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Production and characterization of exopolysaccharides in mycelial culture of *Cordyceps sinensis* fungus Cs-HK1 with different carbon sources☆

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ABSTRACT

The effects of different carbon sources (sugars) on the production and molecular properties of exopolysaccharides (EPS) were evaluated in the mycelial liquid culture of a medicinal fungus *Cordyceps sinensis* Cs-HK1. Galactose or mannose was used (at 5 g·L^{-1}) as a secondary carbon source with glucose (35 g·L^{-1}) at the mass ratio of 1:7. Mannose was consumed notably since the first day of culture, but galactose was not even after glucose was exhausted. The volumetric yield of EPS in culture was increased slightly with the addition of galactose and decreased with mannose. The monosaccharide composition of EPS was also different, *e.g.*, on day 8, the glucose contents of EPS were 76% with the addition of mannose, 59% with galactose, compared with 62% with glucose only. The molecular weight distribution of EPS was also affected by the secondary carbon source, being generally lower compared with that with glucose only. The results suggested that the addition of galactose improved the total yield of EPS in culture while mannose can improve the yield of glucan constituent of EPS.

1. Introduction

Edible fungi or mushrooms are nutritious and healthy foods, and some also have medicinal properties and are classified as medicinal fungi. Polysaccharides (PS) represent a major class of bioactive molecules from edible and medicinal fungi which have notable antitumor, immunomodulatory and other medicinal properties [1,2]. *Cordyceps* (*Ophiocordyceps*) *sinensis*, generally called the Chinese caterpillar fungus, is a precious medicinal fungus in Chinese herbal medicine with a wide range of health benefits and bioactivities [3,4]. Since natural *C. sinensis* in the form of a caterpillar-fruiting body complex is very rare in nature and difficult to cultivate artificially, mycelial fermentation has become the main source of *C. sinensis* fungal materials. Cs-HK1 is a fungus isolated from the fruiting body of a natural *C. sinensis* and has been identified as a *Tolypocladium* fungus and a relative of *C. sinensis* [5]. The mycelial culture of Cs-HK1 has been established and optimized for mycelial growth with a liquid medium containing glucose as the major carbon source and a few other components [5]. In addition to mycelial biomass, a significant amount of exopolysaccharides (EPS) has been produced by the Cs-HK1 mycelial culture in the liquid medium. Microbial EPS produced by liquid fermentation have found wide industrial applications especially in the food and biomedical fields such as xanthan and gellan as food additives, scleroglucan as laxative tablet coating, and dextran as plasma expanders [6]. More recently there has been increasing interest in the pharmaceutical applications of microbial EPS [7].

The crude EPS isolated from Cs-HK1 mycelial culture medium by ethanol precipitation was composed of polysaccharides and polysaccharide–protein complexes in a wide molecular weight range. Some of the completely and partially purified EPS fractions have shown antioxidant and immunomodulatory activities [8–10]. The main components of EPS of Cs-HK1 are (1 → 3)-β-D-glucan [11] and galactomannan–protein [10] while the yield of EPS was about 3.2 g·L^{-1}. However, (1 → 3)-β-D-glucan in EPS of Cs-HK1 was hardly dissolved in water and even after being dissolved in the water, the solution viscosity was very high and difficult to handle [11]. The galactomannan–protein complex fractionated from EPS of Cs-HK1 showed stronger antioxidant activity with high water solubility, though its yield in the culture was much lower than (1 → 3)-β-D-glucan. Carbon source is one of the most important nutrients, and glucose is a common and favorable carbon source for biomass growth and EPS production in most microbial fermentations [12,13]. The addition of other monosaccharide sugars may be utilized by the fungal cells and converted to uridine-diphosphate (UDP)-monosaccharides for EPS synthesis [14]. Galactose and mannose may be transferred to UDP-galactose and

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UDP-mannose during the fermentation process and further synthesize EPS. In this study, galactose and mannose were each added as a secondary carbon source for production of EPS in the Cs-HK1 mycelial culture.

2. Experimental

2.1. Fungal species and mycelial culture conditions

Cs-HK1 was previously isolated from the fruiting body of a natural *C. sinensis* and its stock culture was maintained on solid potato–dextrose–agar medium at 4 °C [5]. Cs-HK1 mycelial culture was routinely maintained in a liquid medium consisting of 40 $g \cdot L^{-1}$ glucose, 10 $g \cdot L^{-1}$ yeast extract, 5 $g \cdot L^{-1}$ peptone, 1 $g \cdot L^{-1}$ KH_2PO_4 and 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$ in shake-flasks at 150 rpm and 20 °C. In the experiments on the effects of a secondary monosaccharide carbon source, 5 $g \cdot L^{-1}$ of galactose or mannose was added to the liquid medium containing 35 $g \cdot L^{-1}$ of glucose. To ensure the yield of EPS, sufficient glucose was added together with galactose or mannose at a mass ratio of 7:1. The Cs-HK1 mycelial liquid fermentation was carried out in 250 ml Erlenmeyer flasks each containing 50 ml of the liquid medium for an overall period of 8 days. On each day of the culture period, three flasks were taken out from the shaking incubator for measurement of biomass, EPS and carbohydrate concentrations. All experiments were performed in triplicate and the results were represented by mean ± SD (standard deviation).

2.2. Determination of biomass and EPS in fermentation liquid

The fermentation liquid in the culture flasks was centrifuged at 10000 $r \cdot min^{-1}$ (~14980 *g*) for 20 min to separate the biomass from the liquid medium. The precipitated biomass was washed twice with distilled water and freeze-dried to give the biomass dry mass. For isolation of crude EPS, the supernatant liquid collected from the centrifuge was subject to ethanol precipitation by adding three volumes of 96% (v/v) ethanol to each volume of the liquid medium. The precipitated EPS was collected by centrifugation at 10000 $r \cdot min^{-1}$ (~14980 *g*) for 20 min, and then freeze-dried on an ALPHA 1–4 LD2 freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) at condenser −60 °C and 1.2×10^4 MPa for 48 h. The moisture content of EPS samples after the freeze drying was negligible and applied for further analysis.

2.3. Determination of sugar consumption in fermentation medium

Concentration of glucose in the liquid medium was determined with a Biochemistry Analyzer (YSI Inc., Yellow Springs, OH, USA). Concentrations of galactose and mannose were determined by the 1-phenyl-3-methyl-5-pyrazolone-high performance liquid chromatography (PMP-HPLC) method as reported by Honda and co-workers [15]. In brief, 450 μl of fermentation broth was derivatized with 450 μl PMP solution (0.5 $mol \cdot L^{-1}$ in methanol) and 450 μl of 0.3 $mol \cdot L^{-1}$ NaOH at 70 °C for 30 min. The reaction was stopped by neutralization with 450 μl of 0.3 $mol \cdot L^{-1}$ HCl, followed by extraction with chloroform (1 ml, 3 times). The extract solution was then applied to HPLC analysis. HPLC analysis was performed on an Agilent 1100 instrument consisting of a G1312A Binpump and a UV detector with a ZORBAX Eclipse XDB-C18 column (5 μm, 4.6 mm × 150 mm) at 25 °C.

2.4. Analysis of EPS composition and MW distribution

The total carbohydrate content of the crude EPS was determined by the anthrone–sulfuric acid assay using glucose as a standard and total protein content determined by the Lowry method using bovine serum albumin (BSA) as a standard [11]. The monosaccharide constituents of EPS were analyzed by the PMP-HPLC method after complete hydrolysis with 2 $mol \cdot L^{-1}$ trifluoroacetic acid (TFA) (110 °C for 4 h) as described in detail previously [8]. NMR spectroscopy was performed on a Bruker AV400 instrument. For the NMR analysis, the EPS sample (30 mg) was co-evaporated with D_2O (Sigma-Aldrich, USA) twice by lyophilization before final dissolution in a mixed solvent (Me_2SO-*d*6/D_2O in the ratio of 6:1). The MW distribution of EPS was analyzed by high performance gel permeation chromatography (HPGPC) with the same instruments (a Waters 1515 isocratic pump and a Waters 2414 refractive index detector) and conditions as in a previous study [10].

3. Results and Discussion

3.1. Consumption of carbon sources during mycelial fermentation

The consumption of the monosaccharide nutrients is shown in Fig. 1. Glucose was consumed daily with about 32 $g \cdot L^{-1}$ glucose utilized after 8 days of culture (Fig. 1A). The absorption of mannose and galactose was much different as mannose was taken since the first day while galactose was not consumed even after 8 days (Fig. 1B). Carbon source is a major limiting nutrient factor for microbial growth. Although glucose is the common carbon source to achieve high production of biomass and EPS in mycelium fermentation [16,17], other monosaccharides, such as fructose, galactose and xylose, have also been used in the mycelial fermentation [18–21]. Glucose can be directly transferred to UDP-glucose and further to synthesize EPS in mycelial fermentation, while mannose or galactose may be converted to UDP-mannose or UDP-galactose directly or transferred to UDP-glucose firstly by enzyme before the uptake [14]. However, galactose was not utilized due probably to the lack of the related enzymes in the fungus.

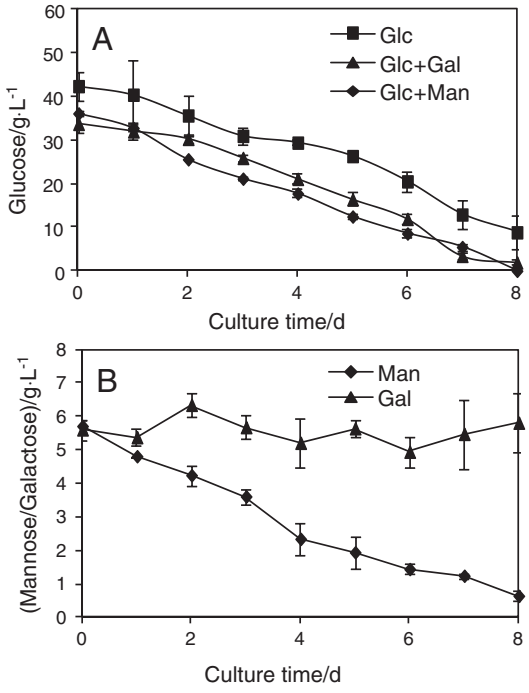

Fig. 1. Time courses of residual carbon sources in Cs-HK1 mycelial cultures: (A) glucose; (B) galactose and mannose. Error bars represent standard deviation (SD) of triplicate flasks.

3.2. Effect of carbon sources on biomass and EPS production

Fig. 2 shows the biomass growth and EPS production by Cs-HK1 fungus on different carbon sources in liquid fermentation. Galactose could not be utilized in Cs-HK1 mycelial fermentation as discussed