum* was also influenced by cultivation time. The longer the mycelia were cultivated, the higher the molecular weight of EPS formed.

Keywords: *Ganoderma applanatum*; Submerged culture; Exopolysaccharide; Endopolysaccharide; Airlift bioreactor

1. Introduction

Many types of polysaccharides could be produced by submerged cultures of higher fungi including mushrooms. The polysaccharide have been studied and used for pharmaceutical purposes due to their diverse biological activities [1–3]. These include anti-tumor and immuno-modulating activities [4,5]. Their mechanism has also been elucidated [6,7]. Most of the polysaccharides mediating biological activities from mycelia were endopolysaccharides (PPS) [1,2] or exopolysaccharides (EPS) [8].

As the production of polysaccharide from mycelia is more efficient than that from fruit bodies, the influence of culture conditions from submerged cultures of mushroom has drown much attention [9–11]. Several investigators have pointed out that both culture medium and environmental conditions affect the production and the physico-chemical characteristics of EPS [10,12,13]. For example, the sugar composition of EPS depends on the medium sources [13], whereas its molecular weight varies with aeration conditions [12]. As the physico-chemical property of polysaccharide is influenced by culture conditions [14], it is very important to optimize culture conditions for the production of a specific type of polysaccharides from submerged culture of a mushroom species. Although much effort has been given to examine the effect of culture conditions on the production of polysaccharide, most reports [9–11] have focused on EPS but not on PPS. It is reasonable to expect that the productivity of the two polysaccharides might be linked to each of other since they utilize the same precursors [15]. Therefore, culture factors that satisfy the production of both the polysaccharides should be considered together.

Ganoderma applanatum is a basidomycete fungus belongs to the family of Polyporaceae. The fruit body has been used as traditional medicine for anti-cancer in China and reported to have diverse physiological activities including anti-tumor [16], anti-virus [17], and immuno-stimulation [18,19]. Moreover, liquid-cultured mycelium of *G. applanatum* has been reported to contain useful anti-tumor polysaccharide. According to Mizuno [20], examination by Sarcoma 180/mice, i.p. or p.o. method has revealed that polysaccharides from *G. applanatum* mycelium have remarkable anti-tumor activities. We also studied the levels of TNF-α, one of the immune cytokine, in the EPS from *G. applanatum* mycelium to examine the biological activity.

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However, despite their potential usefulness, *G. applanatum* mycelium has not been extensively studied for their productivity in culture. In the present study, we tested various culturing factors that might influence the production of PPS and EPS from the submerged culture of *G. applanatum*, determined optimal conditions for the PPS and EPS production, and finally characterize the polysaccharides.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The strain of *G. applanatum* KFRI 646 was obtained from the Korea Forest Research Institute. The stock culture was maintained on potato dextrose agar slant. The seed cultures were grown in 500 ml flasks containing 200 ml fungal growth medium (FGM) containing per liter 25 g glucose, 2 g yeast extract, 1 g glutamic acid, 0.5 mg biotin, 0.1 g thiamine, 2 g KH_2PO_4, 0.5 g $MgSO_4$, 5 ml 0.1 M-$FeCl_3$ and 5 ml 0.1 M-$MnSO_4$ at 25 °C on a shaking incubator at 100 rpm for 7 days. The cultures were then homogenized at 13,000 rpm for 8 s by homogenizer (Ingenieurbüro, X1030 D, German) and used as inoculum.

2.2. Culture conditions

Flask culture experiments were performed in 500 ml flasks containing 200 ml FGM by inoculating seed culture to 5% (v/v) of the total volume. The initial pH of the media was adjusted to 4.5 with HCl and KOH. Fermentations were carried out in the 3 l external-loop type airlift bioreactor [21] for 8–10 days. The 2–3 l volume fermentation was carried at temperatures 24 °C, with an aeration rate of 0.1 vvm. For fermentation FGM was used as basal medium with a change in carbohydrate concentration up to 40 g/l.

2.3. Estimation of mycelial growth and production of exo and endopolysaccharide

Samples collected from various treatments were centrifuged at 6000 rpm for 20 min to separate mycelia from culture solution. The mycelia were washed with distilled water, lyophilized and weighed for dry their weight. We modified the method of Kim et al. [2], for the production of exo and endopolysaccharide. To extract exopolysaccharide (EPS), cultured solution was mixed with four volumes of absolute ethanol, stirred vigorously, and then left overnight at 4 °C for precipitation. The precipitated EPS was centrifuged at 6000 rpm for 20 min, redissolved with distilled water and centrifuged again. The EPS was weighed after lyophilization of the supernatant. To extract endopolysaccharide (PPS), the dry mycelia were washed with 80%-ethanol, submerged in distilled water, autoclaved at 120 °C (1.5 atm) for 1 h and centrifuged. The filtrate was treated by the same procedures used for the extraction of EPS.

2.4. Purification of polysaccharide and determination of molecular weight

To further purify, the extracts of EPS and PPS were redissolved with distilled water, dialyzed at 4 °C for 3 days and lyophilized. The purified EPS and EPS were used to analysis carbohydrate and protein content and determine molecular weight. The molecular weights of the EPS and the PPS were estimated on the basis of the calibration curve obtained by HPLC (TSP, Ca, USA) with Shodex Sugar KB-805 column (0.8 cm × 30 cm, Showa Denko K.K., Tokyo, Japan) using distilled water as a mobile phase (column temperature 50 °C; flow rate 0.8 ml/min) [27]. The column was standardized with dextrans of diverse molecular mass. Detector used was RI (Waters 410, USA).

2.5. Analysis of carbohydrate and protein

Glucose contents in media were analyzed HPLC on a Prevail Carbohydrate ES column (250 mm × 4.6 mm, 5 μm; Alltech Associates Inc., USA), using isocratic elution with acetonitrile/H_2O (72/28, v/v) by ELSD detector (Alltech, USA). Total carbohydrate contents in polysaccharide were determined by phenol-sulfuric acid method [22] using glucose as a standard. Carbohydrate composition of the polysaccharide was analyzed by HPLC on a BP-100Pb^{++} Carbohydrate column (100 mm × 7.8 mm; Alltech Associates Inc., USA), by isocratic elution with H_2O using ELSD 2000 detector after acid hydrolysis with 72% (w/w) H_2SO_4 at 30 °C for 45 min, followed by 4% (w/w) H_2SO_4 for 1 h at 120 °C (1.5 atm) in an autoclave [23]. Total protein was determined by Bradford method with bovine albumin as the standard.

2.6. Measurement of TNF-α levels

Human peripheral blood mononuclear cells (PBMCs) were used to produce tumor necrosis factor (TNF-α) by treatment with EPS size. PBMCs were prepared by method of Jin et al. [35]. The cells was incubated with EPS at concentration of 100 μg/ml at 37 °C under an atmosphere containing 5% CO_2 for 24 h. Bacterial lipopolysaccharides (LPS; Sigma, St. Louis, MO) was used as a positive control at the concentration of 10 ng/ml. Levels of TNF-α (detection range: 15.6–1000 pg/ml) were measured using commercially available ELISA kits (Endogen, Woburn, MA) according to the manufacturer's instruction. The supernatants from the cell culture were tested for the cytokine contents.

3. Results and discussion

3.1. Influences of culture period on the production of mycelial biomass and polysaccharides

To determine optimal period for the production of PPS and EPS, *G. applanatum* was cultured in flasks for 4–24 days. The growth kinetic and time profile for production of PPS and EPS are shown in Fig. 1. *G. applanatum* reached stationary phase after 8 days in culture and death phase after 12 days in culture. Cell biomass also reached highest after 8 days in culture. Contents of PPS in the cell increased as culture period increased. The highest EPS production was obtained from 12-days old culture when culture entered their death phase. The pattern of EPS production was similar to that of the previous report of EPS production from bacteria. [15,24,25]. Whereas cell biomass reached highest at day 8, maxim production of PPS and EPS was at day 12. (The optimal period for the production of cell biomass, PPS or EPS from *G. applanatum* appears to be dependent on its growth kinetic). EPS and PPS were different as before or later death phase, respectively. Thus, production of EPS and PPS has different optima in culture periods.

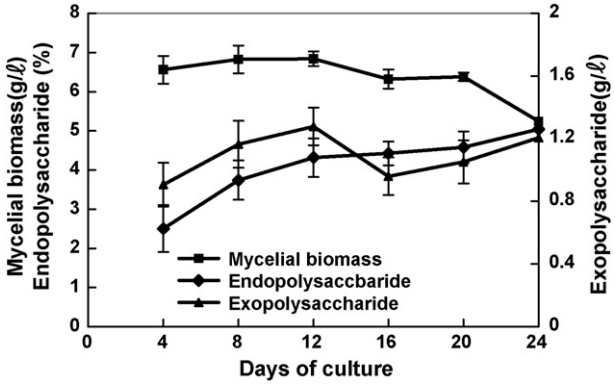

Fig. 1. The time-course change in mycelial biomass, endopolysaccharide and exopolysaccharide production from *Ganoderma applanatum* in shake flask cultures.