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Effect of carbon source on production, characterization and bioactivity of exopolysaccharide produced by *Phellinus vaninii* Ljup

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ABSTRACT

The effect on different three carbon source (i.e. glucose, fructose and sucrose) on production, chemical characterization and antioxidant activity of exopolysaccharide (EPS) produced by *Phellinus vaninii* Ljup was investigated in this study. Amongst carbon sources examined, glucose and sucrose were favorable for the mycelia growth, while the maximum EPS yield was achieved when sucrose was employed. The predominant carbohydrate compositions in EPSs identified were gluconic acid, glucose, mannose and galactose acid. Then, FT-IR spectral analysis revealed prominent characteristic groups in EPSs. EPSs molecule exist as nearly globular shape form in aqueous solution. The variation also affects antioxidant activities by investigated by using hydroxyl and DPPH radical scavenging assay. Sucrose was best carbon source from the viewpoint of antioxidant activity due to the relatively high contents of galactose in the EPS with moderate molecular weight and polydispersity.

Key words: *Phellinus vaninii* Ljup, exopolysaccharide, carbon source, antioxidant activity, composition.

INTRODUCTION

Phellinus vaninii Ljup (also called Yanghuang in Chinese) is a famous Chinese medicinal fungus belonging to Basidiomycetes, Aphyllophorales, Hymenochaetacae, Phellinus (Dai 2010). Bioactive metabolites from *P. vaninii* Ljup can be isolated from fruiting bodies and cultured mycelia, including phenolic compounds, flavonoids, polysaccharides, triterpenoids, terpenes, alkaloid, etc (Cheng et al. 2011, Hu et al. 2014). The ethyl acetate extract of mycelial fermentation by *P. vaninii* Ljup showed strong antiproliferative activity *in vitro* (Hu et al. 2014).

The submerged culture for exopolysaccharide (EPS) production by medicinal fungi are proved to have many properties, such as immunomodulation, anticancer, antioxidant, hypolipidemic and hyperglycemic activities (Mahapatra and Banerjee 2013, Wasser 2002). Many investigators have attempted to obtain the optimal submerged culture for EPS from several fungi (Tang et al. 2008, Xiong et al. 2012). It is demonstrated that the yields and compositions of the EPS were affected by culturing conditions, especially when using different carbon sources which might also affect

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the antioxidant property (Fan et al. 2009, Xiong et al. 2012). However, no data are currently available concerning the effect of carbon sources on the change in chemical characterization and bioactivity of exopolysaccharide of fungal polysaccharides. Therefore, the aim of the study was evaluation of effect of three carbon sources (glucose, fructose and sucrose) on features and antioxidant potential of *P. vaninii* Ljup EPS produced during submerged cultivation.

MATERIALS AND METHODS

CHEMICALS

The carbon sources (glucose, fructose and sucrose) and chemicals for medium culture were of reagent grade, while other chemicals were of analytical grade.

FUNGI AND MEDIUM CULTURE

Phellinus vaninii Ljup was isolated from Wanda mountains of Heilongjiang province and kept in the Forest Microbiological Research Center of Northeast Forestry University (deposit no. 199537). Stock cultures were maintained on potato dextrose agar (PDA) slants. Before the seed culture, *P. vaninii* Ljup was transferred onto another petri dish with the media of 40 g/L glucose, 4 g/L malt extract and 20 g/L agar by punching out 5 mm of the agar plate culture with a cutter and cultivated for 8 d at 26 °C. Seed cultures on the flask were obtained 250 ml Erlenmeyer flasks filled with 50 mL of media (40 g/L glucose, 4 g/L malt extract and 4 mmol/L KH_2PO_4, pH 7). The fermentation media were inoculated with 4% (v/v) of liquid four-day old seed culture, and incubated at 28 °C in a 5-L stirred-tank (Infors, Switzerland). Fermentations were conducted in the same medium as seed culture under the conditions of temperature 28 °C, aeration rate 2 vvm, agitation speed 160 rpm, pH 7, working volume 3 L and cultivation time 8 d. To study the effect of different carbon sources on EPS production, the glucose in the agar plate and submerged culture medium was replaced by fructose or sucrose. All experiments were performed in triplicate to ensure the trends observed were reproducible.

EPS QUANTIFICATION AND MYCELIAL DRY WEIGHT

The culture broth was centrifuged at 9000 rpm for 15 min, and the resulting supernatant was mixed with four vols. of pure ethanol. The precipitated EPS was collected by centrifugation at 9000 rpm for 15 min and then deproteinized using Sevag reagent (1:4 n-butanol/chloroform, v/v). After removing the proteins and Sevag reagent by centrifugation, the aqueous phase was dialyzed against deionized water and lyophilized to yield the crude polysaccharide. The total EPS in the culture medium was determined by the phenol–sulphuric acid method, using glucose as the standard (Dubois et al. 1956). The dry weight of mycelium was measured after washing the mycelial pellet with distilled water three times and drying at 70 °C to a constant weight (Hwang et al. 2003).

PURIFICATION OF EPS

The polysaccharide was re-dissolved in 0.2 M NaCl solution, and applied to a Sepharose CL-6B column (2.4 cm×100 cm, Sigma Chemical Co., St Louis, MO) and eluted with the same solution at a flow rate of 0.6 ml/min. Fractions (5.0 ml/ tube) were collected by a fraction collector (Hwang et al. 2003). The total carbohydrate content in the EPS was also determined by the phenol-sulfuric acid method (Hu et al. 2014).

SEC/MALLS ANALYSIS

The molecular weights of the EPS were estimated by SEC coupled with multi-angle static laser light scattering detection (MALLS; DWAN EOS equipped with a GaAs laser at 690 nm (λ), Wyatt Technology, Santa Babara, CA) and a

Refractive Index (RI) detector (Optilab rEX, Wyatt Technology, Santa Barbara, CA). The EPS samples were dissolved in a 0.1M PBS buffer (pH 6.8) containing 0.04% diaminotetraacetic acid–disodium salt (Na_2EDTA) and 0.01% sodium azide and filtered through 0.025 μm filter membranes (Millex HV type, Millipore Co., Bedford, MA) prior to injection into the SEC/MALLS system. The chromatographic system consisted of a degasser (Degasys, DG-1200, uniflow, HPLC Technology, Macclesfield, UK), a SSI 222D pump (Scientific Systems, State College, PA, USA) single-piston isocratic, pulse-dampened HPLC pump (Model 590 Programmable Solvent Delivery Module, Waters Co., Milford, MA), an injection valve (Rheodyne, Inc., Cotati, CA) fitted with a 500 μl loop, and two SEC columns (Shodex OH Pack SB-803 and 805 HQ, JM Science Inc., Buffalo, NY) connected in series. The flow rate was 0.75 mL/min and the injection volume and concentration was 100 mL and 2 mg/mL, respectively. During the calculation of molecular weights of each EPS, the value of dn/dc (specific refractive index increment) was used from the data in literature (Jumel et al. 1996), in which the estimated dn/dc was 0.14 mL/g. Calculations of molecular weight and root mean square (RMS) radius of gyration for each EPS were performed by the Astra 4.72 software (Wyatt Technology). The RMS radii of each polysaccharide were determined from the slope by extrapolation of the first-order Debye plot (Astafieva et al. 1996). The gross conformation of EPS in aqueous solution could be identified from the double logarithmic plot of RMS radius vs. molecular mass of EPS according to the following equations:

Spheres: $r_i^3 \propto M_i \rightarrow \log r_i = k + 1/3 \log M_i$

Random coils: $r_i^3 \propto M_i \rightarrow \log r_i = k + 1/2 \log M_i$

Rigid rods: $r_i^3 \propto M_i \rightarrow \log r_i = k + \log M_i$

where r_i is the RMS radius of an EPS molecule, M_i is the molar mass of EPS, k is the intercept at the Y-axis (RMS radius), and 1/3, 1/2, and 1 are the critical slope values for determining the molecular conformation of each EPS (Hwang et al. 2003, Lim et al. 2005).

MONOSACCHARIDE COMPOSITION ANALYSIS

For the identification and quantification of monosaccharide, EPS fraction (5 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol, reduced with $NaBH_4$ for 30 min at 20 °C and acetylated with acetic anhydride and pyridine at 100 °C for 20 min. The internal standard sugars were prepared and subjected to GC-MS analysis separately in the same way. The alditol acetates of EPS fraction were analyzed by GC-MS (Varian Co., Model: Star 3600 CX, Lexington, MA, USA) fitted with a fused silica capillary column (Na form, 300mm × 0.25 mm, Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector.

FT-IR SPECTROSCOPY

FT-IR Spectroscopy (Bruker Tensor 27) was analyzed using the KBr disc for detecting functional groups. The purified EPS fractions (1 mg) were ground with 300 mg KBr powder and then pressed into pellets for transform IR spectral measurement on a Mattson Instrument from 550 to 4,000 cm^{-1}. Spectra were corrected for wave number dependent signal-detection efficiency of the setup using the white light spectrum of a temperature-calibrated tungsten band lamp.

ANTIOXIDANT ACTIVITY ASSAYS

For the evaluation of antioxidant activity of EPS produced by *P. vaninii* Ljup for reductive ability, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity and OH (hydroxyl) radical

scavenging activity were determined according to the methods of Wang et al. (2010) and Eloff et al. (2008), respectively.

STATISTICAL ANALYSIS

Data were expressed as mean ± S.D. (n = 3). The statistical significance was determined by Student's t-test. Experimental data were statistically subjected to analysis of variance (ANOVA) using SPSS (version 11.0, Chicago, IL) to evaluate significant differences at probability level of $p < 0.05$.

RESULTS AND DISCUSSION

EFFECTS OF CARBON SOURCES IN MEDIUM ON BIOMASS AND EPS PRODUCTION

The results of the biomass and EPS of *P. vaninii* Ljup from the submerged culture in a 5-L stirred-tank with various carbon sources are shown in Figure 1. After a fermentation period of 8 days, glucose and sucrose were the most suitable carbon source for biomass production. However, the highest EPS yield of 1.96 g/L achieved when sucrose was employed. The result indicates that *P. vaninii* Ljup has the ability to use different carbon sources, all of which have varied degree of stimulatory effect on biomass and EPS production. Both glucose and fructose are hexoses, which can participate in glycolytic and pentose phosphate pathways. According to biomass and EPS yield in Figure 1, sucrose was shown to sustain high growth rates in cell cultures and carbon conversion efficiency is also high. It has been reported that different carbon sources had different influences of catabolic repression on the secondary metabolism (Tang et al. 2008, Khondkar et al. 2002). Though it is well known that sucrose is a disaccharide combination of the monosaccharides glucose and fructose, the information concerning the metabolic utilisation of sucrose for EPS production is extremely limited.

PURIFICATION AND ANALYSIS OF CARBOHYDRATES

The EPS was obtained from the fermentation broth from submerged culture of *P. vaninii* Ljup by the method of ethanol precipitation. In gel filtration chromatography of the culture filtrate on Sepharose CL-6B, the main fraction of each EPS produced from three different carbon sources was coeluted. The detailed monosaccharide compositions from the carbohydrate composition in the EPS fraction could be worked out from trifluoroacetic acid hydrolysis and GC-MS analysis as illustrated in Table I. As expected, the carbohydrates composition within the EPS varies with the different carbon sources. Nevertheless, the predominant sugars in EPS, identified in this study, were gluconic acid, glucose, mannose and galactose acid. Negligible amounts of rhamnose, ribose and xylose were also detected. This indicates that interconversion among glucose, fructose and sucrose in the microorganism give polysaccharides with the similar composition. It is very interesting to note that the highest content of galactose unit was observed in carbohydrate composition when the sucrose was employed as carbon source (Table I). Kai et al. (2003) found that different carbon sources in the media can influence the hetero-polysaccharides synthesis by *Pestalotiopsis microspora*. When glucose was used as the carbon source, a considerable amount of mannose units were formed. While with xylose as the carbon source, polysaccharides containing large amounts of mannose and galactose units were produced. Smiderle et al. (2012) also reported the similar results when glucose, galactose, xylose or arabinose was used as carbon source to produce polysaccharide by *Pleurotus pulmonarius*. Analyzing EPS monosaccharide composition, it was only observed similarities for the culture medium using mannose and arabinose, having mannose as the major component in both EPS samples. However, limited information is available

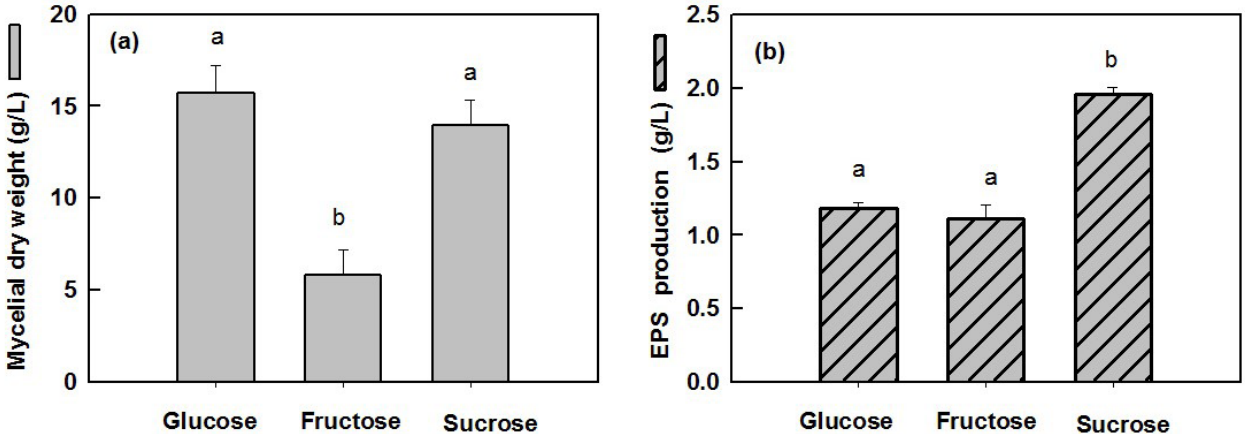

Figure 1 - Effect of three different carbon sources on biomass (**a**) and EPS production (**b**) by *P. vaninii* Ljup in a 5-L stirred-tank. Identical letters indicate no significant differences ($p < 0.05$). Identical letters indicate no significant differences ($p < 0.05$).

TABLE I
Carbohydrate composition in exopolysaccharides produced during submerged cultivation of *P. vaninii* Ljup in the media with selected three different carbon sources.

Carbo-hydrate (%)	Carbon sources in the medium		
	Glucose	Fructose	Sucrose
Rhamnose	nd	1.42±0.08a	nd
Ribose	nd	1.22±0.13a	0.92±0.04b
Xylose	1.52±0.04a	2.05±0.07b	1.84±0.11c
Gluconic acid	19.53±1.23a	35.87±3.45b	20.89±1.98c
Galactose	2.39±0.16a	2.77±0.08b	10.09±0.86c
Glucose	24.28±2.13a	7.77±0.36b	14.79±0.94c
Mannose	30.68±1.87a	42.27±3.94b	36.15±2.17c
Galactose acid	21.60±1.06a	6.63±0.85b	15.32±0.77c

nd refers to not detected. Identical letters indicate no significant differences ($p < 0.05$).

concerning the influence on cellular enzymatic system by different carbon source, detailed discussion on the metabolic process for the EPS formation is difficult currently.

FT-IR SPECTROSCOPY

FT-IR spectroscopy was used as an effective analytical tool to characterize functional groups. Typical IR spectra for the EPS fractions with three different carbon sources have been compiled in Figure 2. All samples exhibited similar characteristic peaks, which indicate that they possess similar functional groups. There is a broad stretching intense characteristic peak at ca. 3279-3285 cm^{-1} typical of -OH groups as well as a weak C–H band at ca. 2922-2928 cm^{-1}. The characteristic band at ca. 1641-1643 cm^{-1} could be correlated to the stretching vibration of the carbonyl group (C=O) of the polysaccharide. Bands at ca. 1024-1030 cm^{-1} suggested the presences of C-O type of linkages (Xie et al. 2010). Each particular polysaccharide possessed the specific bands at 807-810 cm^{-1} and 916-921 cm^{-1}, indicating both α- and β-configurations of the sugar units (Lim et al. 2005).

MOLECULAR AND STRUCTURAL PROPERTIES

The molecular mass values for two eluted fractions were calculated for the portions of peaks, which lie within the peak ranges. The relevant molecular parameters of each EPS are summarized in Table II. In Table II, the molecular weights of EPSs from glucose, fructose and sucrose as carbon source in the medium were estimated to be 6.255×10^5, 3.132×10^5 and 2.469×10^5 respectively. Zhang et al. (2004) suggested that moderate molecular weight on the improvement of the bioactivities of the polysaccharides is important. The high values of polydispersity ratio for all EPSs (4.72-8.95)

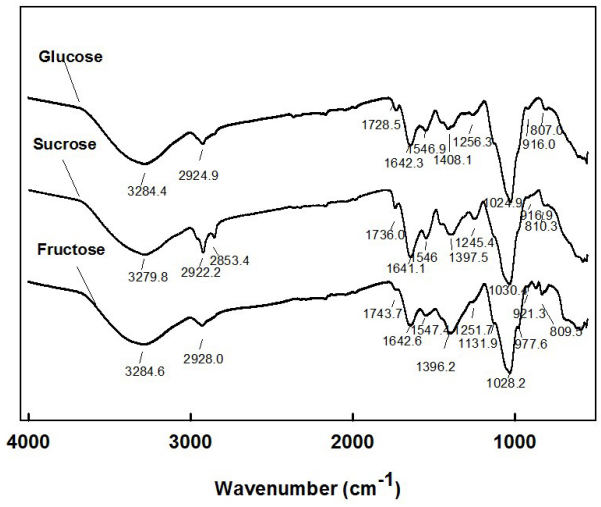

Figure 2 - The FT-IR spectra of the EPS fractions produced by submerged cultivation of *P. vaninii* Ljup in the media with selected three different carbon sources.

TABLE II
Relevant molecular parameters of exopolysaccharides of produced by submerged cultivation of *P. vaninii* Ljup in the media with selected carbon sources in MALLS analysis.

Parameters[a]	Carbon sources in the medium		
	Glucose	Fructose	Sucrose
M_n[a] (g/mol)	7.868×10^4	6.002×10^4	5.236×10^4
M_w[a] (g/mol)	6.255×10^5	3.132×10^5	2.469×10^5
M_z[a] (g/mol)	6.878×10^6	6.128×10^6	4.160×10^6
M_w/M_n[b]	7.950	5.217	4.715
R_n[c] (nm)	34.4	22.1	31.9
R_w[c] (nm)	32.8	19.6	27.8
R_z[c] (nm)	54.4	41.1	51.5

[a] M_n, M_w, and M_z refer number-, weight-, z-average molecular weight, respectively.
[b] M_w/M_n means polydispersity ratio.
[c] R_n, R_w, and R_z refer number-, weight-, z-average square mean radius of gyration, respectively.

mean that these EPS molecules exist much more dispersed in aqueous solution and could form large aggregates (Hwang et al. 2003). This information is important because the functional properties of polysaccharides can be greatly influenced by the molecular weight distribution. For each of these moments of the distribution, the root mean square (RMS) radii of the EPSs were calculated (Table II). These data provide a measure of the EPS molecular size in terms of the RMS distance from the molecular center of gravity to its edge (Hwang et al. 2003). Compared with the peaks of glucose and sucrose, the RMS radii for the peak of sucrose ranged from 27.8-51.5 nm was moderate (Table II). The slope for EPS in the double logarithmic plots of RMS radius versus molecular mass was shown in Figure 3. The values of slope of EPS indicated 0.16-0.25, which implies that the EPS molecule exists as a nearly globular shape in aqueous solution (see SEC/MALLS ANALYSIS). The results indicates that carbon source has more influence on molecular weight of EPS than their conformation.

ANTIOXIDANT PROPERTIES ANALYSIS

In vitro antioxidant capacities of all EPS fractions produced by submerged cultivation of *P. vaninii* Ljup in the media with selected carbon sources were subsequently evaluated using different biochemical methods including DPPH and hydroxyl radical scavenging assays.

DPPH radical method is widely used as an index to evaluate the antioxidant potential of natural compounds (Cheng et al. 2011). In this experiment, scavenging rates of EPS fractions with various carbon sources depicted in Figure 4a. proved that radical scavenging activity was also concentration dependent. The scavenging activities of EPS with sucrose as carbon source were superior to those observed for other two EPSs irrespective of the concentration of the fraction ($p < 0.05$), reaching a maximum of 89.2% at 5 mg/mL. Hydroxyl

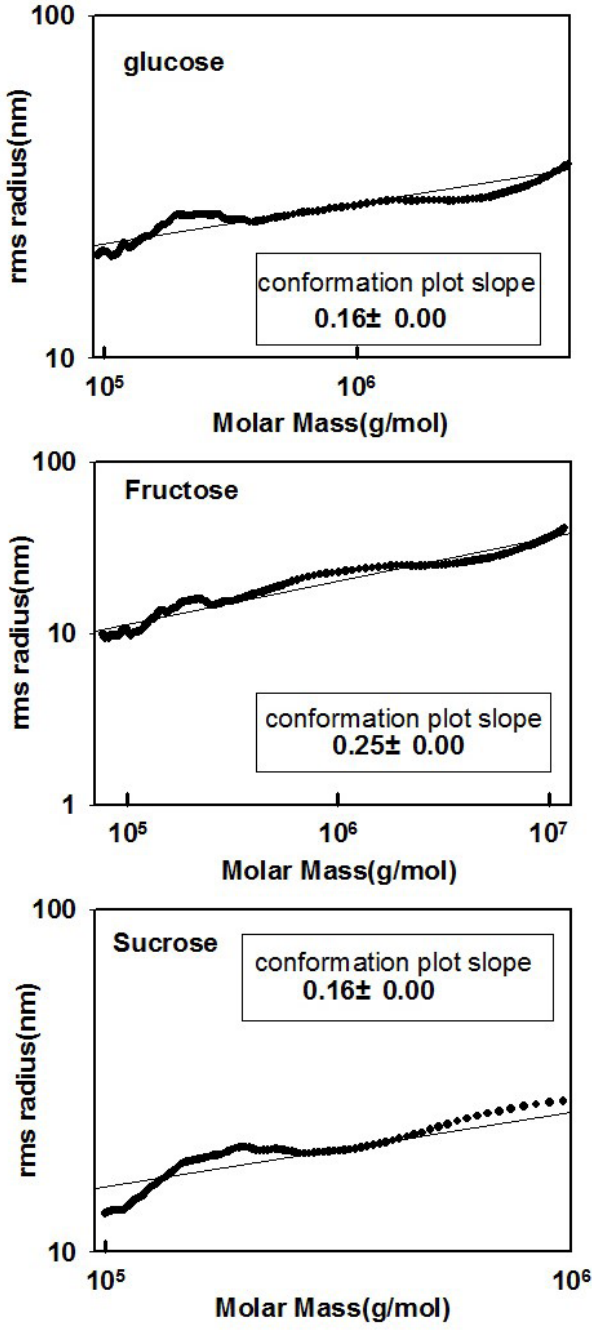

Figure 3 - The double logarithmic plots of root mean square radius vs molecular mass for exopolysaccharides of produced by submerged cultivation of *P. vaninii* Ljup in the media with selected three different carbon sources in MALLS analysis.

radicals can react with most natural compounds functioning and induce severe damage to adjacent biomolecules. The results of hydroxyl radical scavenging activities summarized in Figure 4b showed that hydroxyl radical scavenging activities were improved at increasing concentrations of EPS, in good agreement with DPPH radical scavenging properties. Similarly to previous findings, EPS with sucrose as carbon source exhibited a promising antioxidant activity ($p < 0.05$), with a 88.6%. OH radical scavenging rate at a concentration of 10 mg/mL. Though the EPS was purified by Sepharose CL-6B, some metabolites secreted by *P. vaninii* could bond covalently with EPS and more purification and structural elucidation need be carried out.

One of the mechanisms involved in antioxidant activity may originate from the hydrogen atom-donating ability of a molecule to a radical, which results in terminating radical chain reactions and converting free radicals to unharmful products (Hu et al. 2000). The antioxidant activity of polysaccharides might be attributed to their hydroxyl groups and other functional groups, such as C=O, –COOH and –O–, which can donate electrons to reduce the radicals to a more stable form or react with the free radicals to terminate the radical chain reaction (Leung et al. 2009). The superior antioxidant activities of EPS fraction with sucrose as carbon source as compared to the other two EPS fractions may be attributed to the differing molecular properties, i.e. monosaccharide compositions and molecular size. The larger content of galactose in EPS fraction with sucrose as carbon source could be of relevance to account for the essentially different antioxidant properties. Chen et al. (2006) also investigated the EPS of *Tremella mesenterica* from submerged cultures with various carbon sources and found that xylose and glucose were better carbon sources from the viewpoint of immunomodulatory activity.

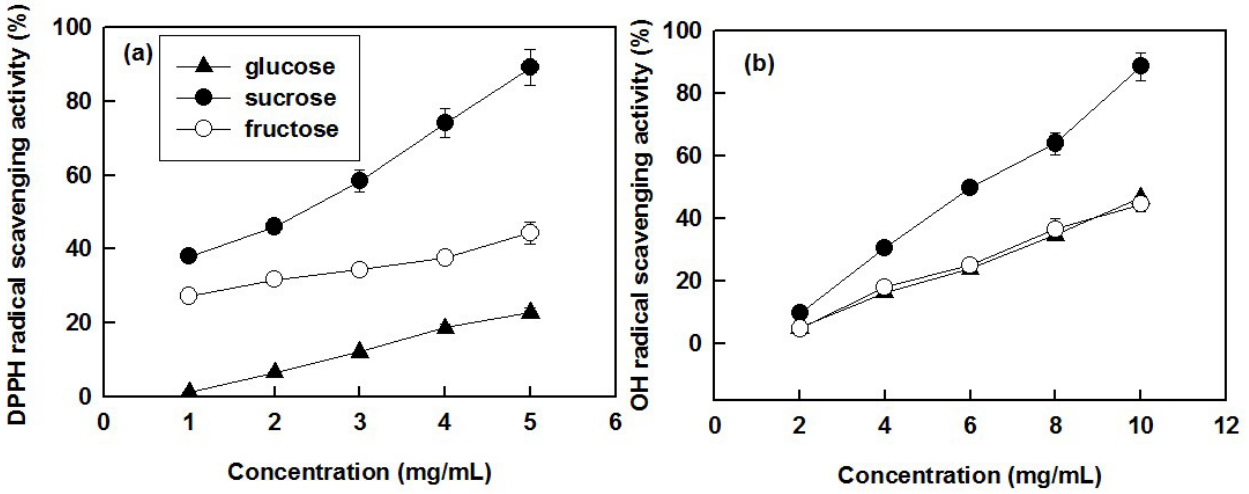

Figure 4 - Antioxidant activity of EPS fractions produced by submerged cultivation of *P. vaninii* Ljup in the media with selected three different carbon sources. The results represent mean ± S.D. (n = 3). DPPH (**a**) and OH (**b**) radical scavenging activity of *P. vaninii* Ljup EPS.

CONCLUSIONS

The effect of three different carbon sources on EPS production from the submerged culture of *P. vaninii* Ljup was investigated and the three EPSs were purified and characterized by FT-IR spectroscopy, GC and SEC-MALLS analysis and their antioxidant activities subsequently compared. The results demonstrated that the molecular properties and antioxidant activities varies with the different carbon sources. Sucrose is better carbon source from the viewpoint of antioxidant activity due to the relatively high content of galactose in EPS and moderate molecular size. Thus, the alternate carbon-source strategy may be successfully used for regulation of biosynthesis and activity by *P. vaninii* Ljup. Further works on the full structure elucidation of EPS fractions from different carbon source by one-dimensional and two-dimensional NMR spectroscopy are in progress in our laboratory.

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