Revised Ms. 28-JYWu

Submitted to CHINESE JOURNAL OF CHEMICAL ENGINEERING

Special Issue: The 6th Global Chinese Symposium of Chemical Engineering, 2014 Hong Kong UST

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

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Supported by The Hong Kong Polytechnic University internal grants.

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 $\cdot L^{-1}$) as a secondary carbon source with glucose (35 g $\cdot L^{-1}$) 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, be 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.

Keywords: *Cordyceps sinensis*; fermentation; carbon source; hydrocarbons; bioprocess; monosaccharide composition

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, 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 as 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 $g \cdot L^{-1}$. 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 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 *Cordyceps sinensis* and its stock culture was maintained on solid potato–dextrose–agar medium at 4 ^{o}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

82 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$ in shake-flasks at 150 rpm and 20 °C. In the experiments on the
83 effects of a secondary monosaccharide carbon source, 5 $g \cdot L^{-1}$ of galactose or mannose
84 was added to the liquid medium containing 35 $g \cdot L^{-1}$ of glucose. To ensure the yield of
85 EPS, sufficient glucose was added together with galactose or mannose at a mass ratio of
86 7:1. The Cs-HK1 mycelial liquid fermentation was carried out in 250 mL Erlenmeyer
87 flasks each containing 50 mL of the liquid medium for an overall period of 8 days. On
88 each day of the culture period, three flasks were taken out from the shaking incubator
89 for measurement of biomass, EPS and carbohydrate concentrations. All experiments
90 were performed in triplicate and the results were represented by mean ± SD (standard
91 deviation).
92
93 *2.2 Determination of biomass and EPS in fermentation liquid*
94 The fermentation liquid in the culture flasks was centrifuged at 10,000 rpm (~14,980
95 g) for 20 min to separate the biomass from the liquid medium. The precipitated biomass
96 was washed twice with distilled water and freeze-dried to give the biomass dry weight.
97 For isolation of crude EPS, the supernatant liquid collected from the centrifuge was
98 subject to ethanol precipitation by adding three volumes of 96% (v/v) ethanol to each
99 volume of the liquid medium. The precipitated EPS was collected by centrifugation at
100 10,000 rpm (~14,980 g) for 20 min, and then freeze-dried on an ALPHA 1–4 LD2 freeze
101 dryer (Martin Christ Gefriertrocknungsanlagen GMB, Germany) at condenser −60 °C
102 and 0.12 Mbar for 48 h. The moisture content of EPS samples after the freeze drying
103 was negligible and applied for further analysis.
104

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 M in methanol) and 450 μL of 0.3 M NaOH at 70 °C for 30 min. The reaction was stopped by neutralization with 450 μL of 0.3 M 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 × 150 mm) at 25 °C.

2.4 Analysis of EPS composition and MW distribution

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 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 M 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 mycelia 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 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 monosaccharaides, 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 mycelia 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.

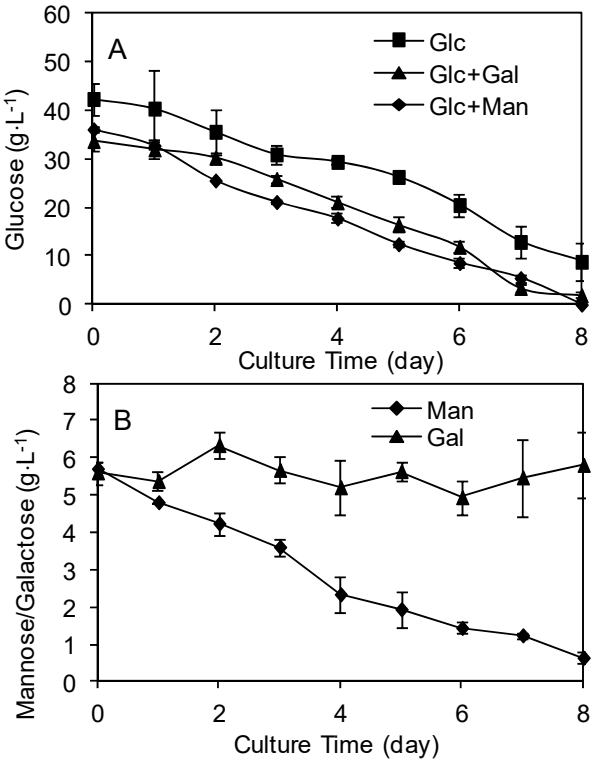


Figure 1 Time courses of residual carbon sources in Cs-HK1 mycelial cultures: (A) Glucose; (B) Galactose and mannose. Error bars represent standard deviation (SD) of triplicate flasks.

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 above. However, the production of mycelium and EPS in the presence of galactose was almost equal to the other two and the yield of EPS was also slightly higher than the other two from day 5 to day 7. A previous study has shown that galactose was consumed to promote the growth of mycelia during fermentation process [22]. In this study, galactose also affected the biosynthesis of EPS although it was not seen utilized. At day 8, the EPS production from the three types

(Glc+Gal, Glc, Glc+Man) were 2.9 $g \cdot L^{-1}$, 2.8 $g \cdot L^{-1}$ and 2.4 $g \cdot L^{-1}$, respectively. As observed in our preliminary experiments (data not shown), galactose or mannose supplied as the sole carbon source in the Cs-HK1 mycelial culture could not support the biomass growth and EPS production.

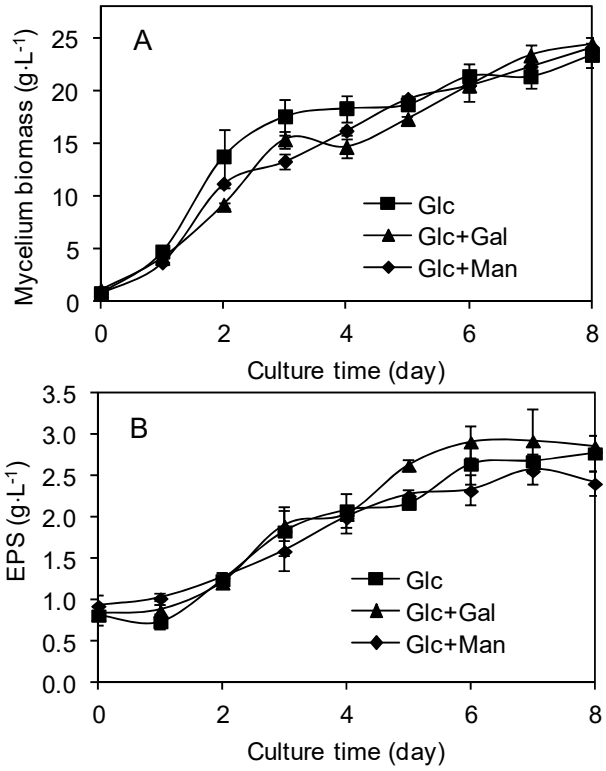


Figure 2 Time courses of biomass growth (A) and EPS production (B) in Cs-HK1 mycelial culture with Glc (40 $g \cdot L^{-1}$), Glc (35 $g \cdot L^{-1}$)+Gal (5 $g \cdot L^{-1}$) and Glc (35 $g \cdot L^{-1}$)+Man (5 $g \cdot L^{-1}$) as the carbon sources. Error bars represent standard deviation (SD) of triplicate flasks.

The Cs-HK1 mycelual culture parameters of biomass growth and EPS production derived from the above time courses (Figures 1 & 2) such as the biomass growth rate, maximum concentrations or yields of biomass and EPS were similar to those reported previously [23].

Table 1 shows the total carbohydrate and protein contents of EPS from Cs-HK1

mycelial culture with three different carbon sources on various days from day 3 to 8. Overall the total carbohydrate contents varied in the range of 14-36% and the protein contents from 15-31%, which are higher in the later days with all carbon sources except for occasional fluctuations.

Table 1 Total carbohydrate and protein contents of EPS from Cs-HK1 mycelial culture with different carbon sources (weight % mean ± SD, n = 3).

Culture day	3	4	5	6	7	8
Carbohydrate content:						
Glc	18.6±3.2	23.5±4.3	26.6±2.5	31.8±0.9	32.5±2.1	36.5±1.9
Glc+Gal	16.9±2.5	27.5±1.5	25.5±3.4	23.9±2.0	29.0±2.0	36.2±1.6
Glc+Man	14.0±1.1	23.0±3.4	21.1±2.1	26.2±1.2	19.2±2.0	27.6±2.3
Protein content:						
Glc	15.3±3.0	24.1±2.4	22.9±3.4	29.0±2.4	30.6±2.5	31.1±3.2
Glc+Gal	22.9±1.0	28.4±2.0	30.7±2.2	27.9±1.4	27.7±2.6	27.8±1.7
Glc+Man	21.7±0.1	27.4±1.5	26.0±1.4	30.1±1.0	32.2±2.5	28.5±2.1

As shown in Table 2, the monosaccharide composition of EPS isolated from the Cs-HK mycelia cultures on different days varied with the carbon sources. Three monosaccharides, Glc, Gal and Man, were found in all EPS samples and Glc represents the major and most abundant monosaccharide constituent accounting for 50-75% molar. The addition of galactose and mannose to the culture did not lead to a notable increase in their content in the EPS or even to a lower content.

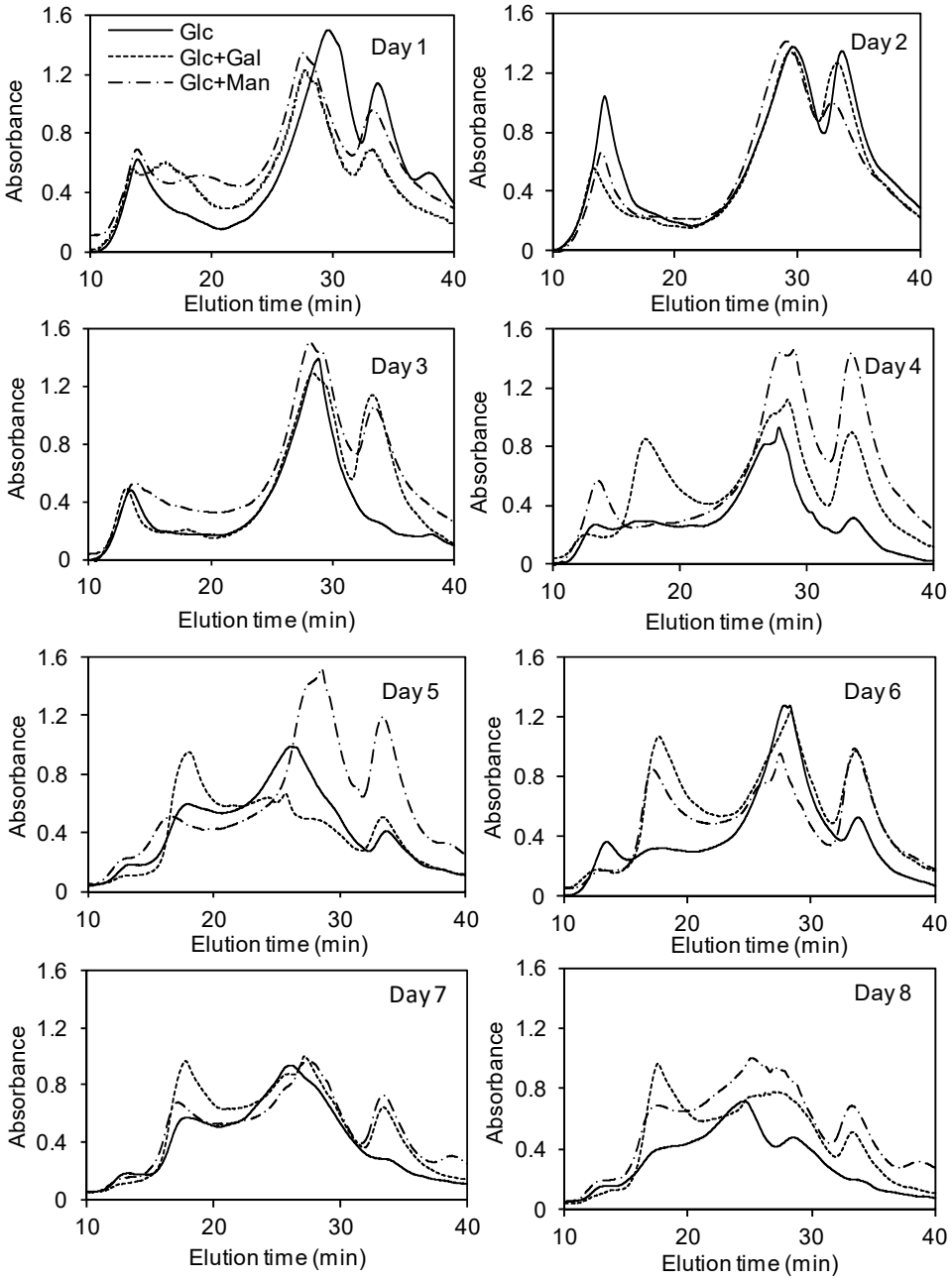
Table 2 Monosaccharide composition of EPS from Cs-HK1 mycelial culture with different carbon sources.

Culture	Carbon source	Content (mole%)			Mol ratio
		Glc	Gal	Man	Glc:Gal:Man
day 3	Glc	71.3	8.2	19.2	8.7:1.0:2.3
	Glc+Gal	68.4	10.2	17.4	6.7:1.0:3.9
	Glc+Man	70.2	9.8	16.2	7.2:1.0:1.7
day 4	Glc	63.7	10.6	23.7	6.0:1.0:2.2
	Glc+Gal	68.0	10.0	17.0	6.8:1.0:1.7
	Glc+Man	67.3	12.7	15.6	5.3:1.0:1.2
day 5	Glc	67.8	8.6	18.7	7.9:1.0:2.2
	Glc+Gal	62.8	12.1	18.1	5.2:1.0:1.5
	Glc+Man	59.4	13.8	19.8	4.3:1.0:1.4
day 6	Glc	59.7	17.7	20.6	3.4:1.0:1.2
	Glc+Gal	66.2	12.8	19.1	5.2:1.0:1.5
	Glc+Man	72.3	8.2	11.3	8.8:1.0:1.4
day 7	Glc	65.0	10.3	19.1	6.3:1.0:1.9
	Glc+Gal	54.0	20.0	20.0	2.7:1.0:1.0
	Glc+Man	71.7	12.6	9.8	5.7:1.0:0.8
day 8	Glc	62.1	11.6	21.9	5.4:1.0:1.9
	Glc+Gal	59.1	15.2	22.7	3.9:1.0:1.5
	Glc+Man	75.5	11.3	10.7	6.7:1.0:0.9

3.3 Effect of carbon sources on EPS molecular properties

Fig. 3 shows the HPGPC MW profiles of EPS collected on all 8 days of culture. In first two days, the production of EPS was slow with three carbon sources and no significant differences were observed among the three EPS. After day 3, the MW profiles of EPS produced started to show notable differences. In comparison of the peak

199 area ratios (Table 3) which represent the relative amounts of different MW fractions of
200 EPS, the amounts of the highest MW (with the shortest elution time of 10-15 min) and
201 the lowest MW EPS (eluted out at 22-32 min) were decreased, and the amount of middle
202 MW EPS (eluted out at 12-22 min) increased with the culture period. Different
203 monosaccharides as carbon source have different pathway in microbial fermentation
204 process [14]. The variation of EPS structural composition could be examined by NMR
205 analysis. The ^{1}H-NMR of EPS from the Cs-HK1 mycelial culture shows different
206 proportions of β-glucan in the EPS produced with three different carbon sources (Fig. 4).
207 The proportion of β-glucan formed with glactose and glucose as the carbon source is
208 higher (the shift around 4.8 ppm & 4.2 ppm) than that in the EPS produced with glucose
209 as the carbon source. Overall the results indicate that the EPS composition and MW
210 distribution are affected significantly by the carbon sources and also vary with the
211 culture period.
212



213

214 Figure 3 HPGGPC MW distributions of EPS from Cs-HK mycelial culture on day 1-8

215 with different carbon sources. The peak area ratios are summarized in Table 3.

216

217 Table 3 The MW distributions of EPS from Cs-HK1 mycelial culture with three carbon

218 sources over 8 days of culture.

Culture	Carbon source	% of total peak area		
		10-15 min	15-22 min	22-32 min

day 1	Glc	34.5	-	65.5
	Glc+Gal	26.8	-	73.2
	Glc+Man	25.8	-	74.2
day 2	Glc	20.0	-	80.0
	Glc+Gal	12.1	4.3	83.6
	Glc+Man	26.4	-	73.6
day 3	Glc	10.2	30.8	59.0
	Glc+Gal	3.9	37.8	58.3
	Glc+Man	12.3	18.9	68.8
day 4	Glc	10.4	16.0	73.6
	Glc+Gal	2.8	47.4	49.8
	Glc+Man	3.0	44.1	53.9
day 5	Glc	2.8	20.6	76.6
	Glc+Gal	1.0	79.2	19.8
	Glc+Man	1.8	13.7	84.5
day 6	Glc	3.9	25.3	70.8
	Glc+Gal	1.1	56.9	42.0
	Glc+Man	1.9	29.7	68.4
day 7	Glc	3.9	18.0	78.1
	Glc+Gal	1.0	48.5	50.5
	Glc+Man	2.5	24.1	73.4
day 8	Glc	5.7	73.9	20.4
	Glc+Gal	0.7	50.6	48.7
	Glc+Man	1.2	36.7	62.1

Glucose and sucrose have been used as the favorable carbon sources for most of microbial fermentations [21,22-24-26]. Although previous studies have also evaluated the use of galactose, mannose and some other mono- and oligo-saccharides for the production of biomass and EPS [27, 28], few have assessed the composition and

structure changes of EPS with these alternative carbon sources. In this study, the HPGPC and NMR analysis of EPS produced with alternative carbon sources show the changes in the molecular composition of EPS affected by galactose and mannose. When galactose and mannose were added as a secondary carbon source, mannose was consumed but galactose was not during the culture process. Galactose has been reported previously with low utilization in the fermentation of *Listeria monocytogenes* [29]. Although galactose was not used by the mycelial culture, it affected the yield and composition of EPS. The mannose consumed did not increase the EPS yield and may be involved in the production of pyruvate [30].

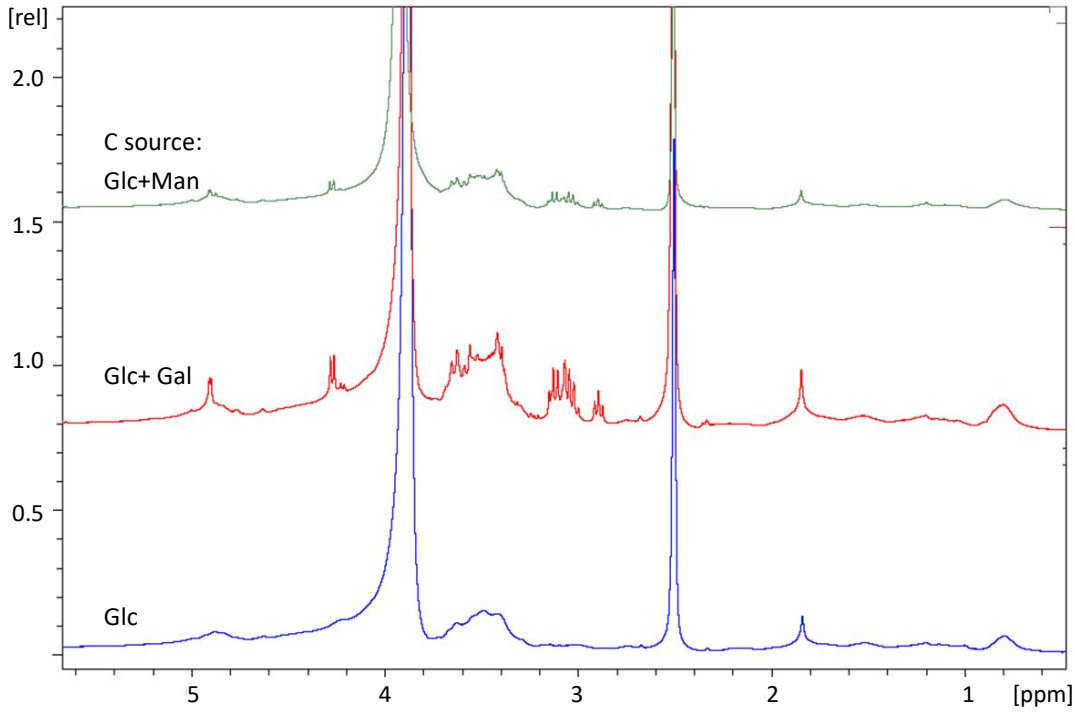


Figure 4 ^{1}H-NMR of EPS collected on the day 3 from the Cs-HK1 fermentation broth on three different carbon sources.

4. Conclusions

The application of galactose or mannose as a secondary carbon source may influence the yield and composition of EPS in fungal mycelia culture. However, the influence appears to be irrelevant to their utilization or consumption during the mycelial fermentation process. The results suggest that galactose and mannose can be applied to vary the molecular properties of EPS composed of glucose, galactose and mannose. Variation of the carbon sources may be effective in the generation of new polysaccharide structures. Further studies should be conducted to investigate the physiological mechanisms such as the metabolism and biosynthetic pathways for the EPS with various carbon sources.

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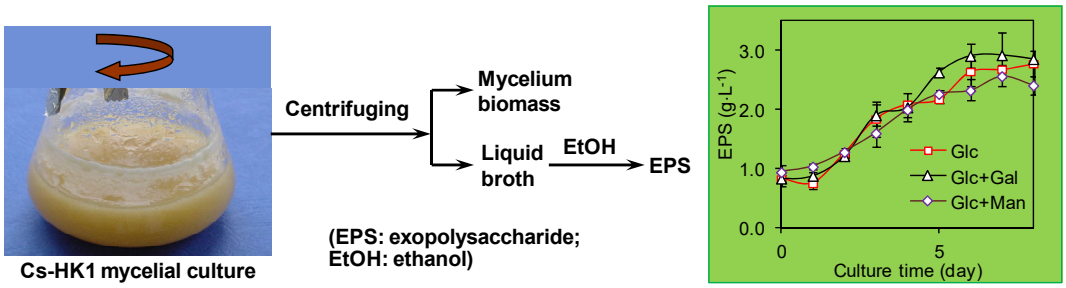
330 Graphic abstract



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