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# Exopolysaccharides from *Pleurotus pulmonarius*: Fermentation Optimization, Characterization and Antioxidant Activity

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**Summary**

Culture conditions for exopolysaccharide (EPS) production by *Pleurotus pulmonarius* in submerged culture are optimized. The suggested medium composition was as follows: 60 g/L of xylose, 6 g/L of soy extract, 5 mM of $KH_2PO_4$ and 5 mM of $MgSO_4$. Under the optimized culture conditions in a 5-litre stirred tank fermentor, the maximum concentration of EPS was 6.36 g/L. Furthermore, the morphological parameters (*i.e.* average diameter, circularity, roughness and compactness) of the pellets and the broth viscosity are characterized. It has been proven that mycelial morphology and broth viscosity may be the critical parameters affecting the EPS yield. After deproteinization using Sevag method, a group of EPS (designated as fraction) was obtained from the culture filtrates by gel filtration chromatography. FT-IR analysis of the purified EPS revealed prominent characteristic groups corresponding to polyhydric alcohols. GC analysis showed that the purified EPS were mainly composed of galactose and glucose. Furthermore, thermogravimetric analysis indicated that the degradation temperature of the purified EPS was 217 °C. Finally, the antioxidant activity of the EPS fraction was investigated and the relationship with molecular properties was discussed as well.

*Key words*: fermentation optimization, *Pleurotus pulmonarius*, morphological and rheological properties, exopolysaccharide production, thermogravimetric analysis, antioxidant activity

## Introduction

The genus *Pleurotus* is widely cultivated and commercialized in many countries. It contains many biologically active materials, such as polysaccharides, proteins, enzymes, dietary fibres and vitamins (*1,2*). The mushroom *Pleurotus pulmonarius* has been studied and it has been concluded that its polysaccharides have different levels of antitumour activities (*3*).

Mushrooms or polysaccharides from edible and medicinal mushrooms have been found to have several physiological activities, such as anticomplementary, antioxidant and antitumour (*3–5*). The submerged culture and fruiting body are the main sources for obtaining polysaccharides from medicinal mushrooms. However, the submerged culture for efficient production of exopolysaccharides (EPS) has received greater interest than the extraction from fruiting body, because submerged culture is a biotechnological tool capable of supplying interesting metabolites on a large scale and in a short period of time (*3*). It has also been reported that EPS obtained from submerged culture always exhibit similar biological activity to that of the polysaccharides extracted from a fruiting body (*6*).

In this study, the optimization of mycelial biomass and EPS production by *P. pulmonarius* in a submerged

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culture is evaluated. Furthermore, the physicochemical characterization of purified EPS is investigated using GC, FT-IR and TGA. Finally, the antioxidant activity of *P. pulmonarius* EPS *in vitro* is analyzed. The results provide a theoretical and practical basis for medicinal EPS production with higher yield and greater bioactivity.

## Materials and Methods

### *Microorganism and media*

*Pleurotus pulmonarius* was purchased from the Henan Province Microbiological Culture Collection Center (HPMCC no. 198716), Henan, PR China, and was used throughout the study. The stock culture was maintained on potato dextrose agar (PDA) slants. The slants were incubated at 25 °C for 8 days and stored at 4 °C. The seed culture was grown in a 250-mL flask containing 50 mL of basal medium (0.3 % peptone and 3 % glucose) at 26 °C on a rotary shaker incubator (120 rpm) for 4 days. The flask culture experiments were performed in a 250--mL flask containing 50 mL of the medium inoculated with 4 % (by volume) of the seed culture. *P. pulmonarius* was initially grown on PDA medium in a Petri dish, then 5 mm of the agar plate culture were cut out and transferred into the seed medium (*7*).

### *Bioreactor fermentation*

The fermentation medium was inoculated with 4 % (by volume) of the seed culture and then cultivated in a 5-litre stirred tank bioreactor (Infors AG, Bottmingen/Basel, Switzerland) equipped with a pH electrode. Unless otherwise specified, the fermentation was conducted under the following conditions: temperature of 25 °C, aeration rate of 2 vvm, agitation speed of 120 rpm, initial pH=5.0, working volume of 3 L and cultivation time of 12 days. All experiments were performed in triplicate to ensure the observed trends were reproducible.

### *Mycelial biomass and EPS quantification*

Samples collected periodically from the bioreactor were centrifuged at 9000×*g* for 15 min, and the resulting supernatant was filtered through a membrane filter (0.45 μm; Millipore, Merck, Darmstadt, Germany). The mycelium dry mass was measured after repeated washing of the mycelial pellet with distilled water and drying at 70 °C overnight to obtain constant mass. The resulting culture filtrate was mixed with four times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitated EPS were centrifuged at 10 000×*g* for 15 min and the supernatant was discarded (*8*). The precipitated EPS were treated with Sevag reagent (1:4 *n*-butanol/chloroform, by volume) to remove most of the proteins. After removing the proteins and Sevag reagent by centrifugation, the aqueous fraction was precipitated again with four volumes of dehydrated ethanol at 4 °C overnight. The precipitate was collected by centrifugation, washed successively with acetone, then dissolved in water and lyophilized to yield the crude EPS. The content of crude EPS was estimated by a phenol-sulphuric acid method using glucose as the standard (*9*).

### *Purification of exopolysaccharides*

The crude EPS were dissolved in 0.2 M NaCl buffer and loaded onto a Sepharose CL-6B column (2.4×100 cm; Sigma Chemical Co., St Louis, MO, USA). The column was eluted with the same buffer at a flow rate of 0.6 mL/min. Protein concentration was determined according to the Bradford method using bovine serum albumin as the standard (*10*). The total carbohydrate content in the EPS was determined by phenol-sulphuric acid method (*9*). The protein moiety in the EPS was monitored by measuring the absorbance at 280 nm, whilst the carbohydrate moiety was monitored at 480 nm.

### *Gas chromatography analysis*

Carbohydrate composition of the trimethylsilyl glycoside derivatives dissolved in cyclohexane was analyzed by GC-MS (Star 3600 CX; Varian Co. Lexington, MA, USA). The trimethylsilyl glycoside derivatives were obtained by hydrolysis of pure EPS with 2 mol/L of trifluoroacetic acid at 110 °C for 2 h. The GC was fitted with a fused silica capillary column (sodium form, 300× 0.25 mm, Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector.

### *Fourier transform-infrared spectroscopy*

Fourier transform-infrared (FT-IR) spectroscopy (Bruker Tensor 27; Bruker Optics, Inc., Billerica, MA, USA) was employed using the KBr disc for the analysis and detecting of functional groups. The sample pellets were prepared by mixing the freeze-dried EPS (1 mg) with 300 mg of KBr. FT-IR spectra were recorded on a Mattson spectrometer (Mattson Instruments, Inc., Madison, WI, USA) from 400 to 4000 $cm^{-1}$. The spectra were corrected for wavenumber-dependent signal-detection efficiency of the setup using a white light spectrum of a temperature--calibrated tungsten band lamp.

### *Thermogravimetric analysis of EPS*

Thermogravimetric analysis (TGA) of the EPS was done using TA Q5000IR TGA apparatus (TA Instruments, New Castle, DE, USA) and 15 mg of freeze-dried EPS. The TGA curve plots the TGA signal, converted to percentage mass change on the y-axis against the reference material temperature on the x-axis.

### *Hydroxyl radical scavenging activity in vitro*

The scavenging activity of hydroxyl radical was determined based on the method described by Zhao *et al.* (*11*). Various concentrations of EPS (0.1–0.4 mg/mL, 1 mL) were incubated with a solution containing phenanthroline (7.5 mM, 1 mL), phosphate buffer (20 mM, pH=7.4, 0.5 mL), $FeSO_4$ (0.75 mM, 1.5 mL) and $H_2O_2$ (3 %, 0.5 mL) at 37 °C for 1 h. The absorbance was measured at 536 nm using UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The scavenging activity was calculated using the following equation:

$$\text{Hydroxyl radical scavenging (or inhibition) rate} = [(A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100 \quad /1/$$

where $A_{sample}$, $A_{control}$ and $A_{blank}$ are defined as the absorbances of the sample, control (without EPS) and blank (without $H_2O_2$ and EPS), respectively.

### *ABTS radical scavenging activity*

Trolox equivalent antioxidant capacity assay measures the ability of a compound to eliminate or scavenge radicals compared with Trolox [(*S*)-(2)-6-hydroxy-2,5,7,8--tetramethyl-chroman-2-carboxylic acid] as an antioxidant reference (*12*). The ABTS kit was bought from Biyutian Institute of Technology, Haimen, PR China. Briefly, the ABTS radical cation solution was diluted with PBS (pH= 7.4) to an absorbance at 734 nm of 0.70±0.05 for the assay. A volume of 10 μL of the EPS solution was added to 200 μL of the diluted ABTS radical cation solution, and after 5 min the remaining amount of ABTS was determined at 734 nm using a spectrophotometer. The ABTS radical cation scavenging activity was expressed as Trolox equivalent antioxidant capacity (TEAC) and the TEAC values of the EPS samples were derived from the calibration curve generated with Trolox in the concentration range of 0–1.5 mM.

### *DPPH radical scavenging activity*

The antioxidant activity of the EPS based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the method described by Eloff *et al*. (*13*). Different amounts of EPS were dissolved in distilled water to make solutions of different concentrations. A volume of 2 mL of 0.1 g/L of DPPH in 50 % ethanol was added to 2 mL of the EPS solution. The absorbance was measured at 517 nm after 20 min of incubation at 25 °C. Ethanol 50 % was used instead of DPPH for the blank, while distilled water was used instead of the sample for the control. The scavenging activity of the sample against DPPH radical was calculated according to the following equation:

$$\text{DPPH radical scavenging activity} = [1-(A_{sample}-A_{blank})/A_{control}]\times 100 \quad /2/$$

where $A_{sample}$, $A_{control}$, and $A_{blank}$ are defined as the absorbances of the sample, blank (without DPPH) and control (without EPS), respectively.

### *Statistical analysis*

Data were expressed as mean values±S.D. ($N$=3). The statistical significance was determined by Student's $t$-test. Results of the experimental design were statistically analyzed by the analysis of variance (ANOVA) using SPSS software (v. 11.0; SPSS Inc., IBM, Chicago, IL, USA). Probability values of $p<0.05$ and $p<0.01$ were regarded as statistically significant and highly significant, respectively.

## Results and Discussion

### *Effect of the initial pH and temperature*

In order to investigate the effect of initial pH on the EPS production, *P. pulmonarius* was cultivated in a medium with different initial pH (3.0–9.0) in shake flask cultures. The mycelial yield was similar between pH=4 and 9, whereas the maximum EPS production was achieved at pH=5.0 (Fig. 1a). To determine the optimal temperature for the EPS production, this organism was cultivated at various temperatures. The optimal temperature was found to be 25 °C (Fig. 1b), which is consistent with a number of findings that many kinds of mushrooms have relatively low temperature optimum, ranging from 20 to 25 °C (*7,8,14*).

### *Effect of carbon, nitrogen and mineral sources*

To find a suitable carbon source for EPS production by *P. pulmonarius*, various carbon sources were tested at a concentration of 30 g/L for 8 days in the basal medium. As shown in Table 1, each carbon source was added to the basal medium instead of dextrose. Although sucrose and lactose gave good mycelial biomass, they led to low EPS yield. The highest EPS levels were obtained in xylose medium. These results indicated that the nutritional requirement for EPS production in *P. pulmonarius* was not always consistent with that of mycelial biomass. In the present study, xylose was selected as a suitable carbon source and its optimum concentration was found to be 60 g/L (data not shown). On the contrary, Smiderle *et al*. (*15*) evaluated different carbon sources (*i.e.* glucose, galactose, xylose and arabinose) and found that galactose was the best carbon source to produce EPS from *P. pulmonarius*, which indicated that optimal carbon source

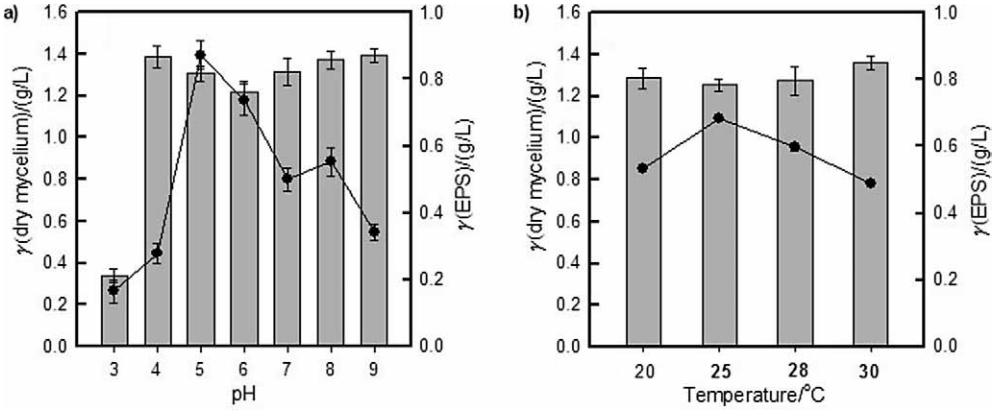


**Fig. 1.** Effect of: a) pH and b) temperature on the mycelial growth and EPS production by *P. pulmonarius* in shake flask cultures (▭) mycelial growth, (●) EPS production

Table 1. Effect of carbon, nitrogen and mineral sources on the mycelial growth and EPS production by *P. pulmonarius* in shake flask cultures

| Source | $\gamma$(dry mycelium) g/L | $\gamma$(EPS) g/L | Final pH |
|---|---|---|---|
| Carbon (3 %) | | | |
| glucose | 0.653±0.026 | 0.251±0.073 | 7.23 |
| fructose | 1.112±0.074 | 0.569±0.066 | 5.98 |
| maltose | 0.813±0.038 | 0.524±0.054 | 7.74 |
| sucrose | 1.287±0.064 | 0.548±0.056 | 8.18 |
| xylose | 1.120±0.059 | 1.082±0.094 | 5.57 |
| lactose | 1.460±0.026 | 0.564±0.049 | 7.32 |
| Nitrogen (0.3 %) | | | |
| beef extract | 1.440±0.104 | 0.767±0.041 | 5.39 |
| yeast extract | 1.480±0.034 | 0.622±0.015 | 5.60 |
| polypeptone | 1.375±0.074 | 0.855±0.017 | 5.27 |
| peptone | 0.753±0.042 | 0.587±0.026 | 7.09 |
| soy extract | 3.210±0.201 | 0.871±0.028 | 5.51 |
| potassium nitrate | 0.260±0.021 | 0.201±0.015 | 6.30 |
| ammonium sulphate | 0.313±0.028 | 0.243±0.027 | 5.30 |
| Minerals (5 mM) | | | |
| $K_2HPO_4$ | 1.826±0.1460 | 0.873±0.075 | 5.02 |
| $MgSO_4$ | 1.566±0.0963 | 0.614±0.042 | 7.01 |
| $CaCl_2$ | 0.142±0.0182 | 0.570±0.068 | 7.59 |
| $MnCl_2$ | 0.673±0.0443 | 0.591±0.053 | 4.99 |
| $FeSO_4$ | 0.567±0.8026 | 0.483±0.086 | 4.01 |
| Control | 1.453±0.1121 | 0.529±0.086 | 7.08 |

Fermentation was carried out for 8 days at 25 °C with initial pH=5. Values are mean±S.D., *N*=3

varies among fungal species, even different strains of the same species need different carbon source for metabolite production.

To investigate the effect of nitrogen sources on the EPS production, cells were cultivated in basal medium containing various nitrogen sources at a concentration of 3 g/L. Amongst seven nitrogen sources examined, soy extract was the most effective for mycelial growth and EPS production (Table 1). In comparison with organic nitrogen sources, inorganic nitrogen sources resulted in relatively lower mycelial growth and EPS production.

The influence of various mineral sources on the EPS production was examined at a concentration of 5 mM. Among various mineral sources examined, $KH_2PO_4$ and $MgSO_4$ yielded good EPS production without adverse effects on biomass production and thus were recognized as favourable bioelements. Similar observations were made by Kalogiannis *et al*. (*16*) and Hwang *et al*. (*17*) in fermentations of other mushrooms. They suggested that $KH_2PO_4$ could improve EPS productivity through its buffering action, and that essential phosphates were favourable for mycelial growth in submerged cultures of mushrooms. Magnesium was reported to be involved in the synthesis of polysaccharide metabolism and it stabilizes the plasma membrane (*18–20*).

### *The effect of C/N ratio*

The C/N ratio is one of the important factors affecting the fruiting body formation in mushroom cultures. The effect of C/N mass ratio on the EPS production was investigated using the medium with xylose and soy extract. As shown in Table 2, the C/N ratio of 10:1 was favourable for both mycelial growth and EPS production. This finding was in accordance with previous results of EPS accumulation by *Ganoderma lucidum* (at a C/N ratio of 10) reported by Yuan *et al.* (*21*).

Table 2. Effect of C/N mass ratio on the mycelial growth and EPS production by *P. pulmonarius* in shake flask cultures

| C/N ratio | $\gamma$(dry cells) g/L | $\gamma$(EPS) g/L | Final pH |
|---|---|---|---|
| 1:1 | 2.840 | 2.368 | 7.41 |
| 10:1 | 3.000 | 3.745 | 5.62 |
| 20:1 | 2.113 | 1.973 | 5.14 |
| 30:1 | 1.987 | 1.819 | 5.37 |

Fermentation was carried out for 8 days at 25 °C with initial pH=5. Values are mean±S.D., *N*=3

### *Results of fermentation in a stirred tank bioreactor*

The typical time courses of mycelial growth and EPS yield in a 5-litre stirred tank bioreactor under optimal culture conditions are shown in Fig. 2. The maximum mycelial yield was obtained at 6.84 g/L after 5 days (Fig. 2a), and the optimal yield of EPS was 6.36 g/L after 7 days of fermentation (Fig. 2b). As expected, the concentration of residual sugars decreased as the fermentation progressed with corresponding increases in biomass and EPS production (Fig. 2c). However, no drastic change in the pH value was recorded during fermentation (Fig. 2d).

### *Characterization of broth viscosity and mycelial morphology*

The apparent viscosity of the whole broth during fermentation was investigated. After day 2, the viscosity of the fermentation broth at 160 rpm increased rapidly as the cells entered their exponential growth. This continued up to day 6 (42.59 mPa·S), then the microorganisms entered their stationary phase and the viscosity of the broth was reduced accordingly (data not shown). Typical morphological changes during the entire fermentation period were observed. The cells were found to form mainly pellets during the entire culture period. The pellet diameter increased rapidly and the outer hairy regions of the pellets became fluffier. However, as the fermentation proceeded, especially on day 9, the outer hairy region of the pellets was shaved off. According to the fungal morphology, the average diameter, roundness, compactness, and roughness of the pellets during cultivation of *P. pulmonarius* were analysed and are shown in Fig. 3. The average diameter and compactness of the pellets in-

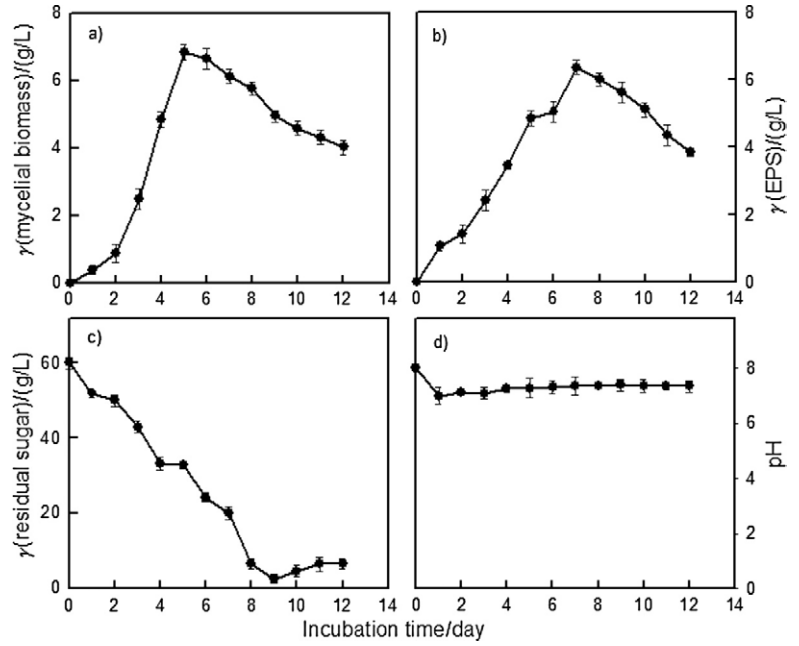


**Fig. 2.** Time profiles of: a) mycelial biomass, b) EPS, c) residual sugar, and d) pH in the submerged culture of *P. pulmonarius* in a stirred tank fermentor

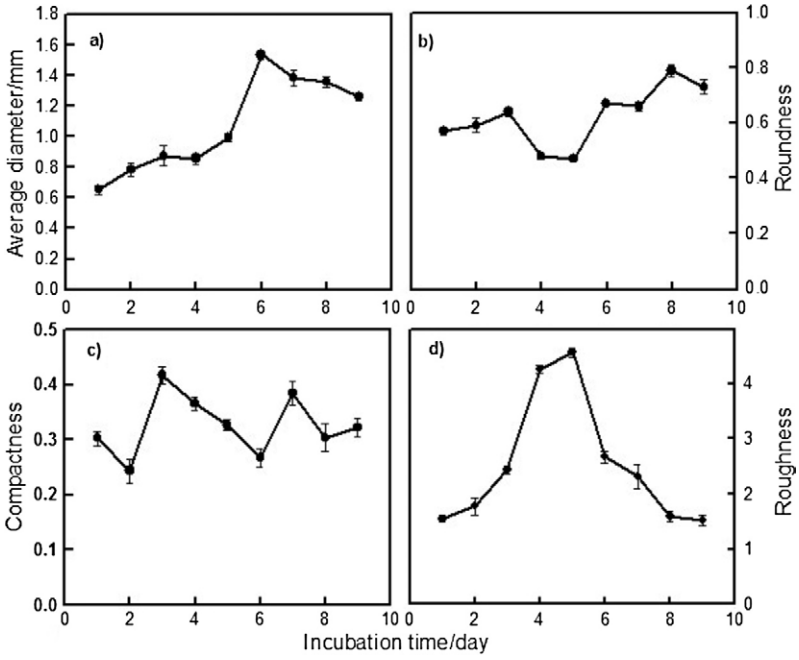


**Fig. 3.** Morphology of *P. pulmonarius* pellets growing in a stirred tank fermentor: a) average diameter, b) roundness, c) compactness, and d) roughness

creased during the first 5 and 8 days of fermentation, respectively, and then they vibrated, but no drastic changes in the circularity and roughness were revealed during the course of fermentation. Further investigation of the EPS accumulation in the mycelial pellets during fermentation revealed that viscosity ($R^2$=0.902, $p<0.01$ for the first 12 days of fermentation), mycelial growth ($R^2$=0.856, $p<0.01$ for the first 8 days of fermentation), and pellet morphology, *i.e.* average diameter ($R^2$=0.837, $p<0.05$ for the first 5 days of fermentation) were significantly positively correlated with EPS content. These results clearly indicate that the viscosity and mycelial morphology change were coincidental with fungal biomass and EPS production, respectively. Park *et al*. (22) also investigated the compactness of the pellet form of *Cordyceps militaris* and found that it was related to EPS production.

### *Purification of EPS and analysis of carbohydrates*

In gel filtration chromatography of the culture, only one fraction of EPS, which consisted of polysaccharides and proteins, was coeluted, as shown in Fig. 4. The de-

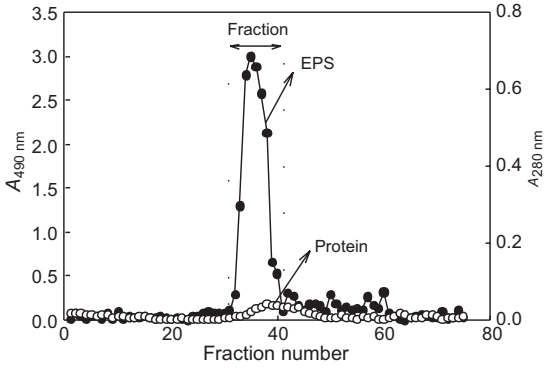


**Fig. 4.** Elution profiles of the EPS fraction (5 mL/tube) in Sepharose CL-6B column. Elutes were analyzed by measuring the absorbance at 490 nm for carbohydrates (●) and at 280 nm for proteins (○)

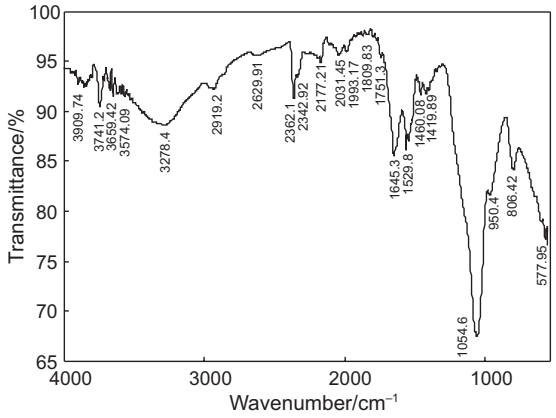


**Fig. 5.** The FT-IR spectrum of the purified EPS produced by submerged culture of *P. pulmonarius*

tailed carbohydrate compositions in the EPS fraction are presented in Table 3. The EPS fraction consisted of nine monosaccharides, mainly galactose (58.35 %) and glucose (30.72 %) in carbohydrate moiety. The results were not totally consistent with EPS fermentation by *P. pulmonarius* reported by Smiderle *et al*. (*15*); the EPS sample showed manose, galactose and glucose as major monosaccharide components, probably due to their different cultivation medium.

Table 3. Carbohydrate composition of the purified EPS fraction from the submerged culture of *P. pulmonarius* in a stirred tank bioreactor

| Carbohydrate | *w*/% |
|---|---|
| talose | 5.84 |
| fucose | 0.02 |
| ribose | 0.53 |
| arabinose | 2.78 |
| xylose | 1.24 |
| mannose | 0.20 |
| galactose | 58.35 |
| glucose | 30.72 |
| altrose | 0.32 |

### *Results of FT-IR spectroscopy*

FT-IR is an effective analytical method for detecting functional groups and characterizing covalent bonding information. Typical IR spectrum for the purified EPS fraction is presented in Fig. 5. A continuous absorption beginning at the region of approx. 3278.4 cm$^{-1}$ is characteristic of a carbohydrate ring. Strong bands at 2919.2 cm$^{-1}$ were assigned to the stretching vibration of the methylene group (C–H), usually present in hexoses. A characteristic absorption band appearing at 1645.3 cm$^{-1}$ was assigned to the stretching vibration of the carboxyl group (C=O). A small peak around 1529.8 cm$^{-1}$ corresponding to an amino group was also observed in the spectrum of the EPS. The stretching vibration peak of 1054.6 cm$^{-1}$ suggested the presence of C-O-H link bond position (*23*). In addition, the absorption band at 806.42 cm$^{-1}$ was characteristic for mannose (*24*). This was in accordance with the results of carbohydrate analysis in Table 3.

### *Results of thermogravimetric analysis*

Polysaccharides are used as materials for food technology, tissue engineering, pharmaceutical excipients and drug carriers. The increasing importance of polysaccharides has motivated scientists to study their physicochemical properties to find their property-based applications. For most of the applications, it is important to know the thermal stability of a material to be used. Thermal stability can conveniently be determined by thermogravimetric analysis (*25*). TGA measures the variation of heat flux in a sample with variation of temperature. The TGA analysis of purified EPS fraction was carried out dynamically (mass loss *vs.* temperature) and the experimental results indicated that the degradation temperature of EPS was 217 °C. About 90 % of the EPS was stable up to 202 °C. This fact implies that EPS should not be exposed to temperature ranges close to 217 °C, in order not to compromise the physical integrity of the evaluated material. The mass of EPS was dramatically lost around 400 °C, it continued gradually to decrease and the final residue was 17.97 % of initial mass.

### *Antioxidant activity of P. pulmonarius EPS*

In this experiment, the *in vitro* antioxidant capacity of purified *P. pulmonarius* EPS fraction was evaluated using different biochemical methods including hydroxyl, DPPH and ABTS radical scavenging assays. The results of hydroxyl radical scavenging assay are shown in Fig. 6a. The hydroxyl radical scavenging rate of *P. pulmonarius* EPS at 3 g/L was 41.03 %. The TEAC scavenging rates of EPS reached 0.24 mM (Fig. 6b). Furthermore, the DPPH scavenging rate of EPS at 2.0 g/L reached 8.4 % (Fig. 6c). These results indicate that the EPS has potential antioxidant capacity. This might be attributed to its hydroxyl group and other functional groups in the fraction of *P. pulmonarius* polysaccharide, such as –COOH, C=O 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 (*26*).

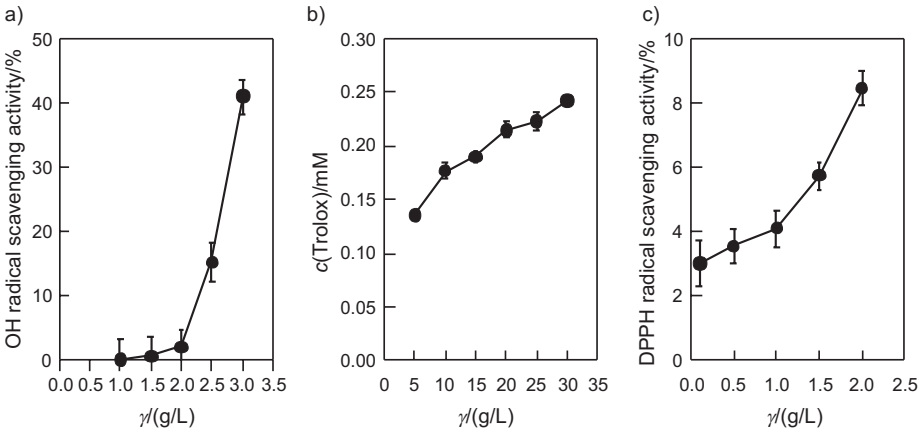


**Fig. 6.** Antioxidant activity of polysaccharides purified from *P. pulmonarius*: a) hydroxyl, b) ABTS and c) DPPH radical scavenging activity. The results represent mean values±S.D., *N*=3

## Conclusion

In this work, the optimization of submerged culture conditions for EPS production by *P. pulmonarius* was addressed. One pure fraction of EPS was obtained by gel filtration chromatography and characterized by GC, FT-IR spectroscopy and thermogravimetric analysis. Furthermore, the antioxidant capacity experiments showed that the pure EPS can be a valuable compound for functional food or medicine production. In order to understand the relationship between the structure and the bioactivity of polysaccharides better, further investigations of physical and other structural features including molecular mass and related glycosidic linkages are in progress in our laboratory.

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